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AIM AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, DOI: 10.3748) is a weekly, open-access, peer-reviewed journal supported by an editorial board of 1096 experts in gastroenterology and hepatology from 60 countries.

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

EDITORIAL

Safety of the long-term use of proton pump inhibitors

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Abstract

The proton pump inhibitors (PPIs) as a class are remarkably safe and effective for persons with peptic ulcer disorders. Serious adverse events are extremely rare for PPIs, with case reports of interstitial nephritis with omeprazole, hepatitis with omeprazole and lansoprazole, and disputed visual disturbances with pantoprazole and omeprazole. PPI use is associated with the development of fundic gland polyps (FGP); stopping PPIs is associated with regression of FGP. In the absence of *Helicobacter pylori* infection, the long-term use of PPIs has not been convincingly proven to cause or be associated with the progression of pre-existing chronic gastritis or gastric atrophy or intestinal metaplasia. Mild/modest hypergastrinemia is a physiological response to the reduction in gastric acid secretion due to any cause. The long-term use of PPIs has not been convincingly proven to cause enterochromaffin-like cell hyperplasia or carcinoid tumors. PPIs increase the risk of community acquired pneumonia, but not of hospital acquired (nosocomial) pneumonia. There is no data to support particular care in prescribing PPI therapy due to concerns about risk of hip fracture with the long-term use of PPIs. Long-term use of PPIs does not lead to vitamin B12 deficiencies, except possibly in the elderly, or in persons with Zollinger-Ellison Syndrome who are on high doses of PPI for prolonged periods of time. There is no convincingly proven data that PPIs increase the risk of *Clostridium difficile*-associated diarrhea in persons in the community. The discontinuation of PPIs may result in rebound symptoms requiring further and even continuous PPI use for suppression of symptoms. As with all medications, the key is to use PPIs only when clearly indicated, and to reassess continued use so that long-term therapy is used judiciously. Thus, in summary, the PPIs are a safe class of medications to use long-term in persons in whom there is a clear need for the maintenance of extensive acid inhibition.

INTRODUCTION

The risk of minor adverse effects from proton pump inhibitors (PPIs) is low, approximately 1%-3%, with rates of withdrawal from clinical research studies being 1%-2%, with no significant differences noted between the PPIs[1-6]. The risk of symptomatic adverse effects with the PPIs is low as well. In pooled data from published trials involving 2812 patients, omeprazole was

reported as causing headache (2.4%), diarrhea (1.9%), nausea (0.9%), and rash (1.1%), a profile similar to that of cimetidine and ranitidine[7]. In a prospective follow-up study of 5669 patients on lansoprazole, the most common reported adverse effects were diarrhea (4.1%), headache (2.9%), and nausea (2.6%). A similar profile has been reported for pantoprazole: diarrhea, 1.5%; headache, 1.3%; dizziness, 0.7%; pruritus, 0.5%[8]; rash, 0.4%[9]; and nausea, 0.015%. Compiled data from 3556 patients taking rabeprazole for up to one year demonstrated that the most common adverse effect was headache with an incidence (2.4%) similar to placebo (3.1%).

Serious adverse events are rare, with case reports of interstitial nephritis with omeprazole, hepatitis with omeprazole and lansoprazole[10-12], and disputed visual disturbances with pantoprazole and omeprazole[13,14]. An anticipated physiological effect of acid suppression with PPIs is an elevated serum gastrin concentration, which occurs with all PPIs. Gastrin elevation may be higher with omeprazole than with pantoprazole, and higher with lansoprazole than with omeprazole[15-17], and higher with rabeprazole than with omeprazole[18]. This variation in PPI-associated elevation of gastrin concentration is not clinically relevant.

Some persons with dyspeptic conditions such as gastroesophageal reflux disease (GERD) may need to be maintained long-term on PPIs. For this reason, we have reviewed the literature on possible long-term adverse effects of PPIs. Narrative or qualitative reviews, when compared to their systematic review cousins, trade off depth in favor of breadth. After all, reviews can be comprehensive and primary, or narrow in scope and heavily guarded against various biases. Narrative reviews remain, despite a heavy battering by hordes of high quality randomized controlled trials and mathematically endowed structured reviews, on their pedestal as a premier venue for medical educators and historians. This review fits in that important tradition, and purports to fill a need for a comprehensive review on the safety of long-term use PPIs. Mulrow published criteria for minimizing bias in narrative reviews[19]. Deeks summed up these as being rigorous, informative, comprehensive, and explicit[20]. Collins and Fauser, in their editorial, enforced the view of the importance of "balancing the strengths of systemic and narrative reviews"[21]. To achieve this balance and complement a primarily journalistic approach, a search of PubMed, Google Scholar and UpToDate for articles published since 1999 on the topic of "PPI" and "safety" (and related MESH terms) was conducted to identify English language meta-analysis, publications in one of the top biomedical journals in this field (NEJM, Annals, Lancet, JAMA, American Journal of Gastroenterology, Gastroenterology, American Journal of Gastroenterology, Alimentary Pharmacology and therapeutics, Drugs, BMJ) as well as major North American and European guidelines. Checklists have been proposed for systematic and qualitative research[22,23], aiding in the review. We present this information on the long-term safety of PPIs with a series of questions, a summary of the literature, and our proposed answer.

THE USE OF PPIS IS NOT ASSOCIATED WITH AN ALTERATION OF GASTRIC HISTOLOGY

In *Helicobacter pylori* (*H. pylori*) negative persons PPIs do not worsen pre-existing gastritis[24,25], and may even improve pre-existing gastritis[26]. PPIs do not cause atrophic gastritis[27,28].

In contrast, in *H. pylori* positive persons, *H. pylori* is associated with antral or body acute or chronic gastritis, atrophy and metaplasia[29]. *H. pylori*-associated chronic gastritis may progress to gastric atrophy, intestinal metaplasia, and gastric cancer[25,27,30,31], or may not[32]. *H. pylori* and PPIs may cause progression or acceleration from gastric antrum-predominant chronic gastritis to body-predominant chronic gastritis, and it is controversial whether gastric body-predominant atrophic gastritis (gastric atrophy) is a risk factor for gastric cancer[33]. *H. pylori* eradication may cause regression of gastric atrophy or intestinal metaplasia[26,27,29,34-37] or may not[33,38-44].

Thus, the long-term use of PPIs has not been convincingly proven to cause or accelerate the progression of pre-existing chronic gastritis, corpus gastric atrophy or intestinal metaplasia.

PHYSIOLOGICAL HYPERGASTRINEMIA DOES NOT CAUSE GASTRIC CARCINOIDS OR CANCER

H. pylori infection (without use of PPIs) itself increases serum gastrin concentration[29,45].

PPIs modestly increase serum gastrin concentration in persons who are *H. pylori*-negative or positive[46,47]. While PPI's may increase apoptosis[48], PPIs do not increase risk of gastric or esophageal cancer[49-51].

It is controversial whether the hypergastrinemia associated with the use of PPIs increase enterochromaffin-like (ECL) cell numbers, as well as linear or micronodular hyperplasia - Yes[52-54] in *H. pylori* positive persons[55], and No[43,56,57]. Hypergastrinemia associated with Zollinger-Ellison Syndrome (ZES), rarely is associated with an increase in ECL cell growth or ECL carcinoid[58]. Furthermore, there is only one published report in world literature of a ZES patient treated with PPIs for associated gastric hypersecretion, who developed gastric cancer[59]. This is probably a chance association.

Thus, mild/modest hypergastrinemia is a physiological response to a reduction in acid secretion due to any cause. The long-term use of PPIs has not been convincingly proven to cause ECL cell hyperplasia or carcinoid tumors. Even when hypergastrinemia is marked and prolonged (such as with ZES or MEN-1), gastric carcinoids are rare.

THE USE OF PPIS IS ASSOCIATED WITH THE DEVELOPMENT OF FUNDIC GLAND POLYPS

PPI use is associated with parietal cell hyperplasia, and an

up to fourfold increased incidence of fundic gland polyps (FGP)[60-62]. FGP also occur in the presence of *H. pylori* infection, likely incidentally[63,64]. Eradication of *H. pylori* or stopping long-term use of PPIs is associated with regression of FGP[62,65,66]. FGP in sporadic cases is rarely associated with dysplasia, but never gastric adenocarcinoma[67]. Dysplasia may occur in 25%-44% of gastric polyps in persons with familial adenomatous polyposis[63,68].

In summary, PPI use is associated with the development of FGP. FGP occur in the presence or absence of *H. pylori* infection. The eradication of *H. pylori* or stopping PPI is associated with regression of FGP. FGP may rarely become dysplastic, but almost exclusively this rare event is seen in persons with familial adenomatous polyposis.

PPIs may mask the symptoms of gastric cancer (GC), heal malignant gastric ulcers, or shorten survival in the patient with GC.

PPIs may mask the symptoms or heal early GC, but there is no data on the effect of PPIs on rates of survival[69]. H2RA's may[70] or may not[71] actually produce longer survival in patients with GC.

BIOAVAILABILITY OR METABOLISM OF A FEW OTHER DRUGS

PPIs reduce gastric acid, and thereby reduce the bioavailability of drugs requiring intragastric acidity to maximize their absorption and bioavailability[51]. Examples of such drugs would include ketoconazole, itraconazole and indinipur[72], and may reduce the effects of locally acting drugs such as sucralfate.

PPIs may alter the intestinal first pass metabolism or the hepatic clearance of some drugs, and thereby modify their pharmacodynamics[72]. They have no effect on n-acetyl-transfer or xanthine oxidase activities[73], and may show a rare class action effect on vitamin K antagonists[74]. PPIs have a low drug interaction through phase I / II effects[5,75], and may differ in their possibility of causing drug interactions. Omeprazole and lansoprazole have a high affinity for CYP2C19 and CYP3A4 but these cytochromes contribute little to rabeprazole metabolism. Pantoprazole is completely metabolized by these cytochrome enzymes, but it uniquely has no drug interactions with a wide range of drugs[72,76-78].

PPIs, with the exception of pantoprazole, have been associated with reduced effectiveness of clopidogrel and a resulting 40% increased risk of coronary stent occlusions[79]. There is no consensus yet on how to manage this[80].

Thus, PPIs have an effect in common with all acid lowering therapy to reduce the absorption of acid-dependent medications. The metabolism of PPIs by hepatic cytochrome enzymes varies significantly between drugs.

THE USE OF PPIS AND DEFICIENCIES IN IRON AND VITAMIN B12

Iron

PPIs reduce gastric acidity, and in patients treated long-term with high dose PPIs duodenal absorption of organic and non-organic iron may be reduced[81]. This effect however is small, and PPIs are not associated with an increased risk of latent iron deficiency or iron deficiency[82].

Vitamin B12

PPIs reduce gastric acidity, which is necessary to activate pepsinogen to pepsin to release vitamin B12 from B12-containing foods. PPIs used short-term may minimally reduce the absorption of protein-bound B12 in food[83-85]. In elderly patients who may already have gastric atrophy (possibly from *H. pylori* infection), PPIs used long-term may reduce serum vitamin B12 concentrations[85-87]. Five out of six studies have shown that PPIs used long-term in non-elderly patients do not reduce serum vitamin B12 concentrations, and therefore body B12 stores[81,88-93].

In ZES patients treated long-term with high dose PPIs, the serum concentration of vitamin B12 may be reduced[94]. And yet, in cystic fibrosis (CF) children with reduced secretion of pancreatic bicarbonate and increased duodenal acidity, there is no reduction in the intestinal absorption of B12[76].

Thus, long-term use of PPIs does not lead to vitamin B12 deficiencies, except possibly in the elderly or in persons with ZES who are on high doses of PPI for prolonged periods of time[95,96].

RISK OF OSTOPENIA, OSTEOPOROSIS AND WITH BONE METABOLISM

PPIs alter osteoclastic vacuolar mechanisms which may reduce bone absorption[97], and thereby actually reduce the risk of OP. PPIs have no known adverse effect on vitamin D absorption or metabolism.

What is the link between PPI use and metabolic bone disease? There is a highly variable effect of acid suppression on calcium absorption[98,99]. In persons with achlorhydria due to pernicious anemia, Ca^{2+} absorption is normal or reduced[100,101].

The real question is whether PPI use is associated with an increased risk of osteoporosis/osteopenia, and more importantly with bone fractures. In case controlled studies, PPI use long-term is associated with an increased risk of bone fractures, and this increased risk depends on the duration and dose of chronic use of the PPI[102] (e.g. Manitoba Population Health Research Data Repository[103]). Use of PPI ≥ 5 years can increase the risk of osteoporotic fractures by 1.62-fold (95% CI: 1.02-2.58). Other studies confirm that use of PPI ≥ 7 years increases the risk of osteoporotic hip fractures by 4.55-fold (95% CI: 1.68-12.29) and PPI use for 6-12 mo has been reported to be associated with an increased risk of osteoporotic hip and spine fractures[103-105]. Osteoporotic fractures of the hip and spine may be associated with many factors, which must be carefully taken into account in any case-controlled study which suggests a new association, such as the use of PPIs. However, case control studies on the risk of OP may be criticized on a methodological basis, such as the lack of appropriate stratification of the risk of other

factors known to be associated with an increased risk of OP[106]. The Canadian Association of Gastroenterology (CAG) position paper suggests that "current data would not support particular care in prescribing PPI therapy due to concern about risk of hip fracture"[107].

COMMUNITY ACQUIRED NOSOCOMIAL PNEUMONIA

What is the physiological background to speculating that PPIs might result in pulmonary complications? PPI use is associated with increased intragastric aerobic bacteria, and with the production of acetaldehyde from alcohol[108]. The increased bacterial colonization of the stomach observed with PPI users may be associated with pulmonary micro-aspiration and lung colonization[109,110]. In addition, it is postulated that secretions from the oropharynx may pass by micro-aspiration into the lower lung airways[111]. Furthermore, lung colonization may occur as a result of mechanisms other than micro-aspiration of gastric contents, because different organisms may grow from cultures of gastric juice and from bronchoalveolar lavage[112].

From the clinical perspective, the risk of community acquired pneumonia (CAP) is 0.6 per 100 person years. In persons on PPIs, the odds ratio (OR) is 1.89 (95% CI: 1.36-2.62) for current PPI use and 1.5 (95% CI: 1.3-1.7) for past PPI use (95% CI: 0.9-1.6, and 0.8-1.3, respectively)[113].

In the short-term, PPI use increases the risk of CAP: use of PPI for 2 d, OR = 6.53 (95% CI: 3.95-10.80); for 7 d, OR = 3.79 (95% CI: 2.65-5.42); for 14 d, OR = 3.21 (95% CI: 2.46-4.18); but long-term use of PPIs does not increase the risk of CAP[114], and furthermore meta-analyses have shown that there is no significant association between PPIs and CAP[115].

In contrast, PPIs do not increase the risk of hospital acquired (nosocomial) pneumonia (NP). In fact, there is a reduced risk of NP in patients with nasogastric tubes on a PPI[116]. For ventilated pediatric patients in ICU, there is no increased risk of NP[117-119].

Thus, short-term PPI use increases the risk of CAP, but PPI use does not increase the risk of hospital acquired pneumonia.

CLOSTRIDIUM DIFFICILE-ASSOCIATED PNEUMONIA

There are numerous risk factors for CDAD (use of antibiotics, age, contact with an infected patient or healthcare worker, crowding, lack of cleanliness, post-pyloric tube feeding, patient immunosuppression)[120]. These factors must be taken into account for the attribution of risk, e.g. before assigning a possible role to a new factor, such as PPIs. Some observational studies show an association between PPI use and risk of CDAD[121-132]. For example, for PPI use and CDAD in chronic renal failure patients, the AOR is 5.7 (95% CI: 1.3-39.1) ($P = 0.02$)[133]. In meta-analyses of studies of CDAD and PPIs, the AOR is 1.96 (95% CI: 1.28-3.00). Some of these reports involve a

hypervirulent strain of *C. difficile*, and after correcting for other factors such as antibiotic use, there is no association with PPIs[134]. The bottom line is that there is no convincingly proven data that PPIs increase the risk of CDAD[134,135].

SMALL BOWEL CONTAMINATION SYNDROME AND ENTERIC INFECTIONS

It is thought that PPIs have a minor effect on altering the intestinal bacterial microbiota[136]. Observational studies have suggested that PPIs may[137] or may not[138] increase risk of enteric infections.

Thus, PPIs do not have a convincingly proven adverse effect on the enteric microbiota, and if such an effect does exist, there is no proven clinically important adverse effect[139,140].

The use and subsequent withdrawal of PPIs may be associated with an exaggeration of, or new onset of, acid-related symptoms. PPIs are a medication that is generously prescribed for a variety of symptoms that are thought, and not necessarily confirmed, to be acid-induced. One reason for this is the relatively low number of adverse effects that have been shown in the short- or long-term. One study suggests that symptoms that commence following the discontinuation of PPIs due to rebound acid hypersecretion may be as troublesome as the symptoms that the PPIs were being used to treat in the first place[141]. Because of these rebound symptoms, there may be a need for further and continuous PPI use. As with all medications, the key is to use PPIs only when clearly indicated, and to reassess continued use so that long-term therapy is used judiciously.

The risk of false-negative urea breath tests (UBT) for the diagnosis of an *H. pylori* infection is lower for pantoprazole[142,143]. While it is recommended that acid suppression therapy should be discontinued prior to a UBT, the false-negative effect is lower for pantoprazole.

The biological plausibility is poor for the possibility that PPI use is associated with an increased risk of colorectal cancer or adenomatous polyps, and there is no clinical data to suggest this possibility.

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EDITORIAL

Double balloon enteroscopy and acute pancreatitis

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Abstract

Double balloon enteroscopy (DBE) is a new technique, first published and introduced into clinical practice in 2001 by Yamamoto, the inventor of this outstanding method. DBE allows complete visualization, biopsy and treatment of the small bowel. Nowadays, we have some experience of this method for evaluation of the complication rate. Severe complications are described in 1%-1.7% of patients. Acute pancreatitis is a rare complication of the investigation. The incidence of acute pancreatitis after diagnostic DBE is 0.3% in most studies. More than 50 cases of acute pancreatitis have been described in the literature so far. On the contrary, hyperamylasemia after DBE seems to be a rather common condition. Association with acute pancreatitis is supposed to be possible, but not obligatory. The causal mechanism of post-DBE acute pancreatitis is uncertain, and there are several theories in the literature. The most probable cause seems to be a mechanical straining of the endoscope with over-tube on the pancreas or in the papillary area.

Key words: Double balloon endoscopy; Gastrointestinal endoscopy; Small intestine; Hyperamylasemia; Acute pancreatitis

INTRODUCTION

Double balloon endoscopy (DBE) is a method of enteroscopy that was introduced 8 years ago. Despite 8 years of experience, the complication rate is still under evaluation. Acute pancreatitis is the most feared complication in oral DBE. The cause of acute pancreatitis is uncertain. The aim of this paper is to provide an in-depth overview of possible risk factors for acute pancreatitis in DBE.

HISTORY

The small intestine was inaccessible to endoscopic methods for a long time. Too far from the mouth and the anus, it seemed to be unreachable for the endoscopist. The history of endoscopy investigation of the small bowel is quite short but accompanied by long-lasting skepticism.

In 1999, Mosse and Swain still stated in their work: "Enteroscopy remains the procedure in the gastrointestinal tract that is most inaccessible to endoscopy, and technical limitations severely impair the ability to advance and examine the small bowel reliably or completely"[1].

In 2000, Oates and Morris[2] published in their article: "It is now more than 25 years since small bowel enteroscopy was first described. For several reasons, this technique developed more slowly than other more usual forms of endoscopy. The small bowel disease is relatively rare in comparison with other gastrointestinal diseases. Also, there

was lack of initial design agreement in different types of enteroscopes. Finally, commercial interests of the manufacturers of endoscopes were mainly focused on the more conventional, large volume markets. Problem areas remain, but with advancing technology and more professional interest in this area, these will be addressed during the next few years"[2].

Attempts to observe the entire gastrointestinal tract began even with early fibroscopes, but only two applicable methods were developed in addition to intra-operative enteroscopy: the ropeway method described by Deyhl *et al*[3] and Classen *et al*[4] in 1972 and the sonde endoscope described by Tada *et al*[5] in 1977. The first successful total enteroscopy was performed in March 1971 by Hiratsuka, using the ropeway method[6]. Both methods were soon abandoned due to the complexity of the technique, patient discomfort, and the long time needed to complete the procedure (and the high rate of complications of ropeway enteroscopy).

Push enteroscopy using a long endoscope was regarded as the gold standard then, but most of the small intestine remained beyond its reach. The first procedure was performed by Parker using a colonoscope in 1983[7]. Push enteroscopy can definitely not evaluate the non-resected small bowel in its entire length. Nowadays, push enteroscopy is reserved exclusively for investigation of the duodenum and oral end of the jejunum[8].

Recent innovations and introduction of two new methods (wireless capsule endoscopy and DBE) have made observation of the entire small intestine possible[6]. Both of these techniques are now available in clinical practice and are complementary: capsule endoscopy for screening and DBE for further diagnostics and/or therapy.

Capsule enteroscopy was invented by Swain and initial experiences in 4000 patients were published by Fritscher-Ravens and Swain in 2002[9]. The introduction of DBE for investigation of the small bowel in 2001 was a milestone in gastrointestinal endoscopy because it allows us to carry out therapeutic interventions as well as diagnostic procedures in the small bowel[10,11].

Double balloon (push-and-pull) enteroscopy (Fujinon, Inc., Saitama, Japan) represents an endoscopic method that enables us to investigate a substantial part or even the entire small bowel. The device was developed by Yamamoto and colleagues, introduced by him into clinical practice, and their first experiences were published in 2001[12]. Several subsequent studies[13-18] have suggested that this method is feasible for diagnostic and therapeutic purposes. Nevertheless, DBE is still under evaluation and its yield and safety aspects must be further determined[19-22].

Single balloon enteroscopy (SBE) is a modification of DBE, and is another system for small bowel enteroscopy (Olympus, Tokyo, Japan). The endoscope (XSIF-Q160Y) consists of a high-resolution enteroscope with a working length of 200 cm. The device is equipped with a transparent silicone overtube with a silicone balloon attached to its distal part. In contrast to the DBE device, there is no balloon attached to the enteroscope, and therefore stable position of the device must be maintained by hooking the distal tip of the enteroscope on the small-bowel wall[23].

Balloon-guided enteroscopy (BGE) is another modification of the DBE method. The advantage of this novel push-pull technique is that it is cheaper than other balloon-assisted methods (DBE and SBE). The device can be used with standard endoscopic equipment. The BGE device comprises a double balloon added onto a disposable element and an air supply unit (NavAid ASU; Smart Medical Systems, Ra'anana, Israel) to control the inflation and deflation of the balloons. The disposable BGE element is slipped over the tip of a standard endoscope and then fixed to the endoscope. At the tip of the endoscope is an inflatable stabilizing balloon. During diagnostic or therapeutic interventions through the endoscope's instrument channel, the advancing catheter with its front balloon deflated can be pulled back and "parked" in its dedicated channel in the BGE device[24].

Spiral enteroscopy is a new technique for deep small-bowel intubation that uses a special overtube (Endo-Ease Discovery SB; Spirus Medical, Stoughton, MA, USA) to pleat the small bowel. Any type of enteroscope can be passed through the overtube, which has helical spirals at its distal end and rotates independently from the endoscope. The enteroscope can be locked in the overtube, which allows the option of spiral enteroscopy, or unlocked and advanced through the overtube[25,26]. Now, it is necessary to gain more data on its usefulness and safety and to compare this method with balloon-assisted enteroscopy[27].

Intra-operative enteroscopy is still a useful method for a specific group of patients (in case of failure of DBE, adhesions, multiple small transmural lesions unresolvable by endoscopic methods, such as carcinoids, and blue rubber bleb nevus syndrome), and therefore, it is necessary to be able to use this method in carefully considered cases[28-31].

PARTICULARITY OF DBE

As DBE is a lengthy procedure, a large volume of air is usually insufflated, which leads to significant distension of the small bowel (with the formation of distended bowel loops and acute angulations with increasing amounts of gases intraluminally). CO_2, unlike air, is rapidly absorbed from the bowel. Preliminary data indicate that bowel insufflation with CO_2, instead of air, enhances patient comfort and decreases the need for sedation[23,31]. We have used CO_2 insufflation in DBE procedures regularly from 2007. We had no complications with hyperinflation, and the comfort of the patient rapidly increased. This type of insufflation is helpful for easier and deeper insertion of the endoscope, because the absorption of CO_2 is 150 times faster than absorption of air in the bowel. Indeed, a recent randomized double-blind trial showed that insufflation with CO_2 is safe, reduces patient discomfort, and significantly improves intubation depth[32].

Combination of water with simethicone is used rou-

tinely to do away with bubbles in the intestine. During withdrawal of the endoscope and during therapeutic interventions, spasmolytics might improve visualization of the small-bowel mucosa by reducing motility of the small bowel[23]. We administer intravenous crystalloids, mostly saline solution, to all patients during DBE lasting over 30 min. Conscious sedation is thought to be sufficient for DBE[23]. It seems to be much better in DBE in comparison with general anesthesia, according to our experience. Abdominal pain is an important warning signal, and it is necessary to terminate the procedure immediately in such cases. Intense pain may be a sign of inadequate pressure on the pancreas and poses a high risk of post-DBE pancreatitis[21,22]. We use small intravenous repetitive doses of midazolam and pentazocine for conscious sedation (batchwise). The duration of the procedure and discomfort for the patient caused by oral passage of the overtube requires deep analgosedation. The procedure requires an experienced endoscopist and the possibility of fluoroscopy if needed, especially during the learning period[33].

COMPLICATIONS

DBE has been reported as a safe endoscopic technique. In initial series on DBE, no complications during or after the procedure were reported[34-37]. Recently, the overall complication rate is stated as being about 1.7%[38,39].

A complication of endoscopy is defined as any event that negatively changes the health status of the patient, and that occurs during the 30-d period after the investigation. Complications are usually categorized as minor when requiring up to 3 d of hospitalization, moderate when requiring 3-10 d and major or severe when requiring > 10 d, and/or an endoscopic, radiological or surgical intervention, and/or contribute to the death of the patient[38,40]. Procedure-related mortality is defined as mortality within 30 d of DBE[38]. Complications are divided into two main categories, those directly attributed to the procedure and those secondary to anesthesia or conscious sedation[23]. The most common complications secondary to anesthesia or conscious sedation are respiratory depression, aspiration, and pneumonia, with a frequency of < 1%[23].

Until now, no standards or definitions for complications during or after DBE have been established. Potential complications during or after DBE might be: perforation, bleeding, balloon dislocation, sedation-related, segmental enteritis after argon plasma coagulation[41], intestinal necrosis after epinephrine injection[42], paralytic intestinal ileus[43], and acute parotitis[44].

Recently, post-DBE pancreatitis has been recognized as a complication[39,45]. In diagnostic procedures *via* the anterograde approach, pancreatitis is the most common and most severe complication[23]. The very first post-DBE acute pancreatitis was reported by Honda *et al*[46] in 2006.

An international symposium held in Atlanta, GA, USA in 1992 has established a clinically based classification system for acute pancreatitis[47,48]. The goal was to establish international standards for definition of acute pancreatitis and its complications, to facilitate valid comparisons of severity of illness and results of therapy, and also to establish criteria for patient selection in randomized prospective trials. According to the Atlanta symposium, acute pancreatitis is defined as an acute inflammatory process of the pancreas that may also involve peripancreatic tissues and/or remote organ systems. Mild acute pancreatitis is defined as pancreatitis associated with minimal organ dysfunction and uneventful recovery. Severe pancreatitis is defined as pancreatitis associated with organ failure and/or local complications (necrosis, abscess, or pseudocyst). Criteria for severity included organ failure (particularly shock, pulmonary insufficiency, and renal failure) and/or local complications (especially pancreatic necrosis but also including abscess and pseudocyst). Early predictors of severity within 48 h of initial hospitalization included Ranson signs and APACHE Ⅱ (Acute Physiology and Chronic Health Evaluation Ⅱ) points[47-49].

In the Atlanta symposium, a uniform threshold was not established for serum amylase and/or lipase for the diagnosis of acute pancreatitis. In recently published articles, the threshold varies from ≥ 2 to ≥ 4 times the upper limit of normal. Criteria for severe pancreatitis include organ failure and/or local complications. This broad definition describes a heterogeneous group of patients with varying levels of severity. For example, the prognosis of pancreatic necrosis is more serious than a pseudocyst or pancreatic abscess. Also, almost all patients with necrotizing pancreatitis without organ failure survive, whereas those with multisystem organ failure do not[49].

Bollen *et al*[50] have revised the Atlanta symposium in their review. The authors propose the following recommendations for revision of the classification of acute pancreatitis. (1) The diagnosis should incorporate two of the following three items: upper abdominal pain, amylase and/or lipase levels ≥ 3 times the upper limit of normal (as this cut-off is used most frequently in the literature), and computed tomography (CT) or magnetic resonance imaging findings compatible with acute pancreatitis; (2) Persistent organ failure (for at least 48 h) should have an important role in defining the severity of acute pancreatitis; and (3) Decisions should be made as to which predictive scoring system, including cut-off value, should be used to define predicted severe acute pancreatitis, based on a systematic review of the available data.

Progress in the field of acute pancreatitis is hampered greatly when various author groups use their own idiosyncratic definitions[50].

According to the literature on post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis [American Society for Gastrointestinal Endoscopy (ASGE) guidelines], post-DBE pancreatitis is defined as newly developed or worsened abdominal pain after the procedure, with a serum amylase ≥ 3 times the upper limit of normal as the upper limit 24 h after the procedure and requiring at least 2 d of unplanned hospitalization after the procedure[51]. According to these guidelines, the severity of the disease has been classified as follows: mild, requiring 2-3 d

hospitalization; moderate, 4-10 d hospitalization; and severe, > 10 d hospitalization, and/or the occurrence of pseudocyst and/or the need for surgery[52]. The duration of pain after the procedure is crucial for defining post-endoscopy pancreatitis[53].

We found the definition from UpToDate 2009 to be fundamental. Acute pancreatitis is an acute inflammatory process of the pancreas. It is usually associated with severe acute upper abdominal pain and elevated blood levels of pancreatic enzymes. Acute pancreatitis can be suspected clinically, but requires biochemical, radiologic, and sometimes histological evidence to confirm the diagnosis. Clinical, biochemical, and radiologic features need to be considered together since none of them alone is diagnostic of acute pancreatitis. Acute pancreatitis is an important cause of acute upper abdominal pain. Because its clinical features are similar to a number of other acute illnesses, it is difficult to base a diagnosis only on symptoms and signs. The disease varies in severity and the diagnosis is often missed at the extreme ends of the spectrum[54].

It is not usually difficult to recognize severe pancreatitis. The mild form may pose a problem. Abdominal pain and hyperamylasemia after DBE need not mean pancreatitis. On the other hand, a lot of mild pancreatitis could be missed in patients with DBE performed on an outpatient basis.

The complication rate of diagnostic procedures is low (0.4%-0.8%) according to the literature[10,11,38,45]. The overall complication rate of therapeutic DBE is 3%-4%. However, difficult therapeutic endoscopic procedures (e.g. resection of large polyps) may increase the risk to 10%[10,11,23,38]. The perforation rate is significantly elevated in patients with postsurgical anatomical changes undergoing diagnostic retrograde DBE examinations[55].

The overall complication rate was reported to be about 1.7% in a recent international multicenter survey of 2362 DBE procedures. The complications were rated minor in 0.9%, moderate in 0.3% and severe in 0.6% of procedures. The complication rate is significantly higher in therapeutic procedures in comparison with diagnostic ones (4.3% *vs* 0.8%). An exception to the rule is acute pancreatitis, the most common complication in diagnostic DBE procedures. Acute pancreatitis was reported in 0.3% of DBEs[38,39].

A recent report from the National German DBE Register showed an overall complication rate of 1.2% in a large series of 3894 DBE procedures. The incidence of acute pancreatitis was also 0.3% in that study[11].

A study by May *et al*[41] evaluated the acute complication rate of DBE in 353 patients. Only therapeutic procedures were evaluated, with a complication rate of 3.4%. No acute pancreatitis was reported[41].

In general, DBE is associated with a higher complication rate compared with standard endoscopic procedures.

OUR OWN EXPERIENCE

We began with DBE in our department in March 2006.

All of our investigations were performed using EN-450T5 and EN-450P5 endoscopes (Fujinon, Inc., Saitama, Japan). Being aware of the possibility of acute pancreatitis as a complication of DBE, we started with our prospective study on hyperamylasemia after DBE right from the start of DBE in 2006. This prospective study is still continuing. All patients were admitted to our department and followed prospectively for at least 2 d after oral DBE.

Urine and serum amylase, serum pancreatic lipase and C-reactive protein (CRP) were investigated before and 4 h and 24 h after DBE. Abdominal pain was evaluated using a three-step scale. Hyperamylasemia (exceeding the upper normal limit) and marked hyperamylasemia (reaching $\geqslant$ 3 times the upper limit of normal) were distinguished.

Normal ranges of the following values in our laboratory were: serum amylase 0.44-1.67 μkat/L (i.e. 28-100 U/L); urine amylase 0-7.67 μkat/L (i.e. 0-460 U/L); serum lipase 0.22-1 μkat/L (i.e. 13-60 U/L); and serum CRP 0-5 mg/L.

Acute pancreatitis was diagnosed in accordance with clinical signs (abdominal pain, fluid sequestration and lack of peristalsis), CRP level and CT scan in addition to the above.

Risk factors for acute pancreatitis and the importance of hyperamylasemia associated with DBE have not been satisfactorily resolved yet. The aim of our prospective study was to clarify the relationship between oral DBE and amylasemia and lipasemia, and to address the possible role of the learning curve for DBE for risk of acute pancreatitis.

A total of 138 DBEs were carried out from March 2006 to November 2009 in 60 men and 56 women under deep conscious sedation (midazolam and pentazocine). The mean time of DBE was 110 min (range 12-270 min), and the mean number of push-and-pull cycles was 15 (range 1-47).

Amylase was set after 128 DBEs; elevation was found in 61 (48%), and marked hyperamylasemia in 27/128 (21%). Abdominal pain was recorded in 19/96 (20%). Elevated lipase levels were found in 55/94 (59%); including 38/94 (40%) with $\geqslant$ 3 times the upper limit of normal. We observed elevation of CRP after DBE in only 18/100 (18%). Peak values of serum amylase and lipase levels were found 4 h after DBE, and peak values of CRP at 24 h after the procedure.

Total panenteroscopy (i.e. investigation of the entire small intestine by one oral approach in one session) was accomplished in 12 DBEs (9%). In this subgroup, we found only four patients with hyperamylasemia and two with marked hyperamylasemia. We had no complications in this subgroup. Compared to the total number of patients, in this subgroup, there was a younger mean age and longer duration of DBE (mean 148 min *vs* 106 min; $P = 0.010$), but there was no acute pancreatitis and no significant difference in amylase and lipase and/or abdominal pain.

In all DBEs, we did not identify any risk factor for abdominal pain and/or elevated pancreatic amylases (sex, age, previous abdominal surgery, panenteroscopy, indica-

tion or endoscopic finding, type of endoscope, number of push-and-pull procedures, diagnostic or therapeutic procedure).

Three patients (2.1%) developed acute pancreatitis after DBE, one necrotizing and two edematous. Two had inflammatory affection of the pancreatic tail, the third of the head region. All of them had abdominal tenderness during the procedure.

Subsequently, we divided our patients into two groups (the first 69 and second 69 procedures) to assess possible influence of the learning curve. Diagnostic and therapeutic DBEs (using Fujinon EN 450P5 or 450T5) were proportionally included in both groups. Neither group differed in age, sex, diagnoses or previous surgery. Differences in amylase and lipase (4 or 24 h after minus basal values before DBE) were used as a major indicator.

The difference in abdominal pain, DBE duration, amylase or lipase was not significant in either of the two groups. Marked hyperamylasemia ($\geq$ 3 times above the upper limit; in 21% after DBE) was not associated with marked pain. There was a weak but significant correlation between amylase difference and abdominal pain ($P = 0.003$), the number of push-and-pull cycles ($P = 0.018$), and negative correlation with age ($P = 0.029$). Lipase difference 4 h after DBE weakly correlated with abdominal pain ($P = 0.034$). Our three cases of acute pancreatitis were numbers 24, 50 and 57 of 138 DBE procedures, without any evidence of further consequences. DBE lasted 12-270 min (mean: 110 min). Shorter ($\leq$ 120 min, 64%) and longer (> 120 min; 50/138, 36%) DBEs did not differ in terms of post-DBE abdominal pain ($P = 0.784$). There was a borderline significant difference between longer and shorter DBEs for amylase ($P = 0.047$) but not lipase ($P = 0.225$) differences 4 h after DBE.

Elevation of amylase and lipase is often associated with DBE, but acute pancreatitis is however a rare complication. Duration of DBE is not a risk factor for post-DBE acute pancreatitis. Abdominal pain during DBE should be considered as a possible risk factor for acute pancreatitis. That is why we prefer conscious sedation to general anesthesia in oral DBE. The initial learning curve for DBE is not associated with higher amylase or lipase in our setting, and it does not signify a risk factor for post-DBE acute pancreatitis[21,22,56]. The most important point of our study was that it was prospective, solely on an inpatient basis, and all consecutive patients who underwent oral DBE were included.

CLINICAL OUTCOMES

Our center has long-term experience (since 1994) with push-enteroscopy[57] and intraoperative enteroscopy[28-31]. We have never registered acute pancreatitis as a complication of either push-enteroscopy or intraoperative enteroscopy in our setting. However, acute pancreatitis as a complication of push-enteroscopy, caused by an overtube, has been described previously by other authors[58]. Acute pancreatitis has even been described after uneventful upper and lower gastrointestinal endoscopy[59-61]. Blackwood *et al*[62] have detected asymptomatic hyperamylasuria in 6.6% of patients undergoing gastrointestinal endoscopy[62].

Pelletier *et al*[63] have studied the prevalence of hyperamylasemia 2 and 24 h after upper gastrointestinal endoscopy in 50 consecutive patients. In the 2-h sample, hyperamylasemia was observed in nine patients (18%), and in the 24-h sample, in five patients. Pelletier *et al*[63] have concluded that the cause of hyperamylasemia may be due to hypersalivation during the procedure. In our opinion, hypersalivation cannot affect the serum amylase level in such a way (most of the saliva runs out of the mouth during endoscopy and is not swallowed). Furthermore, it cannot affect abdominal pain or pancreatic lipase elevation[21,22,56].

DBE was initially described as a safe procedure[6], with the rate of severe complications ranging from 0 to 1.4%[14,18,64]. However, abdominal pain lasting 1-2 d occurred in 9% of patients in one study[13], or even in 20% according to another[14]. Abdominal discomfort reducing within 72 h was reported in three out of six patients after a DBE procedure[65].

There have been 51 published cases of post-DBE acute pancreatitis on PubMed to date.

Eisen and Schreiner[45] have presented a study of 275 consecutive patients who underwent DBE at two tertiary referral hospitals. The most common complication of DBE was abdominal pain which was seen in 20% of cases. This was typically self-limiting, yet a systematic analysis was not performed. Three cases of pancreatitis occurred (1%), two of which were mild and one of which was of intermediate severity[45]. It is hard to say how many patients with self-limited abdominal post-DBE pain had mild acute pancreatitis, because of inadequate follow-up.

Honda *et al*[46,66] described one and Groenen *et al*[67] two (one necrotizing) cases of acute pancreatitis after DBE. Heine *et al*[14] and Jarbandhan *et al*[52] from the same Dutch group have studied 603 DBE procedures (441 oral DBEs) on an outpatient basis, with six cases of post-DBE acute pancreatitis (1% of all DBEs, i.e. 1.4% of oral DBEs); all cases of pancreatitis were diagnosed after oral procedures. None of the cases of pancreatitis were in the head of the pancreas[52]. In a retrospective analysis of 378 DBEs by Zhong *et al*[68], two patients (0.7%) suffered from abdominal pain with an unspecified elevation of serum amylase. Unfortunately, it was not stated if they required hospitalization[68]. Möschler *et al*[10,11], in a German multicenter retrospective study, have reported 3894 DBEs with an overall complication rate of 1.2%. They quoted nine cases of acute pancreatitis with one lethal disease course after oral DBE, with a complication rate of 0.34%[10,11]. A retrospective study by Gerson *et al*[55] in nine United States centers collected data from 2478 DBEs, with a total of 0.9% major complications (22 DBEs), among which there were six cases of acute pancreatitis (0.2%). Surprisingly, one case of acute pancreatitis was reported after an anal DBE procedure[55]. Another multicenter study by Mensink *et al*[38] investigated 2362 DBEs. The majority of these (87%) were performed on an inpatient basis. The overall complication rate was 1.7%;

Figure 1 DBE, panenteroscopy *via* oral approach (plain radiograph) in supine position. In comparison with the normal situation (A, white outline), the duodenum is shifted markedly to the left, straightened and shortened. A-C: Normal anatomy; D: After B Ⅱ resection.

six patients suffered from acute pancreatitis (0.3%). The location of the pancreatitis was the body and/or the tail of the pancreas in four patients, the entire pancreas in two, and the head of the pancreas in one[38]. One case of post-DBE acute pancreatitis that affected the pancreatic tail has been reported at the Mayo Clinic (Rochester, MN, USA)[69], along with another case from Osaka, Japan[70], and Rotterdam (one case of pancreatitis of unknown location from 135 patients, 0.7%)[71]. In a recent study by Sunada and Yamamoto, four cases of acute pancreatitis in 1092 DBEs (0.37%) were diagnosed, although the severity and location were not mentioned[72].

In agreement with the Dutch study, we believe that post-DBE pancreatitis is underestimated in retrospective studies on an outpatient basis. In particular, retrospective questionnaire-based surveys might be at risk from an inaccurate report or inclusion bias[52]. As the distinction between clinically mild pancreatitis and hyperamylasemia with transient abdominal discomfort is somewhat arbitrary, it seems likely that an under-diagnosis of post-DBE pancreatitis might have occurred, especially in outpatients.

There was no association with sex, duration of the procedure, or type of endoscope in most of the studies mentioned above.

Surprising results have been reported by Pata *et al*[73] in their study of 48 oral DBE procedures. Pancreatitis was observed in six patients (12.5%). Acute pancreatitis was diagnosed when amylase and/or lipase reached ≥ 3 the upper limit of normal in the presence of pancreatic-type abdominal pain[73]. The question is whether the diagnostic criteria of acute pancreatitis are sufficient in the case of post-DBE pancreatitis. Asymptomatic hyperamylasemia may occur in nearly half of DBE procedures[21,22,27,56,66,74]. Honda *et al*[66] have investigated 13 patients who underwent DBE. Hyperamylasemia occurred in six of them after the procedure, and one of these developed acute pancreatitis. In agreement with our results, this study demonstrates that latent hyperamylasemia without the development of pancreatitis occurs after peroral DBE more frequently than was previously thought[66]. It may be that some other criteria for the diagnosis of post-DBE acute

pancreatitis should be added: ultrasound and/or CT scanning, peristalsis weakening, or compartment sequestration syndrome. Time will tell if the significance of post-DBE and post-ERCP enzyme elevation is absolutely comparable. Pata *et al*[73] have verified only two reported cases of pancreatitis using CT.

The causal mechanism of post-DBE acute pancreatitis is uncertain, and there are several hypotheses. Nevins assumes local trauma to the pancreas during the procedure or release of as-yet-undefined inflammatory mediators[59]. May *et al*[75] assume that the length of the examination time is an important factor, because they did not observe pancreatitis in more than 500 DBEs and had a strict maximum examination time limit of 2 h[75]. However, data from other authors (including our own data) do not support this assumption. In our study, in agreement with other studies[66,70], we found hyperamylasemia and pancreatitis in patients with shorter procedures, and we had fewer patients with hyperamylasemia and no case of pancreatitis in the subgroup of 12 patients with panenteroscopy *via* an oral approach (mean time: 148 min)[21,22,56]. We therefore believe, like Honda *et al*[76], that it is the technique of DBE itself, with the shortening of the small bowel, that may be a factor in pancreatitis after peroral DBE, rather than the length of the examination time (Figure 1)[21,22,56].

Honda *et al*[66] think that, in peroral DBE, the duodenum and proximal jejunum are markedly shortened, and the duodenum is sometimes found to be nearly straight from the pyloric ring to the ligament of Treitz on fluoroscopy. In these conditions, the pancreas body and/or tail may be subject to severe strain, with traumatic injury and/or ischemia. Intraluminal pressure in the duodenum may increase in such a way as to disturb the secretion of pancreatic juice. This mechanism might be associated with the occurrence of hyperamylasemia and pancreatitis after peroral DBE[66]. This hypothesis is supported by the finding that, in most cases in which early CT was performed, pancreatitis was located in the tail or body-tail region of the pancreas[52].

Heine *et al*[14] state that pancreatitis probably results from prolonged mechanical stress on the organ due to re-

peated stretching by the endoscope[14]. Sunada and Yamamoto suggest that the possible mechanism is mechanical torsion of the pancreatic body during insertion of the endoscope. Therefore, for oral insertion, extreme shortening should not be performed, and counter-clockwise rotation is preferred[72].

Another possibility is ischemia of the pancreas, which could induce acute pancreatitis. This has been confirmed in an animal model[77].

Groenen *et al*[67] hypothesize that acute pancreatitis is caused by an increase in intraluminal duodenal pressure during the endoscopic procedure caused by inflation of the two balloons, which leads to reflux of duodenal fluids into the pancreatic duct[67]. A closed duodenal loop has indeed become an established animal model for acute pancreatitis[78,79]. However, the question is whether the duodenal intraluminal pressure during DBE really can reach such a high level. On the other hand, the pancreatitis caused by reflux should affect the whole pancreas, with diffuse swelling, not only in the body or pancreatic tail.

Some endoscopists prefer to inflate the balloons after passing the ligament of Treitz[52,71], or after two insertions (being at least 50 cm distal from the papilla of Vater)[39]. Unfortunately, precise description of this technique and its control (by fluoroscopy?) was not incorporated in their work. Frankly, it is difficult to believe that by pushing the endoscope with the overtube, without pulling back and straightening out the looping, they were able to pass the ligament with a 129-cm long device (the balloon on the tip is 7 cm long and the rest of working part of the overtube is 129 cm). On the other hand, the papilla is about 80-90 cm from the incisors when inserting the endoscope, therefore, both balloons are probably beyond the papilla of Vater. However, even with this safe technique of endoscope insertion, the Dutch group had a post-DBE acute pancreatitis rate of 1.4%, using the oral approach[52].

Other possible causes are direct trauma to the ampullary area or a direct obstruction of the pancreatic duct by the insufflated balloon[67]. In our opinion, this hypothesis seems also to be unlikely.

Another consideration is the question of whether the elevation of amylase is always a result of pancreatitis or whether it could be of intestinal origin and related to manipulation of the gut[80,81]. However, this mechanism could hardly explain the elevation of pancreatic lipase.

The method of insertion and withdrawal of the endoscope might be a factor in the origin of post-DBE pancreatitis. However, in accordance with the literature[52], we did not find any differences in technique between our center and others that are performing DBE in Europe, and we did not see any differences even observing the masterful work of Professor Yamamoto during his live workshops at our endoscopy unit.

Differences in definitions of post-procedural complications offer a likely explanation for the difference in reported post-DBE pancreatitis[52].

In our opinion, mechanical stress on the pancreas seems possible, and the increased level of pancreatic li-

pase could be a correlate of this. We considered a possible influence of our learning curve on the incidence of hyperamylasemia in our patients, but we did not confirm this by subsequent analysis. The second point of our reflection was the type of endoscope. We used the thicker and stiffer EN-450T5 endoscope, so we were afraid of more forceful pressing of this device onto the pancreas. However, three cases of acute pancreatitis after peroral DBE using the EN-450P5 have been reported in the Netherlands[14] and one after DBE using the EN-450T5[66], and there were no differences in the type of endoscope in large studies[38].

Another question is prevention of post-DBE pancreatitis. We use parenteral hydration during after the oral procedure. The usual dose is 1 L of saline solution during a 2-h procedure. We believe that hydration could improve blood supply to the splanchnic region, pancreatic microcirculation, and post-procedure recovery. The use of proteinase inhibitors such as gabexate mesylate for the prevention of post-endoscopic pancreatitis has been disappointing[53,82]. There have been some studies of intravenous nitroglycerin[83], ulinastatin[84], somatostatin[53,82,85,86], rectal diclofenac[84], and other drugs for the prevention of post-procedure pancreatitis, but the results have not been significant.

CONCLUSION

Acute pancreatitis is a feared complication of oral DBE (51 cases of acute pancreatitis have been described in the literature to date, one of them fatal). Acute pancreatitis is the most common complication seen after diagnostic oral DBE (complications of therapy itself prevail in therapeutic procedures). Hyperamylasemia and elevation of pancreatic lipase after DBE seems to be a common occurrence. Association with acute pancreatitis is possible, but not obligatory. The diagnosis of acute pancreatitis is complex. It can be suspected clinically, but requires biochemical, radiological, and sometimes histological evidence to confirm the diagnosis. The complication rate of acute pancreatitis is reported at about 0.3% of DBEs according to large studies, almost solely after oral DBE. Drawbacks and possible bias of those studies are that they were mostly retrospective, a substantial number of DBEs were performed on an outpatient basis, and the follow-up was inadequate. Nowadays, it is clear that the oral DBE procedure is of higher risk in comparison with the anal one. It would be more precise to count the pancreatitis risk from oral procedures separately. By including anal procedures in determination of post-DBE pancreatitis rate, we obtain much lower and biased numbers. The presumable number of cases of acute pancreatitis after oral DBE is 1.5%-2%. In all patients with abdominal pain during the procedure and/or after oral DBE, diagnosis of acute pancreatitis should be considered and treatment should be provided in good time, the same as in post-ERCP pancreatitis. From the results of our study, we established the following rules in our clinical practice. Conscious sedation seems to be more favorable than general anesthesia due to monitor-

ing of the patient's pain during the procedure. Intense pain during the procedure may be a sign of inadequate pressure on the pancreas and pose a high risk of post-DBE pancreatitis. CO_2 insufflation during DBE is highly recommended as it prevents over-inflation of the small bowel, however, a possible preventive relationship to post-DBE pancreatitis has not been determined yet.

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REVIEW

Progress in laparoscopic anatomy research: A review of the Chinese literature

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Abstract

The development of laparoscopic surgery has generated the new field of study, laparoscopic anatomy. This article reviews the reported literature on laparoscopic anatomy and explores how it has evolved along with advances in abdominal surgery. In addition, the principal concerns in current laparoscopic anatomy research are discussed, including: (1) types of special adjacent anatomical structures; and (2) special surgical planes and anatomical landmarks. Understanding of systematic laparoscopic anatomy can provide the junior surgeons a clear procedural approach, and would benefit laparoscopic surgeons in training.

INTRODUCTION

The current surgery relies heavily on the use of laparoscopy due to its minimally invasive nature. In the field of abdominal surgery, the traditional approach of open surgery and related surgical anatomy are well established, while laparoscopic surgery and correlative laparoscopic anatomy are still under study.

Since laparoscopic surgery produces less trauma, and yields rapid recovery and superior cosmetic effects, it has become increasingly popular among both patients and physicians. However, laparoscopic surgery is not yet advanced enough to overcome its disadvantage of the lack of tactile sense. This impedes the sensitivity of applied laparoscopy to define the important anatomic features, structure locations, and boundary and texture of excision target tissues. Hand-assisted laparoscopy was developed to give surgeons more control and sensitivity while using laparoscopic instrumentation and digital visualization tools, which has been applied widely in abdominal surgery. Unfortunately, the lap-disc sealing and access device is costly and not extensively used in operations.

A second compelling disadvantage in the laparoscopic procedure is the loss of hand flexibility of the surgeon due to limited availability of space in the patient's body, thus increasing the technical difficulty of the procedure.

However, a systematical knowledge of laparoscopic anatomy can help surgeons to address this challenge. Clarified anatomical structures in the high-resolution and high-magnified view are of great significance in miniscule anatomical procedures, with little bleeding in the laparoscopic field of sight in the correct surgical plane while dissection was performed. Thus, gaining a thorough understanding of the laparoscopic anatomy is crucial to a successful laparoscopic surgery.

In order to provide guidance for laparoscopic surgeons in anatomy and technique, we have reviewed and summarized the current laparoscopic anatomical studies on abdominal surgery published in the literature. Although there are variations in anatomy among individual patients and in procedures used among surgeons, many commonalities exist and can be generalized to aid in the application of laparoscopy.

Systematic literature search was performed on PubMed, Medline, ScienceDirect, SpringerLink, CMCI, VIP, and CNKL to find relevant articles using the terms "laparoscopy", "anatomy", and "minimally invasive". Papers both in English and Chinese concerning anatomical characteristics in laparoscopic procedures in the fields of abdominal surgery were identified. The search results were classified as: (1) anatomical descriptions; (2) anatomical variations; (3) surgical planes and landmarks; and (4) measurements of certain structures. Papers dealing with different types of laparoscopic procedures were summarized according to the above classifications to identify the key points of each procedure.

LAPAROSCOPIC CHOLECYSTECTOMY

Studies on laparoscopic anatomy of laparoscopic cholecystectomy (LC) mainly focused on Calot's triangle, including the cystic artery and cystic duct[1,2]. The cystic artery normally has 1-3 branches into the gallbladder, originates from the proper hepatic artery, and is located within Calot's triangle. The variation of the common structure included origination from the right hepatic artery (Figure 1A), or from gallbladder bed of an aberrant type. The cystic duct is normally connected directly to the right wall of the hepatic duct; variations included connection to the left wall of the hepatic duct through an anterior or posterior approach (Figure 1B). Other structural differences reported in the literature were as follows: cystic duct location in parallel with the common hepatic duct (Figure 1C), presentation as an aberrant bile duct, adhesion and atresia, and an even connection to the right hepatic duct (Figure 1D).

Anatomical landmarks in the LC were mainly reported as Rouviere's sulcus, cystic lymph nodes and arteries[3]. The Rouviere's sulcus is a useful demarcation for the division between the S5 and S6 of the liver. When the gallbladder ampulla is stretched to the head side, the Rouviere's sulcus can be exposed. The Rouviere's sulcus was reported as an accurate landmark for the common hepatic duct plane, as they are on the same transversal level, above which the dissection of Calot's triangle is safe. When the fascial strip in Calot's triangle was flattened, the cystic artery could be identified by its visible pulsation and the presence of an adjacent, cystic lymph node. The cystic artery has been reported to run through the level of the gallbladder neck; exposure of the cystic artery may help surgeons locate the gallbladder neck at the same level. In addition, identification of the cystic lymph node may help define the cystic duct and the cystic artery structures (Figure 1E).

The key concerns in LC anatomy research are how to protect the common bile duct and right hepatic duct, which are injury-prone during the laparoscopic procedure. It is necessary to clarify the anatomy of Calot's triangle and ensure that the superior Rouviere's sulcus is on a safe surgical plane.

LAPAROSCOPIC INGUINAL HERNIORRHAPHY

Studies on the laparoscopic anatomy of herniorrhaphy focused primarily on inguinal hernias. Currently, there are two main approaches for laparoscopic inguinal herniorrhaphy (LIH), i.e. the transabdominal pre-peritoneal and the totally extra-peritoneal. Using laparoscopy, the presence of a direct hernia, indirect hernia or femorocele is readily found according to the position of the hernial ring, as shown in Figure 2A. There are marked differences in anatomical structure between oblique hernia and direct hernia. Thus, the size of mesh used in the operation should be decided according to each individual patient's anatomic data[4].

In the reported LIH procedures, meshes were always secured by either staples or sutures. Three potentially hazardous regions (Figure 2B) were identified and were seriously considered. (1) The so-called triangle of doom, wherein the medial margin is the deferent duct and the lateral margin contains the funicular vessels, should not be subject to staples or sutures because the iliac artery resides in this region; (2) An unexpected source of hemorrhage is identified as the corona mortis (translated as the crown of death), wherein lies the aberrant obturator artery and vein. Branches and distribution of the vascular connection between the obturator system and the external iliac or inferior epigastric systems, are located over the superior pubic ramus[5]; and (3) The triangle of pain, wherein the superior margin is the iliopubic tract (inguinal ligament), and the medial margin is the funicular vessel, is the region where the femoral branch of the genitofemoral nerve and the lateral femoral cutaneous nerve reside. The occurrence of post-operative pain was reported to have originated from staples or sutures placed in this region. It is reported that 15% of the lateral femoral cutaneous nerve was within 0.5 cm of the iliopubic tract or in the vertical plane of the anterior superior iliac spine[6]. To avoid post-operative pain, some surgeons opted against all use of the stapler.

Methods for distinguishing these important structures

Figure 1 Anatomy of laparoscopic cholecystectomy (LC) (blue curves stand for bile duct and gallbladder and red curves for arteries). A: Illustration of a variant cystic artery originating from the right hepatic artery; B: Cystic duct connected to the left wall of hepatic duct *via* an anterior approach; C: Cystic duct parallel with common hepatic duct; D: Cystic duct connected to the right hepatic duct; E: Calot's triangle and Rouviere sulcus in LC.

Figure 2 Illustration of inguinal hernia structures. A: Direct hernia was located medially to the inferior epigastric artery, an indirect hernia was located laterally to the inferior epigastric artery, and a femoral hernia was located at the inferior iliopubic tract; B: The three regions, where staples or sutures should not be placed: the triangle of doom, triangle of pain, and corona mortis; C: Live procedure after the peritoneum was absolutely dissected and removed from the abdominal wall.

are a main focus throughout the laparoscopic anatomy literature[7-9]. In live procedures, the structures could be totally exposed after the peritoneum was completely removed from the abdominal wall, as shown in Figure 2C.

The obvious hazards in LIH are the potential for injury of the deferent duct and unexpected hemorrhea. The major technique to protect the deferent duct in this procedure is skeletonization of the spermatic cord. In a nutshell, surgeons should take heed of one "cord", one "crown" and two "triangles".

LAPAROSCOPIC COLORECTAL RESECTION

Studies on the laparoscopic anatomy of colonic resection have focused on the left colon and rectum. Locating the root of the inferior mesenteric artery (IMA) and protecting the autonomic nerve and ureter are of impor-

tance in total mesorectal excision. The reports identified a particular landmark and a surgical plane.

IMA is located between the horizontal portion of the duodenum and the bifurcation of the abdominal aorta; the pulsation of the aorta made the identification easy. The landmark used to find the root of IMA was the convex stalk on the surface of the abdominal aorta. The superior sigmoid colon mesentery was lifted to the left side, straightening out the IMA to form an included angle, and the margins were defined as the abdominal aorta and the root of IMA[10] (Figure 3).

The important surgical plane that is yet to be defined is known as Toldt's space. During embryonic development, the mid-gut mesentery after succeeding in the volvulus combined with the peritoneal wall layer to form Toldt's fascia. Toldt's space appeared and remained consistently between the lateral left mesocolon and pre-renal fascia, as well as between the medial left mesocolon and

Figure 3 The root of inferior mesenteric artery. The inferior mesenteric artery and vein were identified when the superior sigmoid colon mesentery was lifted to the left side. Toldt's space was found in the included angle of the inferior mesenteric artery and abdominal aorta.

Figure 4 Anatomy of LRG. A: Dissection in the first surgical plane, i.e. the space between anterior and posterior lobes of the transverse mesocolon; B: The main vessels in the space of the posterior layer of dorsal mesogastrium and pancreas when the paries posterior gastricus was turned over to the head side.

pre-aortic fascia. The space also appeared between the mesorectum and parietal layer of the pelvic fascia.

On the medial side, Toldt's space in the angle of the abdominal aorta and IMA was a vessel free area (Figure 3). IMA ran along the surface of the cephalic abdominal aorta, down to the pelvic cavity and pre-sacral space[11,12]. On the lateral side, an obvious yellow-white borderline was visible. The yellow portion is a part of the sigmoid mesocolon, and the white is a lateral abdominal wall. Dissecting from the medial and lateral space frees the colorectal mesentery completely. The surgical planes are between the colorectal mesenteries and the continuous pre-renal fascia.

Dissecting in the Toldt's space might protect autonomic nerves and the ureter because they are all located beneath the pre-renal fascia. The superior hypogastric plexus closely associates with the back of the IMA. No obvious branches of autonomic nerves were found in the loose space between the visceral layer fascia (mesorectum) and the wall layer fascia (parietal pelvic fascia) at the back of the rectum[13].

In summary, the root of IMA and Toldt's space, which guided most total dissections of lymph nodes and protected the autonomic nerve, appear to be the most important anatomic structures in laparoscopic colorectal resection.

LAPAROSCOPIC GASTRECTOMY

Laparoscopy was often used to treat benign gastric diseases during the early stages. Recently, laparoscopic surgery has been increasingly used in radical gastrectomy, and more researches in laparoscopic anatomy have appeared[14,15], focusing on identifying crucial surgical planes and anatomical landmarks.

In LRG, there appeared to be two surgical planes that were important to define. The first was found between the posterior layer of the dorsal mesogastrium and the pancreas. The derivation of the posterior layer of the dorsal mesogastrium formed the gastrosplenic ligament, pancreatic fascia, pancreaticoduodenal fascia and the an-

terior lobe of the transverse mesocolon, which was continuous with each other as integrity. The surgical plane could be fixed in the space between the anterior and posterior lobe of the transverse mesocolon (Figure 4A). The plane could be reached more easily for dissection from the shortest distance of connecting fascia from the transverse colon to the descending part of the duodenum. The second surgical plane was identified in the space between the retropancreatic and pre-renal fascia, a safe plane for retropancreatic lymph node dissection. The pre-renal fascia was the post-boundary for ensuring a safe operational plane.

Because of restricted laparoscopic sight and instrument manipulability, the procedure was performed from the caudal end to head. Therefore, the main vessels were identified in the space of the first surgical plane mentioned above. The paries posterior gastricus was gradually turned over to the head side following the opening of the first surgical plane, where each important vessel could be identified with the guidance of the following landmarks (Figure 4B). The pancreas was the largest central landmark. When dissecting in the gastropancreatic ligament near the pancreas head, the gastroduodenal artery and right gastroepiploic artery could be identified. Likewise, when dissecting in the splenocolic ligament near the pancreas tail, the left gastroepiploic vessels could be exposed. After the pre-pancreas fascia was absolutely dissociated, two folds were formed by the pre-pancreas fascia and its related vessels. The hepatopancreatic fold was the landmark used to find the common hepatic artery and proper hepatic artery at the inferior

margin of the pancreatic neck. The gastropancreatic fold was used to locate the left gastric artery.

In LRG, lymph nodes dissection was complicated, which could be achieved by recognizing and skeletonizing the related vessels. In summary, one center (pancreas), two ligaments (gastropancreatic and plenocolic ligaments), and two folds (hepatopancreatic and gastropancreatic folds) are the key landmarks identified in the literature used to find each vessel. These landmarks could be readily defined if the surgical plane (space between posterior layer of dorsal mesogastrium and pancreas) was appropriately identified.

OTHER LAPAROSCOPIC PROCEDURES

Anatomic studies of laparoscopic liver resection were mainly carried out by autopsy. The research priorities were placed on the first and the secundum porta hepatis[16,17]. Laparoscopic procedures were mostly performed as hepatic left lateral lobectomies. Therefore, the important structure of the left triangle ligament was defined as the peritoneal reflection of the diaphragm and the left lateral lobe. Superior hepatic arteries, branches originating from the left gastric artery and inferior phrenic artery, could be found after the left triangle ligament was opened (taking up 80%). The trunk of the superior hepatic arteries crossing the left hepatic veins of the superior posterior margin was an important landmark for locating the hepatic vein, as well as the secundum porta hepatis[18]. The falciform ligament was found as the anterior pathway to reach the secundum porta hepatis by laparoscopy. The left, inferior phrenic artery and middle segment artery of the liver formed a vessel that arched and gave off 6-12 branches to the falciform ligament, finally draining into the left inferior phrenic vein. The falciform ligament provides significant collateral circulation to the liver and is an important landmark for laparoscopic liver surgery[19].

Laparoscopic pancreaticoduodenectomy (LPD) is recognized as an extremely difficult laparoscopic operation due to the emergency status of the laparoscopic surgery. Chinese surgeons[20] developed a method of retroperitoneal laparoscopic dissection to expose the pancreatic body and tail. The retro-pancreatic space was reached using testis (ovarian) vessels as anatomical landmarks. The peritoneum was a landmark for the belly side of the para-renal space, and the splenic artery was a landmark of the upper pancreatic edge. The left renal vein was a landmark of the lower pancreatic edge, the left diaphragm colon ligament (or splenic lower pole) was a landmark for the pancreatic tail, and the intersection point between the right edge of the inferior mesenteric vein and the inferior margin of the pancreas were landmarks for the transition part of the pancreatic neck and body. Lu *et al*[21] developed the duodenal wall approach, in which the lateral wall of the duodenum was dissected and the root of the mesenteric vessel could be seen at the horizontal level of the duodenum; and the

Figure 5 The lesser omentum above the hepatic caudate lobe.

posterior pancreatic segment of the visible portal vein could be located by searching along the superior mesenteric vein.

Laparoscopic fundoplication, routinely used in laparoscopic anti-reflux surgery (LS), is a classical approach for the treatment of gastroesophageal reflux disease. Its related laparoscopic anatomy emphasized the anatomic landmark and surgical plane. The procedure is initiated by opening the lesser omentum above the hepatic caudate lobe, the landmark for finding the right crura of diaphragma. The space between the right crura of diaphragma and the esophagus is the surgical plane for dissociating the esophagus[22] (Figure 5). Special anatomical variations, such as an aberrant left hepatic artery, can complicate the procedure[23].

Anatomic studies on laparoscopic splenectomy focused on the splenic vessels. In most cases, splenic arteries and veins run together along the superior edge of the pancreatic body and tail; the arteries are located above the veins. In some reported cases, the splenic arteries ran along the superior edge of the pancreatic body tail, and the splenic arteries and veins were either found at the back of the pancreas, or the arteries were absolutely absent around the pancreatic body and tail. It was required that two (in most cases), three, and four splenic, lobar branches should be clamped[24,25].

CONCLUSION

What can we learn from the entire body of literature on laparoscopic anatomy?

Researches on laparoscopic anatomy have not been extensively carried out or reported throughout the world. Perusal and a summary of all of the relevant studies indicated that China-based scholars did most of the research work on laparoscopic anatomy. Some works were initially carried out by autopsy, and the results were provided to surgeons for guidance in live procedures. Procedures that were already extraordinarily familiar by surgeons, such as LC and LIH, were initiated in live surgeries.

Studies on the laparoscopic anatomy of abdominal surgery mainly focused on the following aspects: (1)

Simulating laparoscopic surgery on cadavers, or relevantly studying endoscopic anatomy through clinical laparoscopic surgery; (2) Obtaining relevant data by measuring the length, distance, and diameter of the tissue under the study; (3) Calculating the case percentage of special adjacent anatomical structures; and (4) Finding relevant anatomical planes, landmarks and providing relevant intraoperative pictures.

Due to the various surgical approaches, the portion of our study that focused on certain laparoscopic anatomy also varied. During laparoscopic procedure, the size and boundary of target tissues and the distance between two structures in laparoscopy sight were estimated by surgeons based on their experience. These data could not be obtained accurately by the naked eye using the existing laparoscopic technology. Therefore, some data in the autopsy studies could only be used as a reference during live laparoscopic procedures. The key point for laparoscopic surgeons was to understand the special, adjacent anatomical structure and the location of the correct anatomical plane and anatomical landmark. Gaining a definite understanding will guide future prospective studies of laparoscopic anatomy.

Surgical planes and anatomic landmarks

Why could there be less bleeding in the laparoscopic sight if manipulation occurs in certain surgical planes? Each surgical plane and natural space in the human body, are formed by stratum membranosum, like the membranous layer of the superficial fascia present in many regions of the body[26]. The basic research on surgical planes in laparoscopic anatomy should focus on vessel-less stratum membranosum.

Anatomical landmarks are descriptions of neighboring structures crucial to identifying the proper target tissue for resection. Although individual patients vary in their anatomical structure, certain commonalities exist. These commonalities become obvious through the numerous cases and procedures reported. Laparoscopic surgeons must rely on landmarks in each procedure; it is crucial in laparoscopy that detours must be minimized, otherwise an unexpected injury is likely to occur.

Sometimes, the surgical planes and anatomical landmarks can assist each other. In an appropriate plane, it would be easy to identify certain landmarks. Take LRG for example; if the space between the posterior layer of dorsal mesogastrium and pancreas could be found correctly, the landmarks of one center, two ligaments and two folds would be immediately obvious. Likewise, a landmark can help find readily a surgical plane. In LAS, the hepatic caudate lobe was the landmark for finding the right crura of diaphragma, the surgical plane for dissociating the esophagus.

Significance and prospective

Currently, laparoscopy is usually performed using the approach of natural orifice transluminal endoscopic surgery and a single entry port. These techniques require advanced skills of surgeons and a detailed knowledge of laparoscopic anatomy.

In the field of abdominal surgery, laparoscopic surgery has become increasingly popular. Systematic laparoscopic anatomy can provide laparoscopic surgeons with a clear idea to design their operation, and is particularly important for practicing novices. Laparoscopic anatomy is a basic curriculum for laparoscopic surgeons, and will certainly become an important new subject as the use of laparoscopic procedures increases.

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REVIEW

Circulating microRNAs: Novel biomarkers for esophageal cancer

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Abstract

Esophageal carcinogenesis is a multi-stage process, involving a variety of changes in gene expression and physiological structure change. MicroRNAs (miRNAs) are a class of small non-coding endogenous RNA molecules. Recent innovation in miRNAs profiling technology have shed new light on the pathology of esophageal carcinoma (EC), and also heralded great potential for exploring novel biomarkers for both EC diagnosis and treatment. Frequent dysregulation of miRNA in malignancy highlights the study of molecular factors upstream of gene expression following the extensive investigation on elucidating the important role of miRNA in carcinogenesis. We herein present a thorough review of the role of miRNAs in EC, addressing miRNA functions, their putative role as oncogenes or tumor suppressors and their potential target genes. The recent progresses in discovering the quantifiable circulating cancer-associated miRNAs indicate the potential clinical use of miRNAs as novel minimally invasive biomarkers for EC and other cancers. We also discuss the potential role of miRNAs in detection, screening and surveillance of EC as miRNAs can be a potential target in personalized treatment of EC.

INTRODUCTION

Esophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer death worldwide, affecting more male than female[1]. The dramatic geographic difference in incidence is the striking characteristic for EC. The EC incidence in the high-risk northern Chinese exceeds 100/100000, while that in the low-risk western Africa can be 20-fold lower[2]. EC remains the leading cause of cancer-related death in the high-risk areas, especially in Henan Province, northern China[3]. Esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) are the two main forms of EC; each of them has different etiologic and pathologic characteristics. ESCC remains the predominant subtype of EC, especially in China; in contrast, EAC is the most common type in Western countries.

The survival and prognosis of EC patients depend on the stage of the tumor at the time of detection. Despite significant investment and advances in the treatment of cancer, the overall survival for advanced and metastatic cancer is still dismal, with a 5-year survival rate of less than 15% for advanced tumors[1,4,5]. The concept of early detection of esophageal cancer has been established

among clinicians and scientists for years. The 5-year survival rate was more than 90% for early EC[1,4,5], about 50% of cancers extend beyond the primary local region at the time of diagnosis and almost 75% of surgically treated patients have proximal lymph node metastasis[6]. At present, gastrointestinal endoscopy remains the primary screening tool, by which the suspected lesions can be biopsied for histopathological analysis. This invasive test, even though it is proved to increase the detection of early tumor and therefore can prolong the survival of the patient, is generally considered to be inconvenient and painful. Because of its limitation, there is an urgent request for discovery of novel predictive markers for EC.

Micro ribonucleic acids (miRNAs) are a class of single-stranded, evolutionarily conserved non-coding RNAs, only 17-25 ribonucleotides long[7]. The first known microRNA (miRNA), the lin-4, was discovered in 1993 through the study of heterochronic gene lin-14 in worms[8]. Since then, miRNA has shown the great potential regulating roles in many aspects of biological and pathological processes. The miRBase, which is the international registries for miRNAs, clearly described their nomenclature, targets and functions and implications in different diseases[9]. The biogenesis of miRNAs is a multi-step process involving a complex protein system, which includes members of the Argonaute family, Pol Ⅱ-dependent transcription and the RNase Ⅲs Drosha and Dicer[10]. MiRNAs are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation[11,12]. The mechanism is still under debate, but probably related to inhibition of translation and messenger RNA degradation[13]. These small RNAs regulate gene expression at the level of translation through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes[10]. The specific region in miRNA, which is important for messenger RNA target recognition, is referred to as the "seed sequence", and located at the 5- end of the mature miRNA sequence, from bases 2 to 8[11]. With the seed sequence, we can search for the complementary sequences in the 3'-untranslated regions (3'-UTRs) of known genes that exhibit conservation across species[11]. In animals, most miRNAs are thought to form imperfect base pairs with their target mRNA(s) and these interaction sites are enriched in 3'-UTRs[11]. To date, in the human genome, over 700 mature miRNAs have been identified, and the number is still increasing with the future studies. Up to 1000 miRNAs have been predicated by the bioinformatics studies of the human genome[14].

EXPERIMENTAL TECHNIQUES FOR MIRNA ANALYSIS

It is necessary to explore effective tools for detection, quantification, and functional analysis of miRNAs. Oligonucleotide miRNA microarray analysis has been extensively used as high-throughput technique for the assessment of cancer-specific expression levels in a large number of samples[10,13,15]. Another method to gauge miRNA expression level includes utilizing a bead-based flow-cytometric technique by which hybridization occurs in solution and provides a high specificity for closely related miRNAs[16]. The miRNA microarray platform with locked nucleic acid-modified capture probes can improve probe thermostability and increase specificity, so that it can discriminate miRNAs with even single nucleotide differences[17]. Quantitative real-time polymerase chain reaction (PCR) have also been widely applied to miRNA research because of its cost-effectiveness, high throughput and superior detection of low-abundance species[18]. All of these technologies facilitating miRNAs expression profiling are essential for validation of microarray data.

It is also necessary to develop methods of manipulating miRNA expression for elucidating biological significance and manipulating miRNA expression. MiRNA inhibitors such as 2-O-Methyl antisense single-strand oligonucleotides and locked nucleic acid modified oligonucleotides, made the suppression of endogenous miRNA activity and its downstream effect on messenger RNA expression possible[19]. The latest improvement of miRNA inhibition could control miRNA expression *in vivo* by expressing decoy miRNA targets *via* lentiviral vectors[20], with it we can inhibit specific miRNA by expressing various miRNA inhibitors. MiRNA mimicry could be used to identify the cellular processes and phenotypic changes through transfecting specific miRNAs into cell lines[21]. Although these techniques are of different strength and weakness, the large size studies have shown common characteristics of miRNA deregulation in human cancers[13] and shed light on the application of miRNAs as novel biomarkers finally.

MIRNA AND EC

Early studies demonstrated the aberrant expression of miRNA in cancers and the fact that approximately half of miRNA genes are localized in cancer-associated genomic regions or in fragile sites[22] indicates the potential role of miRNAs as a promising class of oncogenes or tumor suppressor gene in human carcinogenesis. MiRNAs still largely intact in conventionally collected, formalin-fixed and paraffin-embedded tissues and even a modest number of miRNAs are adequate to discriminate human tumors according to the developmental lineage and differentiation state of the tumors[16]. Furthermore, compared with messenger RNA (mRNA) profiles, it is easier to use these miRNA expression profiles to classify poorly differentiated tumors[23]. Here, we will focus on the miRNA expression profiles of EC, especially ESCC, to reveal the oncogenic mechanism by miRNA mediated post-transcriptional pathway. We will first discuss the identification of EC specific miRNA signatures for a new class of screening, diagnostic and prognostic biomarkers through miRNA expression profiling of EC; then we will concentrate on how to clarify the functional roles of miRNA in EC and the potential clinical valuation of the circulating miRNAs in EC. We believe that the study of miRNA's role in EC will provide new sight into this disease.

Table 1 Differential expression of miRNAs in EC tissues compared with normal tissue as identified in seven studies

Techniques	Pathology	Population	Overexpressed miRNAs			Underexpressed miRNAs		Ref.
Array profiling	ESCC	Chinese	hsa-miR-25	hsa-miR-424	has-miR-151	hsa-miR-100 hsa-miR-29c	hsa-miR-99a mmu-miR-140	[6]
mirVana miRNA Bioarrays (Ambion)	EAC	Japanese	miR-192 miR-21	miR-194 miR-93	miR-200c	miR-205	miR-203	[25]
	ESCC	Japanese	miR-342	miR-21	miR-93	miR-205	miR-203	
qRT-PCR	ESCC	Cell lines, Chinese	miR let-7d miR-373	miR-330	miR-340			[26]
qRT-PCR	ESCC	Japanese	miR-9 miR-17-5p miR-21 miR-34c miR-107 miR-130a miR-134 miR-151	miR-15b miR-20a miR-25 miR-103 miR-127 miR-130b miR-137	miR-16 miR-20b miR-34b miR-106a miR-129 miR-132 miR-138	miR-133a miR-139	miR-133b miR-145	[27]
Agilent Human miRNA microarray kit version 1.0	EAC	Caucasian	hsa-miR-126 hsa-miR-146a hsa-miR-195 hsa-miR-199a hsa-miR-424	hsa-miR-143 hsa-miR-181a hsa-miR-28 hsa-miR-30a-5p	hsa-miR-145 hsa-miR-181b hsa-miR-199b hsa-miR-29c	hsa-miR-149 hsa-miR-205 hsa-miR-221 hsa-miR494 hsamiR-617	hsa-miR-203 hsa-miR-210 hsa-miR-27b hsa-miR-513 hsa-miR-99a	[28]
miRNA microarray chips version 3	EAC	Caucasian	miR-21 miR-194	miR-192 miR-223		miR-203		[29]
	ESCC	Japanese	miR-21			miR-375		
qRT-PCR	ESCC	Cell lines, Chinese	miR-10					[30]

miRNAs: Micro ribonucleic acids; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; EAC: Adenocarcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction.

Role of miRNAs in carcinogenesis, progression, invasion and metastasis of EC

MiRNA acts as oncomiRNA or tumor suppressors to affect the tumorigenesis if their target mRNAs were encoded by tumor suppressor genes or ontogenesis[24]. Aberrant miRNA expression has been identified and confirmed in EC (Table 1). Comparing the miRNAs expression in ESCC with EAC, we can find that only a few miRNAs expressions were the same in these two diseases, each of which has the different miRNAs expression profiling, indicating that both of ESCC and EAC have distinct etiologic and pathologic characteristics. The miRNAs which are highly expressed in tumor can act as tumor promoter by targeting and inhibiting different tumor suppressor genes such as B-cell CLL/lymphoma 2 (BCL2)[31], *tension homologue* gene (PTEN)[32], large tumor suppressor homolog 2 (LAST2)[25], annexin A1 (ANXA1)[33]. Those genes were all studied in EC tissues and it was suggested that all of them were involved in carcinogenesis of esophagus. Those mature miRNAs with relatively lower expression in cancer may act as tumor suppressor miRNAs. The loss of such miRNAs may enhance the activity of oncogenes.

Apart from identifying different cancer-related miRNAs, efforts have been made to identify their target genes, messenger RNAs and receptors, which will lead to further studies about their contribution in cancer treatment. For example, the putatively identified targets[31,32,34] of miR-21 are BCL2, PTEN, tumor suppressor gene tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4) and maspin. Zhu *et al*[34] found that the biological function of miR-21 is probably due to the simultaneous repression of multiple tumor suppressor genes, including PTEN, TPM1, PDCD4, and maspin. PTEN is capable of restricting growth and survival signals by limiting the activity of the phosphoinositide 3-kinase (PI3K)/AKT pathway[35]. A decrease in functional PTEN causes constitutive activation of downstream components of the PI3K/AKT pathway, leading to tumor progression and metastasis[35]. Thus, down-regulation of PTEN by miR-21 may contribute to transformation and increased tumor cell survival[35]. TPM1 is an actin-binding protein which is capable of stabilizing microfilaments, regulating both microfilament organization and anchorage-independent growth, so its importance in cell transformation has been highlighted[36]. Another important mechanism of cancer development is epigenetic silencing of tumor suppressor genes. DNA hypomethylation, CpG island hypermethylation and histone-modification losses represent epigenetic markers of malignant transformation[37]. Calin *et al*[22] showed that some miRNA (e.g. miR-127) are located in a CpG island, a region that is involved in several types of translocations identified in hematological cancers and deleted by loss of heterozygosity in solid tumors. This phenomenon gives us a clue that the expression of tumor suppressor can be altered in epigenetic silencing mechanism through expression of miRNA.

Dysplasia and Barrett's esophagus are precursors of EC and the progression of these lesions to cancer is a multistep process that involves different sequential DNA aberrations and changes in gene expression. MiRNA expression profiles in tissues of Barrett's esophagus with high-grade dysplasia were significantly different from

their corresponding normal tissues/EAC tissues, but, no significant difference was found between low-grade dysplasia and paired normal tissues[28]. These observations were consistent with the finding of Feber *et al*[25], which suggests that low-grade dysplasia was with a lower malignant transformation rate compared with high-grade dysplasia. Yang *et al*[28] found that seven miRNAs (hsa-let-7b, hsa-let-7a, hsalet-7c, hsa-let-7f, hsa-miR-345, hsa-miR-494, and hsa-miR-193a) were potentially important in the progression from high-grade dysplasia to EAC. Hsa-let-7b, hsa-let-7a, hsalet-7c and hsa-let-7f belong to the let-7 miRNA family, most of their members function as tumor suppressors through negative regulation of the *RAS* gene[38]. Accordingly, RAS mutations and amplifications have been frequently identified in precancerous tissues and EC tissues[39,40]. Oncogene RAS may play a role in cell growth and differentiation[41].

Diagnostic and prognostic value of miRNA for EC

To date, the available data of miRNA expression profile in cancer suggest that miRNA may have diagnostic and prognostic potential. It is crucial to correlate miRNA expression with tumor subtypes or clinical parameters.

Lu *et al*[16] were the first to systematically demonstrate that the specific miRNA expression can be applied not only for diagnosis but also for classification of different tumors, and the accuracy is 70%. Guo *et al*[6] were the first to investigate the expression of miRNA in EC tissues using miRNA microarray techniques. They found that five miRNAs (miR-335, miR-181d, miR-25, miR-7 and hsa-miR-495) correlate with gross pathologic classification (fungating *vs* medullary) and two miRNAs (miR-25 and miR-130b) correlate with differentiation classification (high *vs* middle *vs* low)[6]. The miRNA expression profiling of ESCC is distinct from EAC[25]. Compared with its expression in normal epithelium (NSE), miR-194, miR-192 and miR200c are significantly up-regulated in EAC but not in ESCC[27]. Oppositely, miR-342 is aberrantly expressed in ESCC but not in EAC[25]. Furthermore, the miRNA expression profiling of NSE and SCC samples are more similar to each other than to the EAC samples[26]. The Barrett's esophagus (BE) samples sit between the EAC and NSE and one high-grade dysplasia specimen has a miRNA expression profile similar to the EAC[24]. This makes good biological sense that miRNA expression profiles can distinguish esophageal tumorigenesis and may be proven useful for identifying BE patients who are at high risk for progression to EAC.

As we mentioned before, the survival and prognosis of EC is dismal. Therefore, the discovery of new prognostic markers could be a prodigious advantage for identification of patients that would benefit from more aggressive therapy. The high expression of both miR-103 and miR-107 has been shown to correlate with poor survival in 53 Chinese patients with ESCC either by univariate analysis or by multivariate analyses[6]. In an independent study of 30 Japanese patients with ESCC, overexpression

of miR-129 was found to significantly correlate with a shorter survival and miR-129 was identified as a significant and independent prognostic factor in surgically treated ESCC patients[27]. The largest study so far, found that low expression of miR-375 in EAC patients was strongly associated with worse prognosis, whereas ESCC patients with high expression of miR-21 had poor prognosis[29]. An earlier study conducted by Sugito *et al*[42], measured the expression levels of DICER1, DGCR8, and RNASEN messenger RNA in 73 surgically treated ESCC patients by real-time reverse-transcription PCR, and clarified that high RNASEN expression correlate with poor prognosis in ESCC, indicating that RNASEN may be a good candidate for molecular prognostic marker. Altered expression of these miRNAs is tissue specific, which may provide clues as to the different molecular progression undertaken by these cancers, and opens a potential avenue for targeted therapy to improve prognosis[29].

Circulating microRNAs: Novel minimally invasive biomarkers for EC

Scientists studying on EC are continually dedicated to search for sensitive, and minimally invasive markers which can detect early neoplastic changes, thus identifying EC at an early stage, as well as monitoring the progress of patients with EC and their response to treatment. Existing methods for identification of EC have many inherent deficiencies. Endoscopic biopsy and histopathological examinations are currently the golden standard diagnostic methods for EC. However, it has limitations, including the use of invasive tool and limited number of experienced physicians. Besides, it is difficult to be applied in large-scale studies because of its tedious approaches. To date, there are still no other effective biomarkers established in the routine assessment of EC.

The much desirable biomarker should be sensitive enough and easily accessible to detect the cancer patients at early stage. Recent studies have shown that tumor-derived miRNAs are resistant to endogenous ribonuclease activity so it can be present in human serum in remarkably stable form[43]. Furthermore, the expression level of serum miRNAs is reproducible and consistent among individuals[43,44]. These tumor-derived miRNAs are present in the circulating blood at levels sufficient to be measurable as biomarkers for the detection of tumors and more than 100 circulating miRNAs can be detected in the blood of healthy individuals[43]. Either plasma or serum can be used for identification of these blood-based biomarkers because the miRNAs levels in plasma and serum are strongly correlated[43,45]. Chen *et al*[44] demonstrated that the colorectal cancer patients shared a large number of serum miRNAs with lung cancer patients. Moreover, they identified a unique expression profile of serum miRNAs for colorectal cancers that were not present in lung cancer. Though miRNA research is only emerging recently, it has aroused great interest in clinical and scientific communities. Since the presence of miRNA in serum was first described in patients with diffusive large B-cell lymphoma[46],

a number of studies on other solid cancers (ovarian cancer[47,48], lung cancer[49,50], breast cancer[51], colorectal cancer[52,53]) have been reported about the presence of miRNA in circulation and their potential for use as novel biomarkers. Taylor *et al*[48] demonstrated that the expression of 8 miRNAs in the serum of patients with ovarian cancer were significantly distinct from their expression observed in benign diseases, yet could not be detected in the serum of healthy controls. Another study indicated that miRNA levels were significantly different between lung cancer patients and controls[49]. Both of these studies suggested that there was no significant difference in the miRNA expression between peripheral circulation and tumors tissues[48,49], indicating that circulating miRNA could act as surrogate of tissue miRNA for early diagnosis. And this will initiate a new application that will be widespread in clinical treatment and disease prevention.

Blood-based miRNAs provide a novel class of minimal invasive biomarkers for high-risk subject screening and early diagnosis for cancers. At the time of writing, there was no article about circulating miRNAs profile of EC. We focus on the circulating miRNA expression profiles of ESCC which is the main type of EC, especially in Asia. Checking circulating miRNA expression profiles of ESCC can help identify EC specific circulating miRNAs signatures for a new brand of screening, diagnostic and prognostic biomarkers. Studies can also be performed to clarify the functional role(s) of miRNAs in EC.

Potential in screening, surveillance and treatment of EC

So far, endoscopic biopsy and histopathological examinations are the golden standard for high-risk subject screening and early detection for EC worldwide. We can only identify 1%-2% early carcinomas and 15%-20% precancerous lesions in asymptomatic population aged higher than 35 years in high-incidence areas of EC through endoscopic biopsy and histopathological examinations[3]. Yet nearly 80% asymptomatic populations were in the normal range. In other words, there exists over endoscopic application. Besides, as there are no sufficient experienced physicians in the countryside, the misdiagnosis and missed diagnosis would be inevitable. In addition, standard sterilizing procedure also restricts the wide application of endoscopy in asymptomatic population. Furthermore, the projected cost of mass screening in China for detection of cancers is increasing annually. An urgent and important problem to be solved is to diminish blindness in the screening, minify the range of endoscopy and elevate the detection sensitivity of early carcinoma in high risk subjects. Easily accessible and minimal invasive biomarkers for EC and for other diseases are much desirable. The PCR technique for detection and quantification of miRNA[18] in the blood would be one of the promising methods of screening individuals for EC and other cancers because of its universal application, convenient management, low cost and high sensitivity. Rouet *et al*[54] set up a model of qRT-PCR for serum RNA analysis, suggesting that its cost was only less than £1 for each clinical specimen when

used in a group of 84 patients. Once the accuracy of detection is established, MiRNAs test should be introduced into the national EC and other disease screening programs. The best option might be that annual miRNA blood test in asymptomatic populations in high risk areas of EC, and then the patients with positive test result can choose endoscopic biopsy and histopathological examinations for final diagnosis.

Given the emerging evidence of miRNAs acting as oncogenic or tumor suppressor activities, it is important to seek approaches to interfere with the miRNA[55], and eventually, develop these as novel biomarkers into EC therapies. Scientists are making efforts to explore miRNA and utilize that in cancer. Meng *et al*[32] showed that miRNA expression profiles changed during treatment with gemcitabine, and the sensitivity of cholangiocarcinoma tumor cells to this chemotherapy *in vitro* are increasing with the modulation of some miRNAs. On the contrary, it could be feasible to introduce tumor suppressor miRNA which are tissue-specific to the target gene, and then it may help prevent further progression, or even shrink EC. Yet, recently identified genomic and proteomic biomarkers, tumor cell mutations and microsatellite instability cannot be recommended for clinical use because of insufficient available data[56]. Though recent studies on miRNA in EC are limited, the potential value of miRNAs as prognostic and predictive biomarkers in EC is elegantly highlighted and will be elucidated eventually. However, experimental miRNA therapy need to be validated comprehensively through studies involving different cohorts of patients before introduced into clinical practice.

CONCLUSION

Although much of the work on miRNA is still in its infancy, the previous studies have demonstrated the significant role(s) of miRNA in the initiation and progression of cancer and other diseases. Further exploration is required for better understanding their role in carcinogenesis of EC and for better application in the future. Notwithstanding there are still many questions to be answered about circulating miRNA, there is no doubt that further studies will lead to great progress in the future therapy of EC. The exploration of miRNA will improve our knowledge of the roles of these novel biomarkers, and further demonstrate their true potential in therapeutic methods.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Butyrate stimulates IL-32α expression in human intestinal epithelial cell lines

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Abstract

AIM: To investigate the effects of butyrate on interleukin (IL)-32α expression in epithelial cell lines.

METHODS: The human intestinal epithelial cell lines HT-29, SW480, and T84 were used. Intracellular IL-32α was determined by Western blotting analyses. IL-32α mRNA expression was analyzed by real-time polymerase chain reaction.

RESULTS: Acetate and propionate had no effects on IL-32α mRNA expression. Butyrate significantly enhanced IL-32α expression in all cell lines. Butyrate also up-regulated IL-1β-induced IL-32α mRNA expression. Butyrate did not modulate the activation of phosphatidylinositol 3-kinase (PI3K), a mediator of IL-32α expression. Like butyrate, trichostatin A, a histone deacetylase inhibitor, also enhanced IL-1β-induced IL-32α mRNA expression.

CONCLUSION: Butyrate stimulated IL-32α expression in epithelial cell lines. An epigenetic mechanism, such as histone hyperacetylation, might be involved in the action of butyrate on IL-32α expression.

INTRODUCTION

Interleukin (IL)-32 was originally reported as natural killer (NK) transcript 4, and has been described as a cytokine produced mainly by T-lymphocytes, NK cells, epithelial cells, and blood monocytes[1-3]. The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons[4]. There are four splice variants, and IL-32α is reported as the most abundant transcript[5,6]. Recently, IL-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, tumor necrosis factor (TNF)-α, IL-6,

and IL-8 *via* the activation of p38 mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB and activating protein (AP)-1 signal transduction pathways[3,7]. Recently, we reported the overexpression of IL-32α in the inflamed mucosa of inflammatory bowel disease (IBD)[8]. *In vitro* experiments on human intestinal epithelial cell lines showed that IL-32α expression was induced by IL-1β, IFN-γ and TNF-α through the activation of Akt-phosphatidylinositol 3-kinase (PI3K)[8].

Dietary fiber (nonstarch polysaccharides) and resistant starch escape digestion in the upper gastrointestinal tract, and undergo anaerobic bacterial fermentation in the colon. This process produces short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, as the major by-products[9,10]. The typical concentration of each SCFA has been reported as approximately 10-20 mmol/L[11]. SCFAs have been shown to have significant effects on the colonic epithelium both *in vivo* and *in vitro*. Butyrate serves as the primary energy source for the normal colonic epithelium[12], and stimulates the growth of the colonic mucosa[13]. However, in tumor cell lines, it induces apoptosis and inhibits cell growth[14]. Butyrate has also been reported to modulate gene transcription through the induction of histone hyperacetylation. Since butyrate inhibits histone deacetylation and induces histone hyperacetylation, it is categorized as a histone deacetylase (HDAC) inhibitor[15].

In the present study, we investigated the *in vitro* effects of the SCFAs on IL-32α expression in human intestinal epithelial cell lines.

MATERIALS AND METHODS

Reagents
Recombinant human IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA), and human IFN-γ was obtained from Pepro Tech (Rocky Hill, NJ, USA). SCFAs (sodium acetate, sodium propionate and sodium butyrate) were purchased from Sigma Chemical Co. (Dorset, UK). Biotinylated anti-human IL-32α antibodies were purchased from R&D Systems, and horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Dako Japan (Kyoto, Japan). Antibodies against phosphorylated and total Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). Trichostatin-A was purchased from Tocris Cookson (St. Louis, MO, USA).

Cells
The human intestinal epithelial cell lines HT-29[16], SW480[17], and T84[18] were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured as described previously[16-18].

Real-time polymerase chain reaction
Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method, and was then reverse-transcribed into cDNA using a PrimeScript RT reagent kit

(TAKARA-BIO, Shiga, Japan). The expression of human IL-32α mRNA was assessed by real-time polymerase chain reaction (PCR) analyses. Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan). The oligonucleotide primers used in this study were specific for human IL-32α as follows: 5'-AGCTG-GAGGACGACTTCAAA [nucleotides 192-211, GenBank accession no. BC018782[19,20] and 3'-AGGTGGTGT-CAGTATCTTCA (nucleotides 642-623)]. The PCR was conducted using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). β-actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample.

Western blotting analysis
The intracellular expression of IL-32α protein was determined by Western blotting. Briefly, the cells were washed and lysed in sodium dodecyl sulphate (SDS) sample buffer containing 100 μmol/L orthovanadate. The lysates were homogenized, and the protein concentration was measured by the Bradford method. For Western blotting, 10 μg of protein from each sample was subjected to SDS-PAGE on a 4%-20% gradient gel under reducing conditions. The proteins were then electrophoretically transferred onto a nitrocellulose membrane, and the membrane was blocked with 5% skimmed milk. After washing with PBS containing 0.1% Tween-20 (PBST), the membrane was incubated with a biotinylated anti-human IL-32α antibody at 4℃ overnight. Next, the membrane was reacted with HRP-conjugated streptavidin at room temperature for 1 h. The detection was performed using enhanced chemiluminescence (ECL) Western blotting systems (Amersham Biosciences).

Detection of global histone H3 acetylation
Histone extraction and the detection of global histone H3 acetylation were performed using the Epiquik global histone H3 acetylation assay kit (Epigentek; Brooklyn, NY, USA). The histone protein content was detected by the Bradford method.

Statistical analysis
Statistical significance of the differences was determined using unpaired *t* test (Statview version 4.5). Differences resulting in *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of SCFAs on IL-32α mRNA expression
To evaluate the effects of SCFAs on IL-32α mRNA expression, intestinal epithelial cells (HT-29, Caco-2, T84 cells) were stimulated with 10 mmol/L of each SCFA (acetate, propionate, and butyrate) for 12 h, and then the expression of IL-32α mRNA was determined by real-time PCR. Acetate had no effect, but butyrate significantly increased IL-32α mRNA expression in all cell lines. Propionate increased the expression of IL-32α

Figure 1 Effects of short-chain fatty acids (SCFAs) on IL-32α mRNA expression in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were incubated for 12 h with each SCFA (10 mmol/L). In another experiment, the cells were stimulated with IL-1β (50 ng/mL) in the presence or absence of each SCFA (10 mmol/L). The IL-32α mRNA expression was determined by real-time PCR. The data are expressed as mean ± SD (*n* = 4). [a]*P* < 0.05, [b]*P* < 0.01.

Figure 2 Effects of SCFAs on IL-32α protein secretion in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were stimulated for 48 h with IL-1β (50 ng/mL) in the presence or absence of butyrate (10 mmol/L), and the intracellular IL-32α protein levels were detected by Western blotting.

Effects of SCFAs on cytokine-induced IL-32α mRNA expression

Next, we investigated the effects of SCFAs on cytokine-induced IL-32α mRNA expression. We recently reported that IL-1β, IFN-γ and TNF-α stimulate IL-32α mRNA expression in intestinal epithelial cells[8], and the cells were stimulated with cytokines in the presence or absence of each SCFA (10 mmol/L). In all cell lines, butyrate by itself stimulated IL-32α mRNA expression, and enhanced the IL-1β-induced IL-32α mRNA expression. SCFAs did not modulate IFN-γ- and TNF-α-induced IL-32α mRNA expression in any of the cell lines (data not shown).

Effects of SCFAs on intracellular IL-32α protein accumulation

To confirm the effects of SCFAs on IL-32α protein expression, the cells were stimulated for 48 h with IL-1β and/or SCFAs. Intracellular IL-32α was detected as a 25 kDa protein[8,21]. The effects of the SCFAs were similar to those observed at the mRNA level. The combination of IL-1β and butyrate enhanced IL-32α protein expression additively (Figure 2).

Dose responses

To examine the effects of butyrate on IL-1β-induced IL-32α expression more precisely, HT-29 cells were incubated for 12 h with IL-1β (50 ng/mL) plus increasing concentrations of butyrate. The effects of butyrate on IL-1β-induced IL-32α secretion appeared at the concentration of 5.0 mmol/L, and the most significant difference was observed at 10 mmol/L (Figure 3).

Effects of butyrate on the phosphorylation of Akt

We tested the effects of butyrate on the IL-1β-induced phosphorylation of Akt, which plays a major role in IL-32α induction[8,19]. As shown in Figure 4, there were no effects of butyrate on IL-1β-induced Akt phosphorylation. These observations suggest that the modulation of Akt activity is not involved in the actions of butyrate on IL-1β-induced IL-32α expression.

Effects of trichostatin-A on cytokine-induced IL-32α mRNA expression

Previous studies have demonstrated that butyrate exerts its biological effects through the induction of histone

mRNA only in T84 cells, but in the other cell lines it had no effect (Figure 1).

Figure 3 Dose-dependent effects of butyrate on IL-1β-induced IL-32α mRNA expression in colon cancer cell lines. The cell lines (HT-29, SW480, T84) were stimulated for 12 h with increasing concentrations (0-10 mmol/L) of butyrate in the presence or absence of IL-1β (50 ng/mL). IL-32α mRNA expression was then analyzed by real-time PCR. The data are expressed as mean ± SD (n = 4). [a]P < 0.05, [c]P < 0.005.

Figure 4 Effects of butyrate on Akt phosphorylation. The cells were stimulated in the presence of butyrate (5 mmol/L) and/or IL-1β (50 ng/mL) for 15 min, and then Akt phosphorylation was determined by Western blotting.

hyperacetylation[15]. To examine the relationship between histone acetylation and the effects of butyrate on IL-32α mRNA expression, we tested the effects of trichostatin-A (TSA), a potent histone deacetylase inhibitor[22-24].

The cells were stimulated with cytokines in the presence or absence of TSA (5 μmol/L), and the expression of IL-32α mRNA was then analyzed by real-time PCR. As shown in Figure 5, TSA enhanced the IL-1β-induced IL-32α mRNA expression in all cell lines.

Effects on histone H3 acetylation

We tested the effects of butyrate and IL-1β on histone H3 hyperacetylation in HT-29 cells. As shown in Figure 6, butyrate induced histone H3 hyperacetylation in HT-29 cells (Figure 6).

Figure 5 **Effects of trichostatin-A (TSA) on IL-32α mRNA expression.** The cells (HT-29, SW480, T84) were incubated for 12 h with IL-1β (50 ng/mL) in the presence or absence of TSA (5 μmol/L), and then the IL-32α mRNA expression was determined by real-time PCR. The data are expressed as mean ± SD (*n* = 4). [a]*P* < 0.05.

Figure 6 **Effects of butyrate on histone H3 acetylation.** HT-29 cells were stimulated for 12 h with IL-1β (50 ng/mL) in the presence or absence of butyrate (10 mmol/L). Histone H3 acetylation was then detected by histone H3 acetylation assay kits (Epigentek; Brooklyn, NY, USA). Histone H3 acetylation was expressed as a value relative to medium alone. The data are expressed as mean ± SD (*n* = 4). [a]*P* < 0.05, [b]*P* < 0.01.

DISCUSSION

Among SCFAs, the biological actions of butyrate have been well investigated. Butyrate is easily absorbed in the large intestine, and plays a role as the energy source for intestinal epithelial cells[13]. On the other hand, previous studies have demonstrated that butyrate blocks NF-κB activation in intestinal epithelial cells, and is thought to have an anti-inflammatory effect[25,26]. Based on this hypothesis, some clinical trials have been performed to test the therapeutic effects of butyrate enemas on ulcerative colitis lesions[27-29].

In this study, we examined the effects of SCFAs on IL-32α expression in intestinal epithelial cells. Initially, we hypothesized that SCFAs, in particular butyrate, might suppress IL-32α expression in these cells, since IL-32α expression has been reported to depend on NF-κB activation, and butyrate has been reported to suppress NF-κB activation. However, contrary to our initial expectation, butyrate stimulated IL-32α expression in these cells. This was characterized by two actions: butyrate by itself could stimulate IL-32α expression, and the synergistic effects of butyrate on cytokine-induced IL-32α expression was observed specifically in combination with IL-1β.

Recently, we reported that IL-32 expression mainly depends on the Akt-PI3K signaling pathway and NF-κB activation. As shown in Figure 6, butyrate did not affect Akt phosphorylation, indicating that the Akt-PI3K pathway was not involved in butyrate-mediated IL-32 expression. Furthermore, we have previously shown that butyrate inhibits IL-1β-induced NF-κB activation in HT-29 cells[26,30,31]. This suggests that the modulation of NF-κB activation is not involved in the effects of butyrate on IL-1β-induced IL-32 expression. This concept is supported by the finding that butyrate did not affect IL-32 expression by TNF-α, which is a potent stimulator of NF-κB activation[32,33]. Thus, the mode of action of butyrate on IL-32 expression in intestinal epithelial cells is mediated by different mechanisms from those reported previously.

It is generally accepted that butyrate leads to the development of reversible histone hyperacetylation *via* the inhibition of histone deacetylase activity[14,15]. Reversible histone hyperacetylation is now believed to play an important role in the regulation of chromatin structure[34,35]. Histone hyperacetylation leads to a more relaxed chromatin structure, and thus facilitates transcription factor access to the promoter regions of certain genes without directly initiating gene transcription[23,24]. Based on this knowledge, the mechanisms by which butyrate selectively induces the increase in IL-1β-induced IL-32 gene expression might be explained by an enhancement of transcription by histone hyperacetylation. This was supported by the effects of trichostatin A, a potent histone deacetylase

inhibitor, on IL-32 expression in these cells. On the other hand, although the precise molecular mechanisms remain to be investigated, increased transcriptional activity by histone hyperacetylation might not account for the effects of TNF-α- and IFN-γ-on IL-32 expression. In order to clarify the precise mechanisms by which butyrate specifically stimulates IL-1β-stimulated IL-32 expression, further studies such as the effects of butyrate on signaling pathways to induce IL-32 expression are needed.

Clinical application of this study is limited because the role of IL-32α in inflammatory bowel disease (IBD) is still obscure. Netea *et al*[2] demonstrated recently that IL-32 augments the production of IL-1α, TNF-α, IL-6 and IL-8 by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism. NODs are a family of intracytoplasmic bacterial peptidoglycans which subsequently induce NF-κB activation. Mutations in *NOD2* have been implicated in the pathogenesis of Crohn's disease (CD). Recently, it has been shown that the *NOD2* mutation in CD patients potentiates NF-κB activity and IL-1α processing. Thus, these findings suggest a pivotal role of IL-32 in the pathophysiology of IBD, and CD in particular. Our study also augments the proinflammatory aspect of butyrate.

In conclusion, we observed that butyrate stimulates IL-32α expression in intestinal epithelial cells. Since IL-32α is considered to be a proinflammatory cytokine, our finding might be a part of the proinflammatory picture of butyrate in mucosal inflammatory responses in the intestine. As the next step, the *in vivo* effects of butyrate on mucosal IL-32 expression should be investigated in the future.

COMMENTS

Background
Recently, interleukin (IL)-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8. Dietary fiber (nonstarch polysaccharides) and resistant starch escape digestion in the upper gastrointestinal tract, and undergo anaerobic bacterial fermentation in the colon. This process produces short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, as the major by-products.

Research frontiers
The authors have previously reported that IL-32α is overexpressed in inflammatory bowel disease (IBD). IL-32α was overexpressed in the inflamed mucosa of IBD patients and was strongly induced by IL-1β, IFN-γ and TNF-α. However, the role of IL-32α is yet to be elucidated. In this study, they investigated the *in vitro* effects of butyrate on IL-32α expression in human intestinal epithelial cell lines.

Innovations and breakthroughs
The authors observed that butyrate stimulates IL-32α expression in intestinal epithelial cells. Since IL-32α is considered to be a proinflammatory cytokine, their finding might be a part of the proinflammatory picture of butyrate in mucosal inflammatory responses in the intestine.

Applications
Clinical application of this study is limited because the role of IL-32α in IBD is still obscure. A recent report demonstrated that IL-32 augments the production of IL-1α, TNF-α, IL-6 and IL-8 by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism. NODs are a family of intracytoplasmic bacterial peptidoglycans which subsequently induce NF-κB activation. Mutations in *NOD2* have been

implicated in the pathogenesis of Crohn's disease (CD). Recently, it has been shown that the *NOD2* mutation in CD patients potentiates NF-κB activity and IL-1α processing. Thus, these findings suggest a pivotal role of IL-32 in the pathophysiology of IBD, and CD in particular. This study also augments the proinflammatory aspect of butyrate.

Terminology
IL-32 was originally reported as natural killer (NK) transcript 4, and has been described as a cytokine produced mainly by T-lymphocytes, NK cells, epithelial cells, and blood monocytes. The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons. There are four splice variants, and IL-32α is reported as the most abundant transcript. Recently, IL-32 was defined as a proinflammatory cytokine characterized by the stimulation of secretion of IL-1β, TNF-α, IL-6, and IL-8 *via* the activation of p38 mitogen-activated protein kinases, nuclear factor (NF)-κB and activating protein-1 signal transduction pathways. Recently, the authors reported the overexpression of IL-32α in the inflamed mucosa of IBD. *In vitro* experiments on human intestinal epithelial cell lines showed that IL-32α expression was induced by IL-1β, IFN-γ and TNF-α through the activation of Akt-phosphatidylinositol 3-kinase.

Peer review
Kobori *et al* present an interesting study of a novel pro-inflammatory cytokine IL-32. They investigated the effects of butyrate on IL-32α expression in epithelial cell lines.

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ORIGINAL ARTICLE

Dicoumarol enhances gemcitabine-induced cytotoxicity in high NQO1-expressing cholangiocarcinoma cells

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Abstract

AIM: To investigate whether dicoumarol, a potent inhibitor of NAD(P)H quinone oxidoreductase-1 (NQO1), potentiates gemcitabine to induce cytotoxicity in cholangiocarcinoma cells (CCA) and the role of reactive oxygen generation in sensitizing the cells.

METHODS: Four human cell lines with different NQO1 activity were used; the human CCA cell lines, KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells. NQO1 activity and mRNA expression were determined. The cells were pretreated with dicoumarol at relevant concentrations before treatment with gemcitabine. Cytotoxicity was determined by staining with fluorescent dyes. Oxidant formation was examined by assay of cellular glutathione levels and reactive oxygen species production by using dihydrofluorescein diacetate. Measurement of mitochondrial transmembrane potential was performed by using JC-1 fluorescent probe. Western blotting analysis was performed to determine levels of survival related proteins.

RESULTS: Dicoumarol markedly enhanced the cytotoxicity of gemcitabine in KKU-100 and KKU-OCA17, the high NQO1 activity and mRNA expressing cells, but not in the other cells with low NQO1 activity. Dicoumarol induced a marked decrease in cellular redox of glutathione in KKU-100 cells, in contrast to KKU-M214 cells. Dicoumarol at concentrations that inhibited NQO1 activity did not alter mitochondrial transmembrane potential and production of reactive oxygen species. Gemcitabine alone induced activation of NF-κB and Bcl-$_{XL}$ protein expression. However, gemcitabine and dicoumarol combination induced increased p53 and decreased Bcl-$_{XL}$ levels in KKU-100, but not in KKU-M214 cells.

CONCLUSION: NQO1 may be important in sensitizing cells to anticancer drugs and inhibition of NQO1 may be a strategy for the treatment of CCA.

INTRODUCTION

NAD(P)H quinone oxidoreductase-1 (NQO1 or DT-diaphorase) is a ubiquitous flavoprotein localized widely in body tissues. It is an obligated two-electron reductase that reduces quinones to hydroquinones, thus bypassing the toxic semiquinone intermediates, and these resultant hydroquinones are thus ready for further conjugation and excretion[1]. Several functions of NQO1 have been proposed including xenobiotic detoxification, superoxide scavenging, maintenance of endogenous antioxidant, modulation of p53 and proteasomal degradation[2-4]. It is conceivable that NQO1 functions primarily to protect normal cells from oxidant stress and electrophilic attack. A number of experimental models and epidemiological studies support the concept that intake of dietary phytochemicals confers a cancer chemoprevention effect and these chemicals have been shown to induce increased expression of phase II drug detoxifying enzymes including NQO1[5,6]. The cytoprotective role of NQO1 is supported by reports that disruption of the *NQO1* gene or genetic polymorphism increase the risk of chemical-induced toxicity and carcinogenesis[7,8]. The expressions of *NQO1* and antioxidant enzymes are recognized as an adaptive response to chemical stress[6,9]. On the other hand, analysis of several solid tumors found an over-expression of the *NQO1* gene in cancers of the liver, thyroid, breast, colon, lung, and pancreas[10,11]. Under these circumstances, NQO1 probably functions to protect cancer cells by eliminating oxidant species and making cells resistant to anticancer drugs that induce oxidative injury[12].

Inhibition of NQO1 activity by dicoumarol has been shown to suppress urogenital and pancreatic cancer cell growth and potentiate cytotoxicity of cisplatin and doxorubicin[13,14]. The inhibition of NQO1 with dicoumarol was suggested to stimulate formation of superoxide, oxidative stress and subsequent suppression of pancreatic cancer cell growth and induction of apoptosis[15,16]. However, dicoumarol has been shown to induce formation of reactive oxygen species (ROS) independently from NQO1 activity by inhibition of the mitochondrial electron transport chain[17]. Therefore, the question as to whether inhibition of NQO1 renders cancer cells more sensitive to chemotherapeutic agents is still not clear. More study is necessary to define the role of NQO1 in cancer cells. Sensitizing cancer cells to be more susceptible to radiotherapy or chemotherapy may be an important strategy to overcome resistance in cancer chemotherapy.

Cholangiocarcinomas (CCA) are rare types of liver cancers arising from the biliary duct system. Surgical resection with a histologically free margin is the only chance for cure[18]. Unfortunately, only few patients are eligible for surgery. Furthermore, current chemotherapy and radiotherapy regimens do not substantially improve survival in CCA patients[18,19]. Gemcitabine has been the most important nucleoside analog which has wide spectrum activity against various solid and hematological tumors[20]. It shows some efficacy and is well tolerated in CCA patients[21]. It is essential to gain insight into molec-

ular mechanisms by which cancer cells operate to survive and evade the attack by chemotherapeutic agents.

Because NQO1 appears to be a potential target for exploitation in cancer chemotherapy, we investigated whether dicoumarol, a potent inhibitor of NQO1, sensitized CCA cells to respond to the cytotoxicity of gemcitabine. Furthermore, we examined whether dicoumarol- and gemcitabine-induced cell killing was associated with ROS generation, mitochondrial dysfunction and apoptotic protein expression.

MATERIALS AND METHODS

Human cell line cultures

Three human CCA cell lines established in our institute, KKU-100, KKU-OCA17 and KKU-M214, were derived from human intrahepatic CCA tissues with the histological types of poorly differentiated, well differentiated and moderately differentiated adenocarcinoma, respectively[22,23]. Chang liver cells were also used in the study. CCA cells and Chang liver cells were routinely cultured as previously described[24] in Ham's F12 media, supplemented with 4 mmol/L L-glutamine, 12.5 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), at pH 7.3, 100 U/mL penicillin 100 μg/mL streptomycin sulfate and 10% fetal calf serum. The media was renewed every 3 d, trypsinized with 0.25% trypsin-EDTA and subcultured in the same media.

NQO1 activity assay

NQO1 assay was performed essentially according to a previously published method[25]. Cells were seeded onto 96-well cultured plates overnight. Cells were then lysed with 50 μL of 0.8% digitonin in 2 mmol/L EDTA at room temperature for 10 min. The assay was performed using menadione and MTT [3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] in the substrate coupling reaction and measured as rate-kinetics in a micro-plate reader at a wavelength of 620 nm. The initial velocity of reaction was calculated as NQO1 activity using the extinction coefficient of formazan of MTT of 11 300 L/mol per cm and correction factor for the light path. In the determination of the enzymatic property of NQO1 in each cell line, cells were incubated with varying concentrations of dicoumarol for 10 min before carrying out assays as above.

Cytotoxicity assay

CCA cells (KKU-100, KKU-OCA17, and KKU-M214) and Chang liver cells were seeded onto 96-well cultured plates at a density of 5×10^3 cells/well (KKU-M214 and Chang cells) or 7.5×10^3 cells/well (KKU-100 and KKU-OCA17) for an overnight, then media was renewed with fresh media containing test compound and further incubated for the indicated times. The cytotoxicity was determined by fluorescence microscopy[26]. In brief, cells were washed once with phosphate-buffered saline (PBS) and the following added: 4 μL mixture of acridine orange, ethidium bromide (each 1 μg/mL) and trace amount of he-

moglobin. The cells were examined using a Nikon Eclipse TS100 inverted microscope with excitation and emission filters of 480 and 535 nm, respectively. The microphotographs were taken at predetermined three areas per well in triplicate wells per concentration with a Nikon Coolpix digital camera. The numbers of viable and non-viable cells were counted. The viable cells were colored green with intact nuclei. The non-viable cells included necrotic and apoptotic cells which showed bright orange staining and green fluorescence, with appearance of cell shrinkage and condensation and fragmentation of the nuclei, respectively. The antiproliferation value was calculated as = (number of viable cells in control wells - viable cells in treatment wells)/(number of viable cells in control wells) × 100.

Determinations of glutathione and glutathione disulfide

Total glutathione assay was performed essentially according to Tietze methods[27]. Glutathione disulfide (GSSG) was assayed by the previously described method[28] using 1-methyl-2-vinylpyridinium triflate (M2VP) as a glutathione scavenger. Cell cultures were trypsinized and washed three times with cold PBS buffer and centrifuged at 1500 *g*, 4℃ for 10 min and resuspended in PBS buffer. Cell suspension of 100 μL was reacted with M2VP (33 mmol/L in DI) or without M2VP. The solution was mixed gently and stored frozen at -20℃ until analysis. An aliquot of cell suspension was saved for protein determination by Bradford's dye binding assay.

Measurement of mitochondrial transmembrane potential

To measure the change in mitochondrial transmembrane potential ($\Delta\Psi_m$), the lipophilic cation fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) was used. After treatment with dicoumarol, gemcitabine or the combination at defined period of times, cultured cells were loaded with JC-1 mitochondrial membrane potential assay kit (Clayman Chemical, Ann Arbor, Michigan) by incubation for 30 min at 37℃. After that, cultured cells were rinsed, incubated in JC-1 assay buffer and mitochondrial transmembrane potential was analyzed by a fluorescent plate reader. In healthy mitochondria, JC-1 forms J-aggregates which display strong fluorescent intensity with excitation and emission wavelength at 560 and 595 nm, respectively. In depolarized mitochondria, JC-1 exists as J monomers which show strong fluorescence with excitation and emission wavelength at 485 and 535 nm, respectively. The shift down in ratio of fluorescent intensity of JC-1 aggregates to fluorescent intensity of monomers is used as an indicator of depolarization of $\Delta\Psi_m$.

Determination of formation of reactive oxygen species

Reactive oxygen species (ROS) levels generated from cultured cells were determined by incubating the cells in Hank's buffer supplemented with 15 mmol/L HEPES containing 1.2 μg/mL dihydrofluorescein diacetate (H2-DHFDA) (Sigma-Aldrich, St. Louis, MO) for 30 min at 37℃. H2-DHFDA was taken up into the cells, hydrolyzed and oxidized to the fluorescent product DHF by ROS. The fluorescent signal was determined by a fluorescent plate reader with a setting of the excitation and emission wavelengths at 485 and 520 nm, respectively.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from liver tissues and the four cell lines using Trizol®LS reagent following the manufacturer's instructions. Total RNA (3 μg) was reverse-transcribed in 20 μL containing 0.5 μg of oligo(dT)15 primer, 20 U of RNasin® ribonuclease inhibitor and 200 U of ImProm-II™ reverse transcriptase in 10 × PCR buffer, 3 mmol/L MgCl2, and 1 mmol/L dNTPs. The first-strand cDNA was synthesized at conditions of 42℃ for 60 min. The reverse transcription products served as a template for real-time PCR. PCR amplification was performed using specific primers for the NQO1 and the internal control using FDFT1. The PCR primer sequences were as follows: NQO1; forward primers: 5'GGCAGAAGAG-CACTGATCGTA3', NQO1; reverse primers: 5'TGAT-GGGATTGAAGTTCATGGC3', GenBank accession number BC007659.2, FDFT1; forward primers: 5'TTTA-ACTTCTGTGCTATTCCAC3', FDFT1; reverse primers: 5'TCTCCAGTCTGAACATAGTC3', GenBank accession number NM_004462.3. The real-time fluorescence PCR, based on SYBR Green, was carried out in a final volume of 20 μL containing 1 × SYBR Green PCR Master Mix (DyNAmo™ Flash SYBR® Green qPCR Kit), 0.5 μmol/L of each NQO1 or FDFT1 primer. Thermal cycling was performed for each gene in duplicate on cDNA samples in 96-well reaction plates using the ABI 7500 Sequence Detection system (Applied Biosystems). A negative control was included in the experimental runs. The negative control was set up by substituting the template with deionized H2O and this routinely had a high Ct value which represented the lower detection limit. Real-time PCR was conducted with the following cycling conditions: 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s, 55℃ for 30 s and 72℃ for 45 s. To verify the purity of the products, a melting curve analysis was produced after each run. Upon completion of 40 PCR amplification cycles, there was a dissociation step of ramping temperature from 60℃ to 95℃ steadily for 20 min, while the fluorescence signal was continually monitored, for melting curve analysis. The relative expression ratio (R) of target genes is calculated based on efficiency (E) and Ct deviation and expressed in comparison to a reference gene. The corresponding real time PCR efficiencies were calculated according to the equation $E = 10^{[-1/slope]}$. All data were analyzed using Sequence Detector Software Version 1.4 (Applied Biosystems).

Western blotting analysis of whole cell and nuclear protein extracts

The whole cell lysates and nuclear protein were prepared according to a previous report[29]. Treated KKU-100 and KKU-M214 cells were washed with PBS, collected and lysed at 4℃ with cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium py-

rophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] with vigorous shaking. Following centrifugation at 10 000 *g* for 15 min, supernatant was collected and stored at -80℃ until use. Nuclear protein was prepared by lysis of cultured cells with hypotonic buffer A [10 mmol/L HEPES-KOH pH 7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 0.1 mmol/L EGTA], incubated in an ice bath for 15 min and then 1% NP-40 was added, cells were centrifuged at 12 000 *g*, 4℃ for 15 min, the nuclear pellet was resuspended in ice-cold buffer B (20 mmol/L HEPES-KOH pH 7.9, 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, and 1 mmol/L EGTA), followed by incubation at 0℃ for 45 min. After vortex mixing, the suspension was centrifuged at 12 000 *g*, 4℃ for 30 min. The supernatant containing nuclear proteins was stored at -80℃ for the NF-κB Western immunoblot analysis.

The protein samples were mixed with 5 × loading dye buffer, heated at 95℃ for 5 min and proteins were separated by electrophoresis in 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 50 V for 2 h. The PVDF membranes were blocked for 1 h at room temperature with 5% (w/v) skimmed milk powder in Tris buffer saline (TBS) with 0.1% Tween-20. PVDF membrane was incubated overnight at 4℃ with primary antibodies of rabbit polyclonal IgG NF-κB p65 subunits (dilution 1:500) (sc-109: Santa Cruz Biotechnology), mouse monoclonal IgG Bcl-xL (1:1000) (sc-8392), rabbit polyclonal IgG Bax (1:2000) (sc-493), rabbit polyclonal IgG cyclin D1 (1:1500) (sc-718), and mouse monoclonal IgG1 β-actin (1:2500) (sc-8432) diluted with TBS. The primary antibody was then removed and the blots were extensively washed with TBS/Tween-20. Blots were then incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase goat anti-mouse IgG (sc-2005) and goat anti-rabbit IgG (sc-2004) at 1:5000 dilutions in TBS buffer. After removal of the secondary antibody and washes in TBS buffer, the blots were incubated in the ECL substrate solution (Supersignal® West Pico Chemiluminescent Substrate). Densities of the specific bands of NF-κB, Bcl-xL, Bax, cyclin D1, and β-actin were visualized and captured by Imagequant 350 (GE Healthcare).

Statistical analysis
Data are expressed as mean ± SE of duplicate assays from three independent experiments. An analysis of variance with repeated measurement was used to determine significant differences between each experimental group. The level of significance was set at $P < 0.05$.

RESULTS

Activity and expression of NQO1 in CCA cells
Three CCA cell lines with different histological back-

Figure 1 NQO1 activity and mRNA expression of cultured cells. A: NQO1 activity: Cholangiocarcinoma cells, KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates for assay of NQO1 activity by enzymatic methods; B: Expression of NQO1 mRNA: The cells were cultured in 6-well plates. Total RNA was extracted by Trizol reagent, converted to cDNA and analyzed by real-time PCR using FDFT1 as internal control. Bars represent mean ± SE, each from 3 experiments. [a]$P < 0.05$ *vs* control group.

grounds were employed in this study for assessing the status of NQO1. The CCA cells illustrated varying degrees of NQO1 activity and mRNA expression. KKU-100 and KKU-OCA17 cells had high NQO1 activity, whereas KKU-M214 showed low NQO1 activity. Moreover, Chang liver cells, which were derived from normal liver tissue, showed relatively low NQO1 activity and were comparable to KKU-M214 cells (Figure 1A). Consistently, NQO1 mRNA expression in KKU-100 cells showed higher levels than in the other cell lines (Figure 1B).

Sensitivity of NQO1 to dicoumarol
Dicoumarol is a very potent inhibitor of NQO1 activity. All four cell lines were tested against dicoumarol. NQO1 in CCA and Chang cell lysates were assayed for kinetics of inhibition by dicoumarol. The inhibition of NQO1 activity was rapid, within 10 min, and was apparent with a comparable potency among the 4 cell lines (Figure 2) with IC50 of 0.15 ± 0.07, 0.15 ± 0.07, 0.24 ± 0.14 and 0.10 ± 0.22 μmol/L for KKU-100, KKU-OCA17, KKU-M214 and Chang cells, respectively. Since the enzyme activity in all cell lines was almost completely abolished at a dicoumarol concentration of 10 μmol/L, in subsequent experiments dicoumarol was used at the concentration of 10 μmol/L.

Figure 2 Concentration response of NQO1 inhibition by dicoumarol. KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates overnight. The NQO1 activity was assayed in the presence of the inhibitor dicoumarol at concentrations from 0.1-10 μmol/L. Bars represent mean ± SE, each from 3 experiments. [a]$P < 0.05$ *vs* control group.

Enhancement of cytotoxicity of gemcitabine by dicoumarol

To investigate whether inhibition of cellular NQO1 activity was associated with increased anticancer drug sensitivity, CCA cells were preincubated with dicoumarol at varied concentrations for 4 h followed by an addition of gemcitabine at predetermined concentrations. The concentrations of gemcitabine that caused cytotoxicity of about 20%-30% were used in the experiments. Those for KKU-100, KKU-OCA17, KKU-M214 and Chang cells were 1, 10, 1 nmol/L and 10 μmol/L, respectively. Treatment with dicoumarol alone at concentrations of 0.1-10 μmol/L caused modest cytotoxicity after incubation for 24 h. Dicoumarol at a concentration of 10 μmol/L induced ≤ 10% cytotoxicity in all cell types.

Combination of gemcitabine and dicoumarol produced a markedly enhanced cytotoxic effect, particularly in KKU-100 and KKU-OCA17 cells. The enhanced cytotoxic effect in both cell lines was conceivably more than a simple additive effect of the drug and dicoumarol (Figure 3). The cytotoxicity of gemcitabine in KKU-100 cells was enhanced from 34% to 68% and in KKU-OCA17 cells from 25% to 47% in the presence of dicoumarol. On the other hand, the drug combination produced only an additive cytotoxicity in KKU-M214 and Chang cells, i.e. increased from 23% to 36% and 23% to 40%, respectively. It is noted that KUU-100 and KKU-OCA17 cells are high NQO1 activity cells when compared with KKU-M214 and Chang cells.

Dicoumarol-induced oxidative stress in CCA cells

The interactive effect of dicoumarol with gemcitabine in enhancing cytotoxicity was explored with regard to whether dicoumarol induced cellular stress, rendering the cells more susceptible to gemcitabine. In this study, KKU-100 and KKU-M214 cells were employed as representatives of high and low NQO1 activity cells, respectively. The basal total GSH levels in KKU-M214 were higher than that in KKU-100 cells and significantly increased by the treatment with dicoumarol (10 μmol/L)

Figure 3 Potentiation of cytotoxicity of gemcitabine by dicoumarol. KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells were cultured in 96-well plates overnight. Cultured cells were pretreated with dicoumarol (0.1-10 μmol/L) for 4 h before treatment with gemcitabine at 1 nmol/L, 10 nmol/L, 1 nmol/L and 10 μmol/L, for KKU-100, KKU-OCA17, KKU-M214, and Chang liver cells, respectively. Antiproliferation was analyzed by staining the cells with fluorescent dyes before examination under fluorescent microscope. Bars represent mean ± SE, each from 3 experiments. [a]$P < 0.05$ *vs* control group.

(Figure 4A). On the other hand, glutathione disulfide (GSSG) levels in KKU-100 were dramatically increased with the treatment of dicoumarol, whereas the levels in KKU-M214 were decreased (Figure 4B). This indicates that the two cell types have different oxidative responses after the treatment with dicoumarol.

Determination of the mitochondrial transmembrane potential and formation of reactive oxygen species

Disruption of $\Delta\Psi_m$ is recognized as one of the critical steps leading to apoptotic cell death. The integrity of the inner mitochondrial membrane can be assessed by monitoring of the potential gradient across the membrane using the fluorescent dye JC-1. There was no change in $\Delta\Psi_m$ in cells after treatment with dicoumarol, gemcitabine or the drug combination in both cell types, at an early time (6 h) as well as at 24 h (Figure 5A and B). In contrast, dicoumarol at excessive concentrations (50 and 150 μmol/L) showed a rapidly depolarized $\Delta\Psi_m$, as shown by decrease in the ratio of fluorescent intensity of JC-1 aggregates/JC-1 monomers after 3 h incubation (Figure 5C). The formation of ROS by cultured cells was monitored by a fluorescent dye, DHFDA. It was apparent that there was no increase in ROS formation in cells treated with dicoumarol, gemcitabine or the combination in both cell lines (data not shown), even at a concentration and time where cell apoptosis was evident. High concentrations of dicoumarol also did not induce ROS production, even at those concentrations which caused cell death.

Alteration of survival response proteins induced by gemcitabine and dicoumarol

In order to understand how the combination of gemcitabine and dicoumarol enhanced cytotoxicity in high NQO1 activity cells, proteins related to cell survival were analyzed by Western immunoblotting. Protein p53 is a tumor suppressor protein that responds to various noxious stimuli.

Figure 4 Glutathione redox status in cells treated with dicoumarol. KKU-100 and KKU-M214 cells were cultured and treated with dicoumarol (10 μmol/L) for 4 h, and cells were scraped for assays of (A) total glutathione and (B) glutathione disulfide. Bars represent mean ± SE, each from 3 experiments. [a]$P < 0.05$ *vs* control group.

Dicoumarol or gemcitabine treatment induced increased p53 levels in KKU-100 cells and the level was further increased during the treatment with the drug combination. On the other hand, dicoumarol or gemcitabine or the combination did not alter p53 levels in KKU-M214 cells. Bcl-2 proteins consist of proapoptotic (Bax) and antiapoptotic proteins (Bcl-XL) where they regulate mitochondria outer membrane permeabilization. Bcl-XL levels were induced by gemcitabine in both cell types, however, the combination of gemcitabine with dicoumarol suppressed elevated Bcl-XL levels in KKU-100 cells but not in KKU-M214 cells. On the other hand, the changes in Bax levels were found with a similar pattern in both cell types, i.e. Bax levels were induced with gemcitabine, while drug combination induced no changes when compared with controls. Cyclin D1 levels were decreased upon the treatment with dicoumarol in both cell types, but not by gemcitabine. However, the drug combination apparently suppressed cyclin D1 levels when compared with gemcitabine alone.

The expression of the nuclear p65 subunit of NF-κB was slightly increased after the treatment with gemcitabine and showed little change induced by the co-treatment with dicoumarol in both cell lines. Figure 6 shown the Western analysis of proteins related to survival.

DISCUSSION

NQO1 is one of the attractive targets in development

Figure 5 Assay of mitochondrial transmembrane potential and reactive oxygen in CCA cells. The mitochondrial transmembrane potential was analyzed by using JC-1 fluorescent probe. Fluorescent readings of the J-aggregates and J monomers were used as measurement of mitochondrial transmembrane potential. KKU-100 and KKU-M214 cells were cultured in 96-well black plates. The cultured cells were pretreated with dicoumarol at 10 μmol/L for 4 h, then gemcitabine at 1 nmol/L was added and incubated at various times. A: Incubation for 6 h; B: Incubation for 24 h; C: Other cultured cells were treated with dicoumarol at 50 and 150 μmol/L for 3 h. Bars represent mean ± SE, each from triplicate assay. [a]$P < 0.05$ *vs* control group.

of chemotherapy, since NQO1 may function to protect normal cells, as well as tumor cells, particularly when it is highly expressed[12,30]. Suppression of NQO1 induced by proinflammatory cytokines or genetic defects may render normal cells susceptible to oxidant-mediated cell toxicity and carcinogenesis[7,8,22]. Inhibition of NQO1 activity with dicoumarol at relevant concentrations which produced modest cytotoxicity when administered alone potentiated gemcitabine-induced cytotoxicity in cells with high NQO1 activity. The combination of dicoumarol with gemcitabine

Figure 6 Western blotting analysis of proteins related to survival. KKU-100 and KKU-M214 cells were cultured overnight, pretreated with 10 μmol/L dicoumarol for 4 h before being treated with 1 nmol/L gemcitabine for 24 h. Cultured cells were collected for Western blotting analysis using β-actin as an internal control for equal protein loading. A: KKU-100 cells; B: KKU-M214 cells. Cont: Controls; Dic: Dicoumarol alone; Gem: Gemcitabine alone; Gem + Dic: Combination of gemcitabine and dicoumarol. Values were an average from two experiments of the target protein normalized with the internal control.

enhanced p53 and decreased Bcl-XL expression, and this may be related to the mechanism of cell sensitization and killing.

NQO1 is well known to function as a drug metabolizing and antioxidant enzyme, protecting the cell from oxidative injury[3]. Previous studies suggested that NQO1 modulates p53 expression by interference with 20S proteasome-mediated degradation of p53[3,31]. *P53* is a tumor suppressor gene that upon stimulation by DNA damage or oxidative stress, induces either growth arrest or apoptosis[32]. Our study showed that dicoumarol increased levels of p53 in KKU-100 cells, but these levels were unchanged in KKU-M214 cells. The increased p53 expression, together with decreased Bcl-XL protein expression, was associated with enhanced cytotoxicity of gemcitabine. However, dicoumarol has been previously reported to decrease p53 protein levels and this was associated with either inhibition or induction of apoptosis in myeloid leukemic cells or urogenital cancer cells, respectively[14,31]. The discrepancy in p53 levels induced by the treatment with dicoumarol is not readily comprehensible, although our study used much lower concentrations of dicoumarol. Moreover, treatment of gemcitabine apparently induced the activation of NF-κB and expression of Bcl-XL. This increase of antiapoptotic proteins may be a survival signal engaged in response to gemcitabine[33]. Interestingly, the combination with dicoumarol diminished the expression

of Bcl-XL in KKU-100 cells concurrent with potentiation of the cytotoxicity of gemicitabine, while this did not happen in KKU-M214 cells.

Protein p53 and the Bcl-2 protein family regulate mitochondrial outer membrane permeabilization[34]. Induction of apoptotic cell death is often associated with disruption of the mitochondrial inner membrane which is reflected as dissipation of $\Delta\Psi_m$ and formation of mitochondrial permeability transition pore (MPTP) with subsequent cell death[35]. In the treatment with low concentrations of dicoumarol with or without gemcitabine, $\Delta\Psi_m$ appeared to be well maintained according to the assay of JC-1, despite the drug combination inducing marked CCA cell killing. This suggests no disruption of the mitochondrial inner membrane at the concentrations that potentiate gemcitabine cytotoxicity. Likewise, it was shown that prostaglandin A2 induced human promyelocytic leukemia cell death associated with cytochrome c release, with no change in $\Delta\Psi_m$[36]. However, dicoumarol at the high concentrations caused disruption of $\Delta\Psi_m$ and rapid cell killing which is consistent with many previous reports[14,17].

Dicoumarol has been suggested to trigger cell killing *via* increased formation of ROS[15-17]. We did not observe an increased ROS formation when cells were treated with dicoumarol or dicoumarol with gemcitabine combination. Dicoumarol, even at high concentrations

(150 μmol/L) that rapidly killed both types of CCA cells within 3 h of incubation (data not shown), had no significant effect on ROS formation. This suggests that superoxide formation may not be an important mediator of cell killing by dicoumarol[17]; it is probable that dicoumarol exerts its cell killing mechanism on CCA cells differently from other epithelial cells.

GSH, a tripeptide found abundantly in cells, functions as antioxidant and regulates activities of various redox-sensitive proteins[37]. Although dicoumarol did not increase formation of ROS, it induced oxidant stress in KKU-100 cells, but not in KKU-M214 cells, evidently by increasing and decreasing GSSG levels, respectively. The increased cellular oxidant may be implicated in several signal transduction pathways which eventually lead to enhanced susceptibility of KKU-100 cells. Nonetheless, dicoumarol has been reported to show several different pharmacological activities[15,17,38] and apoptotic cell killing may be dissociated with NQO1 inhibition[39], as some synthetic mechanism-based NQO1 inhibitors have shown lack of association between enzyme inhibition and cytotoxic effect. The discrepancy of the reports may be explicable partly on the grounds that the sensitizing effect of dicoumarol may be dependent on inherent activity of NQO1 in these cell types.

In conclusion, NQO1 may play roles in the sensitivity of CCA cells to gemcitabine. When using dicoumarol at relevant concentrations to inhibit NQO1 activity, dicoumarol enhances gemcitabine cytotoxicity in high NQO1 activity CCA cells. Furthermore, the mechanism of dicoumarol-induced cell killing may not be mediated *via* disruption of mitochondrial function and formation of ROS, but may be related to suppression of the pro-survival response to the chemotherapy. NQO1 is a potential target in the development of chemotherapy for tumors with high enzyme expression.

COMMENTS

Background
NAD(P)H quinone oxidoreductase-1 (NQO1) is a drug metabolizing and antioxidant enzyme which plays important roles in protection of cells against electrophiles and oxidants. Some cancers exhibit high activity of NQO1 which suggests a cytoprotective role in the cancer.

Research frontiers
Inhibition of NQO1 activity may abate the adaptive survival response of cancer cells induced by gemcitabine therapy and render cancer cells more susceptible to the drug.

Innovations and breakthroughs
This report shows that dicoumarol, a potent inhibitor of NQO1 at low concentrations, potentiates cytotoxicity of gemcitabine in cholangiocarcinoma cells which inherently have high NQO1 activity. Disruption of cellular glutathione redox may be associated with modulation of redox-sensitive signaling proteins such as p53 and render enhanced cells susceptible to gemcitabine.

Applications
Inhibition of NQO1 is suggested to be a potential strategy for development in cancer chemotherapy, particularly for cholangiocarcinoma which was used in this study.

Peer review
The methodology and the results are appropriate and support the findings in the literature.

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ORIGINAL ARTICLE

Effect of 5-Aza-2'-deoxycytidine on immune-associated proteins in exosomes from hepatoma

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Abstract

AIM: To study the effect of 5-Aza-2'-deoxycytidine (5-Aza-CdR) on heat shock protein 70 (HSP70), human leucocyte antigen-Ⅰ (HLA-Ⅰ) and NY-ESO-1 proteins in exosomes produced by hepatoma cells, HepG2 and Hep3B.

METHODS: Exosomes derived from HepG$_2$ and Hep3B cells treated with or without 5-aza-CdR were isolated and purified by ultrafiltration centrifugation and sucrose gradient ultracentrifugation. The number of exosomes was counted under electron microscope. Concentration of proteins in exosomes was measured by bicinchoninic acid protein assay. Expression of HSP70, HLA-Ⅰ and NY-ESO-1 proteins in exosomes was detected by Western blotting and immunoelectron microscopy. mRNA expression of p53 gene was detected by reverse transcription polymerase chain reaction.

RESULTS: The mRNA expression of p53 gene was increased in both hepatoma cell lines after treatment with 5-Aza-CdR. The number of exosomes and the concentration of total proteins in exosomes were increased significantly after treatment with 5-aza-CdR (P < 0.05). After treatment with 5-Aza-CdR, immunoelectron microscopy and Western blotting showed that the HSP70, HLA-Ⅰ and NY-ESO-1 proteins were increased in exosomes produced by both hepatoma cell lines.

CONCLUSION: 5-aza-CdR, an inhibitor of DNA methyltransferase, can increase exosomes produced by hepatoma cells and immune-associated protein component of exosomes, which may be mediated by p53 gene upregulation and 5-Aza-CdR demethylation.

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an annual incidence of over half a million. Despite the improvements in surveillance, imaging technology, surgical techniques, and perioperative care, its mortality rate still increases, ac-

counting for 53% of all liver cancer deaths worldwide in China[1]. The most effective treatment modalities available for HCC to date are surgical resection, liver transplantation, and ablative therapy[2,3]. However, they are not indicated for patients who have relapse or are at advanced stage of the disease[4]. Moreover, the most commonly used treatment methods for various cancers, such as radiotherapy and chemotherapy, are often excluded in treatment of HCC due to their intolerable toxicity and insensitivity[5]. It is therefore necessary to develop a novel strategy against the progression and recurrence of HCC.

Exosomes secreted by tumor cells have become a recent hot spot in research of tumor immunology, raising intriguing attention to their immune-stimulating function *in vitro* and tumor model experiments[6,7]. However, acquiring a sufficient number of exosomes with a high quality for more powerful immune-stimulating effects has remained a great challenge for tumor immunotherapy[8-10]. Apart from the p53-dependent pathway, the mechanisms by which tumors secrete exosomes have not been well understood[11].

5-Aza-2'-deoxycytidine (5-Aza-CdR), a DNA methyltransferase inhibitor and a demethylation promoter in CpG regions of many genes, including the *p53* gene, can significantly restore or increase their expression[12,13], including the expression of *p53* by damaging DNA[14]. It has been shown that 5-Aza-CdR can significantly increase the expression of immune molecules necessary for anti-tumor cellular immunity by demethylating DNA, such as human leucocyte antigen (HLA)-Ⅰ, and HLA-Ⅱ, and significantly enhance the therapeutic effect of anti-tumor immunity *in vitro* and in animal experiments[15-17]. However, few reports are available on the effects of 5-Aza-CdR on the secretion of exosomes and the protein level in exosomes. This study was to explore the effect of 5-Aza-CdR on the secretion of exosomes, tumor-associated antigens and immune molecules in exosomes, and its mechanisms by which hepatocellular carcinoma cell lines secrete exosomes, in an attempt to provide preliminary experimental evidence for 5-Aza-CdR-modified exosomes-based anti-hepatoma immunotherapy.

MATERIALS AND METHODS

Materials
HepG2 cell line was generously provided by Professor You-Yong Lu, Beijing Cancer Institute. Hep3B cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS).

Drugs and reagents
5-Aza-CdR, heavy water, cane sugar (analytically pure) and protein A colloidal gold (SPA) were purchased from Sigma Company (Santa Clara, CA, USA). FBS and DMEM culture media were purchased from GIBCo Company (Carlsbad, CA, USA). Western blotting reagents used in this study included rabbit anti-human heat shock protein 70 (HSP70) polyclonal antibody from Abcam (Cambridge, UK), mouse anti-human HLA-Ⅰ monoclonal antibody from Chemicon

(Los Angeles, CA, USA), mouse anti-human NY-ESO-1 monoclonal antibody from ZyMed (San Diego Diego, South CA, USA). Western blotting kit was obtained from Pierce (Rockford, IL, USA), and BCA protein assay kit was from Puli Lai Gene Technology Co., Ltd (Beijing, China).

Instruments
Instruments used in this study included a Himac-CP70G low-temperature ultra-high speed centrifuge and a Hitachi TEM H-7500 transmission electron microscope (Hitachi Corporation, Tokyo, Japan). Electrophoresis devices used in this study included an electrophoresis tank and a trans-membrane tank (Beijing 61 Instrument Factory, China), a GelDoc2000 gel imager (Bio-Rad Corporation, Chicago, IL, USA), a 100 ku MWCO Centriplus centrifugal ultrafiltration tube and a 100 ku MWCO Millipore Amicon high recovery-high-flow tangential flow ultrafiltration centrifuge tube (Millipore Corporation, Bedford, MA, USA).

Cell culture
Human hepatoma cell lines, HepG2 and Hep3B, were maintained at 37℃ in 10% DMEM containing 10% FCS (Gibco Corporation, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Corporation, Santa Clara, CA, USA). HepG2 and Hep3B cells were divided into 3 control groups and 3 experimental groups, respectively, for regular culture. Cell viability was 95% as determined by trypan blue exclusion. Twenty-four hours after inoculation, cells in experimental groups were treated with 5-aza-CdR at a concentration of 1×10^{-6} mol/L and 150 mL of culture supernatant was collected 72 h later from each group, while cells in control groups were cultured without any drug and 150 mL of culture supernatant was collected from each group as controls.

Isolation and purification of exosomes
Exosomes were isolated as previously described[18]. In brief, 150 mL of a medium from confluent cultures (5-7 d) was harvested and centrifuged twice (2000 *g* and 10 000 *g*) to remove cells and debris. Clarified supernatant was then ultrafiltrated using 100 ku MWCO Centriplus centrifugal ultrafiltration tubes to remove big-molecule compounds and reduce the volume of samples before ultracentrifugation on a 30% sucrose/D$_2$O cushion, which was collected and diluted in PBS. Ultrafiltration was then performed using a 100 ku MWCO Millipore Amicon high recovery-high-flow tangential flow ultrafiltration centrifuge tube. Finally, 5 mL of exosomes was obtained and stored at 4℃ for no more than 48 h before use.

Reverse transcription polymerase chain reaction
Total RNA was extracted from HepG2 and Hep3B cells using a Promega's total RNA extraction kit 72 h before and after 5-Aza-Cdr treatment. The sequences of p53 upstream and downstream primers used in this study are 5'-ACCCAGGTCCAGATGAAG-3' and 5'-CACTCG-GATAAGATGCTGA-3', respectively. The length of amplified fragments was 422 bp. The sequences of β-actin upstream and downstream primers used in this

study are 5'-CGGGAAATCGTGCGTGACATT-3' and 5'-GGAGTTGAAGGTAGTTTCGTGG-3', respectively. The length of amplified fragments was 150 bp. RT reaction system contained 2 μg total RNA, 150.5 μg oligo (dT), 5 × PCR buffer, 1 mmol/L dNTP, 20U RNasin, 200 U M-MLV reverse transcriptase. PCR system contained 2 μL reverse transcriptase reaction products, 500 pmol/L of each upstream and downstream primer, 200 μmol/L of each dNTP, 1 U Taq DNA synthesis enzyme, and 10 × PCR buffer. The total reaction volume was 20 μL. Thirty cycles of PCR amplification were performed under the following conditions: predenaturation at 94℃ for 5 min, denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, extension at 72℃ for 30 s, and a final extension at 72℃ for 5 min. Finally, 6 uL of PCR products was electrophoresed on 1.5% agarose gel. The results were recorded using a gel imaging system for semi-quantitative analysis by grey ratio of target gene to β-actin. Reverse transcription polymerase chain reaction (RT-PCR) reagents were purchased from Promega Company (San Luis Obispo, CA, USA) and primers were synthesized in Beijing Bioko Biotech Co., Ltd (Beijing, China).

Marking and counting exosomes under colloidal gold immunoelectron microscope

An antigen-antibody complex was prepared. In brief, 10 μL of exosomes was obtained and mixed with an equal volume of rabbit anti-human HSP70 monoclonal antibody (1:50 dilution), mouse anti-human HLA- I monoclonal antibody (1:100 dilution) or mouse anti-human NY-ESO-1 monoclonal antibody (1:50 dilution), respectively. These samples were dropped onto a copper mesh surface and incubated for 1 h at room temperature. Twenty μL of SPA diluted at 1:15 was dropped onto the hydrophobic membrane to form liquid beads. The copper mesh was floated in SPA droplets with its membrane surface faced down at room temperature for 30 min. Then, 15 μL of uranyl acetate drops was put onto the copper mesh surface and stained at room temperature for 30 s. A SPA-coated copper mesh was taken as a control. Exosomes with black colloidal gold particles on the capsular membrane and cavity were marked as positive under transmission electron microscope.

Quantification of exosome immune molecules under electron microscope

Exosomes and their labeled colloidal gold particles were counted under each camera view of exosome samples. Each protein was represented by the number of colloidal gold particles in 100 exosomes. The data were expressed as mean ± SD for further statistical analysis.

Exosome counting under electron microscope

The area under each photographic view was 1000 nm × 700 nm. The number of exosomes under each field of vision was counted. The average number of exosomes under each camera view was calculated in 10 randomly selected horizons. The number of exosomes in each mL supernatant of the cells was figured out by copper mesh diameter (2.5 mm) and area (1.25 mm × 1.25 mm × 3.14). The cells in each of experimental and control groups were counted three times and averaged for statistical analysis.

Western blotting

Forty microgramme of exosomes was taken and 10% SDS-PAGE electrophoresis was performed at a constant electric power. After electrophoresis, the gels were transferred to NC membrane, incubated with HSP70, HLA- I and NY-ESO-1 antibodies diluted at 1:500 overnight at 4℃. Then, horseradish peroxidase-tagged antibody was added and incubated at room temperature for 2 h. Photography was performed with a Kodak X-OMAT film (Eastman Kodak, Rochester, NY). The light absorption value A of target bands and β-actin was determined using the imaging analysis system. The results were indicated by the absorption ratio of target bands and β-actin, and averaged from three independent experiments.

Determination of protein concentration in exosomes

Exosomes in two cell lines of experimental and control groups were taken to determine the protein concentration with a bicinchoninic acid (BCA) protein assay kit following its manufacturer's instructions.

Statistical analysis

The data were expressed as mean ± SD. Statistical analysis was performed using the SPSS13.0 statistical software. $P < 0.05$ was considered statistically significant.

RESULTS

Exosomes and their related immune molecules identified by immunoelectron microscopy

Immunoelectron microscopy showed that exosomes, secreted from HepG2 and Hep3B cells, had the membranous structure of microcapsules, 30-80 nm in diameter. They were round or oval and their cavities were full of components with a low electron density. After colloidal gold immunoelectron marking, dotted, granular colloidal gold markers could be observed in the capsular membranes and cavities. Each kind of immune molecules had no more than two gold-labeled particles in the control groups. However, after treatment with 5-Aza-CdR, 5, 4, and 3 gold-labeled HSP70, HLA- I and NY-ESO-1 molecules were observed in exosomes, respectively. The number of gold-labeled particles in each immune molecule was significantly greater in experiment groups than in control groups ($P < 0.05$, Figure 1, Table 1).

Tumor antigen and immune molecules in exosomes detected by Western blotting

In the supernatant of exosomes from two cell lines, a clear specific protein band could be detected by Western blotting at the molecular weights of 73, 40 and 18 kDa. After treatment with 5-Aza-CdR, the HLA- I and NY-ESO-1

Figure 1 Immunoelectron microscopy showing colloidal gold HSP70 labeled HepG2 exosomes in control group (A) and in experiment group (B), colloidal gold HLA- I labeled HepG2 exosomes in control group (C) and experiment group (D), colloidal gold NY-ESO-1 labeled HepG2 exosomes in control group (E) and experiment group (F), colloidal gold HSP70 labeled Hep3B exosomes in control group (G) and experiment group (H), colloidal gold HLA- I labeled Hep3B exosomes in control group (I) and experiment group (J), colloidal gold NY-ESO-1 labeled Hep3B exosomes in control group (K) and experiment group (L) (× 140 000).

Table 1 Immune-related molecules of exosomes in control and experimental groups (mean ± SD)

	n	HepG2		*P*	Hep3B		*P*
		Control group	Experiment group		Control group	Experiment group	
HSP70	3	74.2 ± 3.1	99.8 ± 5.2	< 0.01	70.2 ± 5.1	99.9 ± 3.8	< 0.01
HLA- I	3	53.3 ± 2.5	79.6 ± 6.1	< 0.01	40.6 ± 2.7	86.7 ± 3.6	< 0.01
NY-ESO-1	3	38.2 ± 4.2	58.2 ± 4.3	< 0.01	32.1 ± 4.6	70.3 ± 2.9	< 0.01

expression level was significantly higher in experimental groups than in control groups ($P < 0.05$), while the HSP70 expression did not increase significantly (Figure 2).

Exosomes and protein in exosomes under electron microscope

The number of exosomes secreted from both cell lines and the total protein in exosomes were significantly higher after treatment with 5-Aza-CdR than before treatment with 5-Aza-CdR ($P < 0.05$, Table 2).

Change of wild-type P53 gene expression in HepG2 and Hep3B cells after 5-Aza-CdR treatment

The *p53* gene was moderately expressed in HepG2 cells but not in Hep3B cells before 5-Aza-CdR treatment. However, the *p53* gene mRNA was significantly expressed in two cell lines after 5-Aza-CdR treatment (Figure 3).

DISCUSSION

5-Aza-CdR can recover some genes with CpG islands in their promoter regions, or significantly increase the expression of these genes by directly inhibiting the DNA methyltransferase activity[19]. It has been shown that DNA methylation can regulate cancer/testis antigen (CTA), MHC I , MHC II, and a variety of immune adhesion molecules[20,21]. 5-Aza-CdR can significantly increase their expression in tumor cells and improve anti-tumor immune response capacity[17].

Exosomes, originating as vesicles in some late endosomes, would release when these mature endosomes or multivesicular bodies (MVBs) are fused with cell membranes. Exosomes carry a variety of specific proteins, such as antigen-presenting-associated protein, T cell activation-associated protein, and some tumor antigens and antigen

Table 2 Exosomes and their total protein level in hepatoma cells (mean ± SD)							
Subject	*n*	HepG2 con	HepG2 exp	*P*	Hep3B con	Hep3B exp	*P*
Exo No	3	$3.56 \times 10^8 \pm 2.6 \times 10^2$	$4.53 \times 10^9 \pm 3.7 \times 10^2$	0.010	$3.12 \times 10^8 \pm 6.2 \times 10^2$	$4.13 \times 10^9 \pm 3.7 \times 10^2$	0.021
Exo Pro	3	0.92 ± 0.13 mg	1.31 ± 0.16 mg	0.012	0.89 ± 0.10 mg	1.52 ± 0.16 mg	0.011

Exo: Exosome; con: Control group; exp: Experiment group.

Figure 2 Immune molecules Western blotting results (A), semi-quantities results of Western blotting (B). 1: HepG2 control; 2: HepG2 experiment; 3: Hep3B control; 4: Hep3B experiment.

Figure 3 RT-PCR results of *p53* gene expression (A), semi-quantities results of RT-PCR (B). M: Marker; C: Control group; E: Experiment group.

chaperones[7]. Exosomes themselves offer an antigen delivery system and can transfer tumor antigens to antigen-presenting cells, causing anti-tumor responses[22].

For these reasons, use of exosomes in immunotherapy and research requires high-quality exosomes. However, the mechanism underlying exosome secretion has not been well elucidated. Yu *et al*[11] found that activation of wild-type p53 increases exosome production by upregulating a transmembrane protein, TSAP6, which selectively transports proteins to exosomes and adjusts exosome formation. 5-Aza-CdR can increase *p53* gene expression by damaging or methylating DNA[12,14]. In addition, it has been reported that 5-Aza-CdR increases the expression of CTA, HLA-Ⅰ, and HLA-Ⅱ through demethylation, thus improving anti-tumor specific immune response and decreasing the size of transplanted tumors in mice[15,16]. Therefore, this study investigated whether 5-Aza-CdR can increase the number of exosomes and the immune molecule level in exosomes secreted by hepatoma cells through *p53* gene activation and DNA demethylation pathways.

Immunoelectron microscopy and Western blotting in this study showed that exosomes derived from HepG2 and Hep3B cells were rich in HSP70 protein both before and after 5-Aza-CdR treatment. However, after 5-Aza-CdR treatment, electron microscopy showed that the tagged HSP70 proteins in exosomes were increased, while Western blotting showed that they did not, possibly due

to the fact that *HSP70* gene expression is not regulated by DNA methylation. Moreover, HLA-Ⅰ and NY-ESO-1 molecules were clearly regulated by DNA methylation, the expression of these molecules especially that of HLA-Ⅰ in Hep3B cells, was relatively low in control groups. After 5-Aza-CdR treatment, these molecules were significantly increased in both cell lines. The number of exosomes secreted by treated and untreated hepatoma cells was calculated and changes of protein in exosome stock solutions treated and untreated with BCA were observed under electron microscope, showing that after 5-Aza-CdR treatment, the number of exosomes secreted by hepatoma cells and the protein in exosomes are significantly increased ($P < 0.05$). Furthermore, immune electron microscopy and Western blotting revealed that the number of exosomes was increased by almost an order of magnitude. Although there was a difference in *p53* mRNA expression between the two hepatoma cell lines before treatment with 5-Aza-CdR, the *p53* expression was significantly increased in both cell lines, especially in Hep3B cell line after 5-Aza-CdR treatment,. The number of exosomes and the expression of HSP70 protein were slightly higher in Hep3B cells than in HepG2 cells. However, the expression of HLA-Ⅰ and NY-ESO-1 molecules was significantly higher in Hep3B cells than in HepG2 cells, indicating that exosomes secreted by hepatoma cells are regulated by the *p53* gene, while

HLA-I and NY-ESO-1 proteins in exosomes are mainly regulated by DNA methylation. As a "molecular chaperone", HSP70 can actively deliver antigens through receptors on antigen-presenting cell membranes, thus increasing the efficiency of antigen presentation by 104-folds of pure pinocytosis or phagocytosis. Tumor-specific antigens are released from HSP within cells and presented to cytotoxic T cells, which are activated to execute immune responses to different tumors[23]. NY-ESO-1, belonging to the CTA family, is expressed in a variety of tumor tissues. Anti-NY-ESO-1 specific antibodies and sensitized lymphocytes can be found in serum of hepatoma patients[24,25]. Thus, NY-ESO-1 is currently thought to be a tumor-specific antigen with the strongest antigen immunogenicity[26]. However, NY-ESO-1 re-expression in tumor cells is not homogeneous and its low expression level is insufficient to stimulate cell-mediated immunity. To enhance tumor-specific immunity, sufficient tumor-specific antigens or immune-associated molecules are needed.

In conclusion, hepatoma cells secrete more exosomes after 5-Aza-CdR treatment. Moreover, the expression of tumor-specific antigen, NY-ESO-1, and tumor-specific immune stimulating molecule, HLA-I, increase significantly. While 5-Aza-CdR up-regulates *p53* gene expression, it may play a more important role in DNA demethylation. Exosomes secreted from hepatoma cells after 5-Aza-CdR treatment are more able to stimulate anti-tumor-specific immune response, which may be useful for the preparation of a new cancer therapeutic vaccine for hepatoma.

COMMENTS

Background

Human hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. However, no effective curative therapy for it is currently available. It is therefore necessary to develop a novel strategy against HCC. Tumor-derived exosomes, containing tumor-associated antigens and chaperones, are membrane nanoparticles, which make them stable, easy to maintain and susceptible to uptake by immune cells. However, cancer/testis antigens (CTAs) with a strong immunity and immune-associated molecules with adjuvant anti-cancer effects are heterogeneous and insufficient in cancer cells to emit specific immune reactions. Therefore, getting enough quantity and quality of exosomes for a sufficient immune effect remains a great challenge for tumor immunotherapy. 5-Aza-CdR, an inhibitor of DNA methyltransferase, can restore or increase CTAs and HLA-I/II expression in cancer cells by demethylation and induce *p53* gene expression by damaging DNA, while p53 can increase exosome production. The authors hope to get more exosomes with a large number of CTAs and immune-associated molecules by modifying hepatoma cells with 5-Aza-CdR.

Research frontiers

Exosomes secreted by tumor or dendritic cells are a recent hot spot in tumor immunology research. However, insufficient tumor-associated antigens and tumor-derived immune stimulated molecules are still a problem in such a research. Various methods, such as *IL-2* gene transfection and curcumin-modified exosomes, have been used to improve exosome immune stimulation. In this study, hepatoma was treated with 5-Aza-CdR, and more powerful exosomes were produced.

Innovations and breakthroughs

5-Aza-CdR, a DNA methyltransferase inhibitor and a demethylation promoter of CpG in many genes including the *p53* gene, can significantly restore or increase their expression. It has been shown that 5-Aza-CdR can significantly increase the expression of molecules necessary for anti-tumor cellular immunity such as HLA-I and HLA-II, and the therapeutic effect of anti-tumor immunity *in vitro* and in animal experiments. However, no report is available on the effect of 5-Aza-CdR on the number of exosomes in cancer cells. This study showed that 5-Aza-

CdR could increase exosomes in hepatoma cells and immune-related molecules of exosomes. Although its mechanism remains unclear, it may be related to demethylation and the p53-induction effect of 5-Aza-CdR. These findings suggest that 5-Aza-CdR can modify exosomes, thus offering an interesting possibility for developing a cancer vaccine.

Applications

This study has confirmed that 5-Aza-CdR-modified exosomes from hepatoma cells contain more CTAs, chaperones and immune-stimulating molecules, which may be used in preparation of a new cancer therapeutic vaccine *in vitro* for hepatoma.

Terminology

Exosomes, secreted by many kinds of cells including blood and tumor cells, have a membrane structure, 30-100 nm in size, consisting of proteins and RNA, and function as a vehicle for messages from cell to cell.

Peer review

The authors studied the effect of 5-Aza-CdR on the number of exosomes and immune-associated proteins in them produced by hepatoma cell lines, HepG2 and Hep3B, showing that exosomes function as a vehicle for messages from cell to cell, which may be used in preparation of a new cancer therapeutic vaccine for hepatoma.

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Chronic niacin overload may be involved in the increased prevalence of obesity in US children

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Abstract

AIM: To investigate nicotinamide's action on glucose metabolism, and the association between niacin consumption and obesity prevalence.

METHODS: Dynamic nicotinamide's effect on plasma hydrogen peroxide and glucose metabolism was investigated using oral glucose tolerance tests with or without nicotinamide in the same five healthy subjects.

Lag-regression analysis was used to examine the association between the niacin consumption and the obesity prevalence among US children using the data from the Economic Research Service of the US Department of Agriculture and from US Centers for Disease Control and Prevention, respectively.

RESULTS: Compared with the control oral glucose tolerance test, the 1-h plasma hydrogen peroxide (1.4 ± 0.1 μmol/L *vs* 1.6 ± 0.1 μmol/L, P = 0.016) and insulin levels (247.1 ± 129.0 pmol/L *vs* 452.6 ± 181.8 pmol/L, P = 0.028) were significantly higher, and the 3-h blood glucose was significantly lower (5.8 ± 1.2 mmol/L *vs* 4.5 ± 1.1 mmol/L, P = 0.002) after co-administration of glucose and 300 mg nicotinamide. The obesity prevalence among American children increased with the increasing per capita niacin consumption, the increasing grain contribution to niacin due to niacin-fortification, and the increasing niacin-fortified ready-to-eat cereal consumption, with a 10-year lag. The regression analyses showed that the obesity prevalence in the US children of all age groups was determined by niacin consumption (R^2 = 0.814, 0.961 and 0.94 for 2-5 years, 6-11 years and 12-19 years age groups, respectively).

CONCLUSION: The appetite-stimulating effect of nicotinamide appears to involve oxidative stress. Excess niacin consumption may be a major factor in the increased obesity prevalence in US children.

Li D, Sun WP, Zhou YM, Liu QG, Zhou SS, Luo N, Bian FN, Zhao ZG, Guo M. Chronic niacin overload may be involved

INTRODUCTION

The high prevalence of obesity, a major risk factor for type 2 diabetes, has been recognized as a serious global health problem[1]. It is likely that both dietary factors and physical inactivity may contribute to the development of obesity[2,3]. However, the reason why the prevalence of obesity suddenly increased dramatically starting around 1980 in the US is not well understood[4]. It is argued that eating and dietary quality factors may act as a primary driver for the obesity epidemic[2,4]. Therefore, exploring dietary risk factors should be of importance for the prevention and treatment of obesity and type 2 diabetes.

During the past few decades, one of the significant, but relatively overlooked worldwide changes in dietary composition has been the marked increase in the content of niacin (nicotinamide and nicotinic acid). For example, as shown in Figure 1A, the daily US per capita niacin consumption has maintained an increasing trend since the early 1940s, and has reached 33 mg in the early 2000s[5], which is more than two times the recommended dietary allowance (RDA) by the US Food and Nutrition Board (RDA: 14 and 16 mg/d for adult women and men, respectively)[6]. Animal flesh (meat, poultry and fish) and grains are the largest contributors of dietary niacin. Most of the increased niacin consumption comes from grain products due to the implementation of mandatory niacin-fortification (i.e. the addition of niacin to food) started from the early 1940s[7]. Most significantly, a sharp increase in the grain contribution in 1974 due to an update of niacin-fortification standards has made grains the largest contributor (Figure 1B)[8,9]. However, the effect of long-term exposure to excess niacin on human health is poorly understood.

Niacin-fortification has been practiced first in many developed countries to prevent pellagra, a disease due to niacin deficiency[10], and then introduced to developing countries[11]. Soon after the introduction of niacin-fortification, the prevalence of obesity and diabetes has begun to increase rapidly first in developed countries and then in developing countries[12,13], basically in a similar way as the niacin-fortification spread. Thus, there is the possibility that niacin-fortification-induced increase in dietary niacin may be involved in the increased global prevalence of obesity and diabetes. However, there are very limited data regarding the possible adverse long-term consequences of niacin-fortification.

Grains (flour and cereals), the main source of carbohydrate, are the most widely used vehicles for niacin-fortification[11]. As a result, the niacin content in the fortified grain products has been significantly increased. Traditionally, low-fat, high-carbohydrate diets were used to treat type 2 diabetes[14,15]. However, since the implementation of niacin-

fortification in the US from the early 1940s and the significant increase in carbohydrate consumption during the past three decades, more and more studies from the US have found that high-carbohydrate diets increase, instead of decrease, the risk for obesity and type 2 diabetes[12,16,17]. Then, low-carbohydrate diets have been used to treat obesity and diabetes since the late 1990s[18-20]. Whether such a profound change in the effect of carbohydrate diets is related to niacin fortification of grains is not known.

Obesity is characterized by increased appetite and insulin resistance[21,22], whereas niacin is a potent stimulator of appetite and niacin deficiency may lead to appetite loss[10]. Moreover, large doses of niacin have long been known to impair glucose tolerance[23,24], induce insulin resistance and enhance insulin release[25,26]. Evidence suggests that niacin-induced increase in insulin release may be a compensation of pancreatic islet β cells in response to the insulin resistance[25,26]. However, whether excess niacin intake is involved in the increased appetite and the insulin resistance of obesity is unclear. Our recent study found that oxidative stress may mediate excess nicotinamide-induced insulin resistance, and that type 2 diabetic subjects have a slow detoxification of nicotinamide. These observations suggested that type 2 diabetes may be the outcome of the association of high niacin intake and the relative low detoxification of niacin of the body[27]. Based on these lines of evidence, we postulated that excess niacin intake may also play a role in obesity. To address this issue, this study explored the mechanism underlying niacin's action on glucose metabolism, and the association between the US per capita niacin consumption and the obesity prevalence in the US.

MATERIALS AND METHODS

Nicotinamide load test

The present study was approved by the relevant ethics committee, and all the participants gave informed consent. Five healthy young male volunteers aged 20-24 years participated in the two oral glucose tolerance tests (glucose dose 75 g) with (NM-OGTT) or without (C-OGTT) oral co-administration of 300 mg nicotinamide (Lisheng Pharma, Tianjin, China), respectively, with an interval of 4 d. Each test was conducted after an overnight fast, venous blood (1.8 mL) was collected into sodium citrate tubes before, and 1, 2, 3, and 4 h after the administration. The blood samples were separated by centrifugation ($1500 \times g$, 10 min). Aliquots of each plasma sample were placed directly in liquid nitrogen and then transferred to -80℃ for later analysis.

Assays of blood glucose, plasma insulin and hydrogen peroxide

Blood glucose was measured using a glucometer (One-Touch Ultra, LifeScan Inc.). Plasma insulin was measured by radioimmunoassay using commercial kits (Beijing North Institute of Biological Technology, China). Plasma hydrogen peroxide (H_2O_2) concentration was measured using an H_2O_2 Assay Kit (Beyotime Biotechnology, Jiangsu, China).

Figure 1 Trends in per capita niacin consumption and contributions of grains and animal flesh to niacin in the US. A: The trends in total niacin consumed per capita per day in 1909-2004 and in the amounts from grains and animal flesh (meat, poultry and fish); B: Changes in the percentage of grain and animal flesh contribution to dietary niacin. The data on niacin consumption are derived from Ref. 5. The data on the grain and animal flesh contribution to niacin are derived from Ref. 8 (1909-2000) and Ref. 9 (2001-2004). The per capita niacin consumption (A) and the grain contribution to niacin (B) have been suddenly increased since 1974 due to an update in niacin-fortification standards.

Determination of nicotinamide and N[1]-methylnicotinamide

Nicotinamide and N^1-methylnicotinamide were analyzed, as previously described[27], using a high-performance liquid chromatography (HPLC) system that consisted of an LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne 7725i sample injector with a 20-μL sample loop (Rheodyne LLC, Rohnert Park, CA, USA), a Hypersil ODS C18 column (Thermo, Bellefonte, PA, USA) and a Waters 470 fluorescence detector (Milford, MA, USA).

Data sources

The data used for assessing the relationship between niacin consumption and obesity prevalence were derived from the databases of the US Centers for Disease Control and Prevention (CDC) and of the Economic Research Service of the US Department of Agriculture. The data on the prevalence of obesity (body mass index ⩾ 95th percentile for age and sex) in US children and adolescents were from National Health Examination Survey (NHES)

2 (1963-1965, only available for the age group of 6-11 years), NHES 3 (1966-1970, only available for the age group of 12-19 years), the National Health and Nutrition Examination Surveys (NHANES) I (1971-1974), II (1976-1980), III (1988-1994), and the continuous NHANES data collection (1999-2000, 2001-2002, 2003-2004)[28-30]. NHES and NHANES are conducted by the CDC, and include a series of cross-sectional nationally representative health examination surveys. Each cross-sectional survey provides a national estimate for the US population at the time of the survey. Detailed descriptions of the survey methods are available elsewhere[29,30], and on-line (http://www.cdc.gov/nchs/nhanes.htm). The nutrient data on the US per capita niacin consumption in 1909-2004[5], grain consumption in 1909-2007[31], grain contribution to niacin in 1909-2000[8] and in 2001-2004[9] and ready-to-eat cereal (RTE) consumption[32] were derived from the databases of the Economic Research Service (ERS) of the US Department of Agriculture. ERS annually calculates the amounts of several hundred foods

available for human consumption in the US and provides estimates of per capita availability. The estimates of nutrients in the food supply reflect Federal enrichment and fortification standards and technological advances in the food industry[8].

Statistical analysis

The data are presented as mean ± SD. Statistical differences in the data were evaluated by paired Student's *t* test. Lag-regression analysis was used to test the relationships between the consumption of niacin or grain and the obesity prevalence in the US children and adolescents using SPSS software (SPSS Inc., Chicago, USA). Statistical significance was set at $P < 0.05$.

RESULTS

Dynamic effect of nicotinamide on blood glucose metabolism

The methylation of nicotinamide to N^1-methylnicotinamide is a major mechanism to eliminate excess nicotinamide[27]. As shown in Figure 2, in C-OGTT, the plasma concentrations of nicotinamide and N^1-methylnicotinamide were fairly constant (Figure 2A and B), and the 2-h plasma level of H_2O_2, a major reactive oxygen species (ROS), was slightly increased, but without statistical significance compared with the value before C-OGTT (Figure 2C). In contrast, NM-OGTT led to a significant increase in plasma nicotinamide and N^1-methylnicotinamide levels, with a peak at approximate 2 h (Figure 2A and B). In parallel with the rising phase of plasma nicotinamide and N^1-methylnicotinamide, there was a significant increase in 1-h and 2-h plasma H_2O_2 levels in NM-OGTT (Figure 2C). Importantly, although the 1-h and 2-h blood glucose values were similar in the two tests (Figure 2E), however, the 1-h plasma insulin level was much higher in NM-OGTT than in C-OGTT (Figure 2D). As the plasma H_2O_2 returned to the basal level at 3 h in NM-OGTT (Figure 2C), there was a sharp decrease in the blood glucose concentration (Figure 2E). Two of the five subjects in NM-OGTT had reactive hypoglycemia symptoms (i.e. sweating, dizziness, faintness, palpitation and intense hunger) with the blood glucose levels below 3.6 mmol/L. In contrast, no subjects had reactive hypoglycemic symptoms during C-OGTT. These results indicated that nicotinamide overload might induce a biphasic effect, i.e. insulin resistance followed by hypoglycemia.

Association between niacin consumption and obesity prevalence in US children

We next investigated the relationship between the daily per capita niacin consumption and the obesity prevalence in US children and adolescents. There were two historical events in niacin-fortification in the US. The first is the initial introduction of niacin-fortification in the early 1940s and the second is the update of fortification standards in 1974. These two events resulted in significant increases in the per capita niacin consumption respectively in the 1940s and in the mid-1970s. Evidently, the prevalence of

Figure 2 Dynamic effect of nicotinamide on plasma H_2O_2 level and glucose metabolism. A-E: Dynamic changes in the concentrations of plasma nicotinamide (NM), N^1-methylnicotinamide (NMN), H_2O_2, insulin and blood glucose in C-OGTT and NM-OGTT (co-administration of 300 mg nicotinamide and 75 g glucose). For each point, $n = 5$. Values represent mean ± SD, [a]$P < 0.05$, [b]$P < 0.01$ *vs* C-OGTT.

obesity in US children of all age groups increased in parallel with the increase in the per capita niacin consumption with a 10-year lag (Figure 3A, C and E). Lag-regression analysis revealed that the obesity prevalence in the children of all age groups was determined by niacin consumption (Figure 3B, D and F). This relationship was observed in both sexes (Figure 3G and H) ($R^2 = 0.958$ and 0.96 for boys and girls aged 6-11 years, $P = 2.6e-17$ and $1.6e-17$,

Figure 3 Correlations between US per capita niacin consumption and obesity prevalence in US children. A, C and E: The trends in the daily per capita niacin consumption in 1909-2004 (Ref. 5) and in the obesity prevalence in the children aged 2-5, 6-11 and 12-19 years (Ref. 28); B, D and F: The 10-year lag-regression plots of the obesity prevalence in different age groups against daily per capita niacin consumption using the data in A, C, and E; G and H: The obesity prevalence in the age group of 6-11 years of either sex in 1963-2004 (Ref. 29 and 30) increased in parallel with the per capita niacin consumption in 1953-1994.

Figure 4 Correlations between consumption and contribution of grain and prevalence of obesity in US children. A: Trends in US per capita grain consumption in 1909-2007 (Ref. 31) and in the obesity prevalence in the children aged 12-19 years in 1966-2004 (Ref. 28); B: The linear regression plot of the obesity prevalence in 1988-2004 against grain consumption in 1978-1994 using the data in A; C and D: Relationships between the grain consumption (Ref. 31) and the prevalence of obesity in the children aged 6-11 and 2-5 years (Ref. 28). R^2 = 0.902 (P = 7.1e-7) and 0.955 (P = 9.7e-9), for 2-5 years age group and 6-11 years age group; E: Trends in the grain contribution to niacin (Ref. 8 and Ref. 9) and in the obesity prevalence in the children aged 12-19 years (Ref. 28); F: The linear regression plot of the prevalence of obesity in 1966-2004 against grain contribution in 1956-1994 using the data in E.

respectively; R^2 = 0.949 and 0.92 for boys and girls aged 12-19 years, P = 1.2e-17 and 3.4e-15, respectively).

Association between grain consumption and obesity prevalence in US children

Grains have been used as a major vehicle for niacin-fortification. The increase in the daily per capita niacin consumption from grains reflects the fortification level and the trend toward the consumption of fortified grain products. Although the US per capita grain consumption steadily decreased from the late 1930s through the early 1970s (Figure 4A), the grain contribution to niacin

actually significantly increased due to niacin-fortification (Figure 4E). In the early 20th century, high per capita consumption of non-fortified grains was associated with a low prevalence of obesity in US children and adolescents. However, the re-increase in the consumption of the grain products fortified with more niacin since 1974 was followed by a steep increase in the obesity prevalence in the US children of all age groups in the 1980s and 1990s. The lag was ten years (Figure 4A, C and D). Moreover, the obesity prevalence in US children also increased in parallel with the increase in grain contribution to niacin with a 10-year lag (Figure 4E). The regression analyses showed

Figure 5 Relationship between per capita ready-to-eat cereal (RTE) consumption and prevalence of obesity in US children. A: Trends in US per capita RTE consumption in 1970-1997 (Ref. 32) and in the prevalence of obesity in the 2-5 years age group; B: The linear regression plot of the obesity prevalence in 1980-2004 against RET consumption in 1970-1994 using the data in A; C and D: A similar relationship between the RTE consumption and the obesity prevalence in the 6-11 years and 12-19 years age groups.

significant lag-correlations between the grain contribution to niacin and the obesity prevalence of US children aged 2-5 years ($R^2 = 0.726$, $P = 4.8e-7$), 6-11 years ($R^2 = 0.898$, $P = 6.8e-13$) and 12-19 years (Figure 4F).

Association between RTE consumption and the obesity prevalence in US children

ERT, a popular food item for many Americans, especially children, is the most common vehicle for niacin-fortification in the US. The continued upward trend of niacin since the mid-1970s has been primarily due to the increase in the fortification standards of RTE in 1974 and the greater use of enriched grain products[8]. As shown in Figure 5, the yearly per capita consumption of RTE has rapidly increased since 1970, and the obesity prevalence in the US children of all age groups increased in parallel with the increase in the RTE consumption with a 10-year lag. There were significant correlations between the RTE consumption in 1970-1994 and the obesity prevalence in US children aged 2-5 years (Figure 5B), 6-11 years ($R^2 = 0.933$, $P = 2.1e-8$) and 12-19 years ($R^2 = 0.913$, $P = 1.0e-7$) in 1980-2004.

DISCUSSION

The major findings of this study are: (1) nicotinamide overload may induce a biphasic effect on glucose me-

tabolism, characterized by insulin resistance followed by hypoglycemia; and (2) there is a significant association between the US per capita niacin consumption and the obesity prevalence in US children and adolescents. These findings may help explain both the development of obesity and the increased prevalence of obesity.

Insulin resistance is a key feature of obesity[22]. Increasing evidence has indicated that systemic oxidative stress, characterized by elevation of plasma ROS, is an important trigger of insulin resistance[33]. In agreement with this suggestion, this study found that the plasma insulin level increased in parallel with the rise of plasma H_2O_2, the major mediator of oxidative stress, while decline in the H_2O_2 level led to hypoglycemia, which suggested that the insulin sensitivity increased. It seems that the oxidative stress plays a critical role in nicotinamide-induced insulin resistance. The present findings are in agreement with the hypothesis that niacin-induced increase in β-cell secretory capacity is the result of pancreatic islet adaptation to niacin-induced insulin resistance[25,26].

It is generally accepted that obesity results from excess energy intake and physical inactivity. A recent hypothesis suggests that factors favoring a trend toward hypoglycemia might induce excess energy intake and overweight[34]. Interestingly, the present study demonstrated that nicotinamide overload induced a biphasic response: insulin resistance in the early phase characterized by more insulin

Figure 6 Proposed model of the role of niacin overload in obesity development. Excess nicotinamide metabolism may induce a biphasic effect: postprandial hyperinsulinemia followed by postprandial hypoglycemia, in which reactive oxygen species (ROS) generation and scavenging may play a central role.

release due to the enhanced ROS production, and hypoglycemia in the late phase due to the different clearance rates of plasma ROS and insulin. The biphasic response may underlie the increased appetite in obesity: high nicotinamide diet may produce more ROS and decrease insulin sensitivity, which leads to more insulin release. Then, with the relative rapid fall of plasma ROS and the re-increase in insulin sensitivity, the relative high insulin level may lead to hypoglycemia, which may induce hunger, eating behavior change, and subsequent excess energy intake (Figure 6). As such, it is not difficult to imagine that long-term nicotinamide overload-induced insulin resistance may eventually lead to β-cell failure. From this point of view, it seems that long-term excess nicotinamide intake may be a primary cause of obesity and type 2 diabetes. Moreover, our previous study demonstrated that sweat is an effective way to eliminate excess nicotinamide from the body[27]. Thus, physical activity not only increases energy expenditure but also decreases insulin resistance by facilitating the elimination of excess nicotinamide through sweating, which may help explain why sweat-inducing activities are effective in preventing obesity and type 2 diabetes. It should be pointed that excess niacin-induced oxidative stress and abnormal glucose metabolism is expected to involve other blood glucose-controlling hormones, such as glucagon and glucagon-like peptides. Further studies are warranted to examine the effect of excess niacin intake on these hormones.

Since niacin overload may be involved in the development of obesity, determining whether there is niacin overload in the modern diet may be helpful for understanding the global prevalence of obesity. Generally, dietary niacin comes mainly from the following two main sources: niacin-rich foods and niacin fortified foods. In term of the development of obesity and diabetes, the known high-risk foods are those rich in niacin, such as meat[35,36]. Most importantly, niacin fortified-grain products have been the most significant source of niacin. As shown in Figure 1, the daily US per capita niacin consumption has been kept

a rising trend since the implementation of mandatory niacin-fortification, and has been two times higher than the RDA in the early 2000s. The amount of the daily per capita niacin consumption from grains and animal flesh was 3.7 and 6.8 mg, respectively, before the introduction of mandatory niacin-fortification in the 1930s, and has increased to 14.8 and 11.8 mg, respectively, in 2000. The percentage of grain contribution to dietary niacin has been increased four times since the implementation of niacin fortification. It is obvious that niacin-fortification may be mainly responsible for niacin overload, and may play a role in the increased prevalence of obesity in the US. To test this hypothesis, we examined the relationship between US per capita niacin consumption and the prevalence of obesity in US children and adolescents. Indeed, the present results showed that the obesity prevalence in the US children of all age groups increased in parallel with the increase in the per capita niacin consumption and the increase in the grain contribution to niacin, with a 10-year lag. RTE is a popular food item for many Americans, especially children. The update of fortification standards in the US in 1974 has led to a significant increase in niacin content in RTE[8]. The present study also revealed that there was a significant lag-correlation between the obesity prevalence in US children and the RTE consumption. Since the late 1990s, the US per capita niacin consumption has remained relatively stable (around 32-33 mg/d per capita)[9]. According to the regression equations shown in Figure 3, the obesity prevalence in the US children of all age groups should have been near its peak now. Indeed, the most recent NHANES data showed that there was no significant change in obesity prevalence between 2003-2004 and 2005-2006 in US children[37].

Dietary factors play a key role in the development and prevalence of obesity and diabetes[12,16,35,36]. The increasing global prevalence of obesity and diabetes implies that there may be a common change in diet composition worldwide. It should be pointed out that one of the common and major global changes in diet composition in the

last few decades is the significant increase in the content of dietary vitamins due to the spread of food fortification worldwide. The global prevalence of obesity and diabetes has spread in much the similar way as that of niacin-fortification, in contrast, those countries that had not introduced niacin-fortification or that prohibited niacin fortification before the early 1990s, such as Norway[38,39], have a low prevalence of obesity and diabetes in the 1990s, compared with the niacin-fortified countries such as the US and Canada. Thus, the possibility cannot be ruled out that the rapid global increase in the prevalence of obesity in the past three decades may be, at least in part, a man-made event due to niacin fortification.

Obesity is strongly associated with non-alcoholic fatty liver disease (NAFLD), a common disease characterized by an increase in intrahepatic triglyceride content with or without steatohepatitis[40]. As early as in 1964, Rikans *et al*[41] found that excess niacin in high fat diets can induce fatty liver in rats. Moreover, numerous case reports have shown that excess niacin can induce liver injury[6]. Thus, there is the possibility that excess niacin intake may play a role in the development of NAFLD. Investigating excess niacin metabolism and its toxic effects may be of help in gaining insight into the development of not only obesity but also NAFLD.

In summary, the present study demonstrates for the first time that nicotinamide overload-induced biphasic response in glucose metabolism may play a role in the development of obesity, and suggests that the high prevalence of obesity in US children and adolescents may involve a long-term niacin overload largely due to the grain fortification with niacin. It seems that the long-term safety of niacin fortification needs to be carefully evaluated.

COMMENTS

Background
Niacin, a potent stimulator of appetite, may induce insulin resistance at high doses. Global prevalence of obesity which is characterized by increased appetite and insulin resistance has occurred following the spread of grain fortification with niacin worldwide. However, how niacin stimulates appetite and whether excess dietary niacin plays a role in the obesity epidemic are not known.

Research frontiers
Obesity is directly related to diet and physical activity. Therefore, exploring the dietary risk factors for obesity and understanding the underlying mechanism of increased appetite and insulin resistance in obesity are important issues in the prevention and treatment of overweight and obesity.

Innovations and breakthroughs
The present study demonstrated for the first time that excess nicotinamide, when co-administered with glucose, induces biphasic response: insulin resistance in the early phase characterized by more insulin release due to the enhanced reactive oxygen species (ROS) production, and hypoglycemia in the late phase due to the different clearance rates of plasma ROS and insulin. The excess niacin-induced biphasic response may play a role in the development of obesity. This study also revealed for the first time that the obesity prevalence among US children and adolescents increased in parallel with the increase of the per capita niacin consumption with a 10-year lag, in which niacin fortification-induced sharp increase in niacin contents in grain products may play a major role. The present findings may help explain why there has been a sudden sharp increase in the obesity prevalence among US children and adolescents starting around the early 1980s, i.e. about 10 years after updating the niacin fortification standards in 1974.

Applications
Reducing niacin intake and facilitating niacin elimination through sweat-inducing physical activity may be a key factor in the prevention and treatment of obesity.

Peer review
Li *et al* studied in a small (pilot) study the effects of niacin overload on glucose metabolism in relation with a study performed in a large cohort, and this paper would be of interest.

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BRIEF ARTICLE

Quality of life after curative liver resection: A single center analysis

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Abstract

AIM: To evaluate quality of life (QoL) after curative liver resection and identify variables associated with decreased QoL.

METHODS: From October 2001 to July 2004, 323 patients underwent liver resection. At 3-36 mo after discharge, 188 patients were disease free. QoL was assessed using the Short Form (SF)-12 Health Survey with mental and physical component scales (SF-12 MCS and PCS), supplemented with generic questions concerning pain and liver-specific items.

RESULTS: Sixty-eight percent (128/188) returned the questionnaire, which was completed in 75% (96/128) of cases. Median SF-12 PCS and MCS were 46.7 (interquartile range: 34.2-53.9) and 54.1 (42.8-58.2). Fifty percent were pain free with a median symptom score of 1.75 (1.38-2.13). PCS was higher after major hepatectomy [57% (55/96)] compared to minor resection (*P* = 0.0049), which represented an improved QoL. QoL was not affected by sex but by age compared to the general German population. MCS was higher after liver surgery for metastatic disease [55.9 (47.5-58.8)] compared to primary carcinoma [49.6 (36.5-55.1)] and benign disease [49.2 (37.7-56.3)] (*P* = 0.0317). There was no correlation between length of postoperative period and QoL. Pain, deficiencies in everyday life and a high symptom score significantly decreased MCS and PCS.

CONCLUSION: Most patients were only marginally affected even after major liver resection; however, minor complications were associated with decreased SF-12 MCS and PCS and need careful attention.

INTRODUCTION

Over the past few decades, the multimodal concept of

quality of life (QoL) has been appreciated in many areas of medical practice[1]. Social, physical and mental factors contribute to QoL[2,3]. QoL assessment has proven to be a valuable parameter for patients and surgeons and may be helpful in determining the optimal treatment. As an outcome parameter, QoL is considered as important as disease-free and overall survival[4]. For many different procedures in surgery, the effect on QoL has been assessed, including liver resection for primary and secondary cancer, organ transplantation and gastrectomy[5-13].

In various benign and malignant liver diseases, resection is a common procedure with curative intention. While major and minor hepatectomy are both safe procedures, little is known about postoperative QoL in these patients[14-16]. Erim *et al*[17,18] have investigated QoL of live donors who have undergone liver resection for living related liver transplantation. Anxiety and depression increased in live donors, and improved 3 mo after surgery. Furthermore, Kaneko *et al*[19] have published a study on laparoscopic *vs* open liver resection in patients with hepatocellular carcinoma. QoL improved earlier after laparoscopic surgery. Their finding is similar to other studies that have compared various laparoscopic with open surgical procedures[19,20]. Recently, it has been published that QoL returns to baseline after liver resection for malignancies in most cases[21,22]. Martin *et al*[21] have shown in a prospective study of 36 patients that QoL returns to baseline at about 3 mo after liver resection for cancer.

Thus, the present study was designed to assess postoperative QoL of patients who underwent curative liver resection. Curative resection was defined as the absence of recurrent disease at the time of assessment. QoL was compared with that of the general German population, with a special focus on demographics (e.g. age and sex) and medical history (e.g. indication, type of resection, complications and impairment).

Although several studies on QoL after liver resection have been performed, to the best of our knowledge, this is the largest study on QoL of patients who have undergone curative liver resection[19,21,23,24]. In our opinion, this study's value is the exclusion of any bias caused by the palliative situation of the study population. It seems plausible that patients in a palliative situation might be impaired in their physical and especially mental QoL scores. Therefore, we decided to exclude any patients with signs of recurrence or active disease.

MATERIALS AND METHODS

Study population
Patients who underwent curative partial hepatectomy from October 2001 to July 2004 were recruited for this single-center, observational study on QoL after liver resection. While all patients underwent liver resection with curative intention, curative treatment in the context of this study was defined as the absence of recurrent disease at the time of assessment. The study protocol was approved by the ethical committee and all patients provided informed consent.

A total of 323 patients underwent partial hepatectomy in our hospital from October 2001 to July 2004. One hundred and eighty-eight patients were disease-free at the time of assessment and were eligible to participate in the study, and questionnaires were mailed to these patients. A return rate of 68% (128 patients) for the questionnaires, of which in 75% were filled out completely, further decreased the final study population to 96 patients. Thus, the final population consisted of almost 50% of the eligible study population.

Measures
QoL was assessed by the Short-Form (SF)-12 Health Survey, which is the abbreviated version of the widely used generic profile SF-36[25,26]. Both questionnaires have been validated for numerous populations and quantify the overall physical and mental aspects of QoL[27-34]. The SF-12 provides a subset of 12 items from which two summary measures of physical and mental health status (SF-12 PCS and SF-12 MCS) can be derived from subscales for physical function, physical role, pain, general health, vitality, social function, emotional role, and mental health with high validity, reliability and sensitivity[35].

The demographic and postoperative data, and results of the SF-12 Health Survey, a well-established QoL questionnaire, were analyzed, together with a pain assessment on a 0-10 scale and relevant liver-specific items.

Additional illness-specific items were used to assess liver-specific issues, for example, fever, dyspepsia, heartburn, lack of appetite, nausea, vomiting, night sweat and exhaustion. Patients indicated on a scale of 1-5 (1, never; 2, rarely; 3, sometimes; 4, often; 5, very often) the frequency of the individual symptom postoperatively. The calculated mean was used as a symptom score that ranged from 1 to 5. Impairment in everyday activities was measured on a scale of 1-5 (1, none; 2, light; 3, moderate; 4, heavy; and 5, strongest) and patient autonomy was measured as a variable indicating "independent" and "help needed". Pain was assessed using a 0-10 scale.

Surgery
As described by Belghiti *et al*[36], major hepatectomy was defined as resection of three or more segments and minor hepatic resection as resection of two or fewer segments. Thus, our patients were divided into a major and minor hepatectomy group.

Complications
Postoperative complications were stratified into surgical (e.g. bile leak or biloma, pneumothorax, wound infection, liver abscess, bleeding, and surgical dehiscence) and medical (e.g. pleural effusion, renal failure, hepatic failure, pneumonia, cardiac insufficiency, and cholangitis), and were assessed from patient records. Complications were defined as described elsewhere[16].

Study procedures
QoL was assessed at least 3 mo after discharge from hospital (range: 3-36 mo). Medical data were collected from

follow-up examinations as proof of patient eligibility (e.g. no signs of recurrence). In patients with malignant diseases, staging was performed according to the guidelines of the Association of the Scientific Medical Societies in Germany. Only patients without signs of recurrence were eligible for the study. Subsequently, questionnaires were sent to the patients by mail. Furthermore, patients were contacted by telephone to increase the return rate of questionnaires. Clinical data such as indication for liver resection, type of liver resection, medical and surgical postoperative complications were collected and documented in a database in a prospective manner.

Statistical analysis

SAS software (version 9.1; SAS Institute, Cary, NC, USA) was used for statistical analysis. The quantitative parameters SF-12 PCS, SF-12 MCS, pain, symptom score, and age are presented as median with interquartile range. Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance were chosen to compare the quantitative parameters between subgroups of patients. Patients were stratified for age, sex, type of resection and indication for surgery. The relationships between the quantitative parameters were analysed using Spearman's rank correlation coefficient r and its corresponding P value. Two-sided P values were always calculated; $P < 0.05$ was considered statistically significant, and $P < 0.001$ was considered highly significant.

RESULTS

Study population

One hundred and eighty-eight patients underwent partial hepatectomy for benign and malignant hepatic lesions and were recurrence-free at the time of assessment. While 128 patients (68%) returned their questionnaire, it was fully completed in only 96 cases (52% of recurrence-free patients), of which 50% were male. Thus the final study population comprised 96 patients (Table 1). No significant difference could be found in demographics, indications and type of resection between patients with fully and partial filled questionnaires.

Indication for liver resection

The diagnoses are listed in Table 1; almost 21% of patients resected had benign lesions and > 79% had malignant liver tumors. Of the latter, > 72% were metastatic. More than 69% of metastases had spread from a colorectal tumor.

Surgical procedures

The procedures performed are listed in Table 1. There were 55 (57%) major and 41 minor hepatic resections. Patients underwent hemi-hepatectomy or extended hemi-hepatectomy in 45% and > 6% of the cases, respectively. Furthermore, six patients (6%) underwent resection of ≥ 3 segments other than (extended) hemi-hepatectomy. Minor resection was performed in 43% of patients. These patients underwent resection of one segment (24%), or

Table 1 Demographics, indications and type of resection

	n = 96	100%
Median age (yr)	63.4	IQR: 54.5-70.5
Male/female	48/48	50/50
Primary malignant	21	21.9
Hepatocellular carcinoma	12	12.5
Cholangiocellular carcinoma	8	8.3
Gallbladder carcinoma	1	1.0
Metastatic	55	57.3
Colorectal	38	39.6
Other	17	17.7
Benign	20	20.8
Adenoma	4	4.2
Focal nodular hyperplasia	6	6.3
Cysts	1	1.0
Echinococcus	3	3.1
Haemangioma	2	2.1
Other	4	4.2
Major hepatectomy	55	57.3
Right hemi-hepatectomy	35	36.5
Extended right hemi-hepatectomy	3	3.1
Left hemi-hepatectomy	8	8.3
Extended left hemi-hepatectomy	3	3.1
Segmentectomy (*n* > 2)	6	6.3
Minor hepatectomy	41	42.7
Segmentectomy (*n* = 1)	23	24.0
Segmentectomy (*n* = 2)	18	18.8
Concomitant extrahepatic resection	10	10.4

Patients were stratified for demographic data, indications and type of resection. Patients suffering from benign, primary malignant and metastatic liver diseases were included. IQR: Interquartile range.

segmental resection that consisted of *en bloc* resection of two segments or resection of two discontinuous segments (20%).

Morbidity

At least one of both medical and surgical postoperative complications occurred in about 30 (31%) patients (Table 2). Pleural effusion was the most frequent medical complication and occurred in about 9% of all cases. The most frequent surgical complication was bile leakage or biloma, which occurred in 6% of all cases (Table 2). Type of surgery (i.e. major or minor hepatectomy) was not significantly associated with frequency of surgical and medical complications.

QoL and symptom scores

Table 3 shows the SF-12 PCS and SF-12 MCS median scores stratified for demographic items, type of resection, indication and overall morbidity.

The age-dependent distribution of physical and mental SF-12 scores is shown in Figure 1 for patients *vs* the population norm. Patients younger than 40-51 years who underwent liver resection showed significantly lower SF-12 PCS (Figure 1A) and SF-12 MCS (Figure 1B) scores compared to their age-matched normal group ($P < 0.05$).

The mental QoL score was significantly higher ($P < 0.05$) in patients who underwent liver resection for

Table 2 Morbidity and postoperative complications

Complication	Total		Major hepatectomy		Minor hepatectomy	
	n = 96	100%	*n* = 55	100%	*n* = 41	100%
Total morbidity	30	31.2	20	36.4	10	24.4
Surgical complications	19	19.8	14	25.5	5	12.2
Bile leak/biloma	6	6.3	5	9.1	1	2.4
Pneumothorax	3	3.1	2	3.6	1	2.4
Wound infection	2	2.1	2	3.6	-	-
Abscess (liver)	1	1.0	1	1.8	-	-
Bleeding	1	1.0	1	1.8	-	-
Surgical dehiscence	1	1.0	1	1.8	-	-
Other	5	5.2	2	3.6	3	7.3
Medical complications	19	19.8	11	20.0	8	19.5
Pleural effusion	9	9.4	7	12.7	2	4.9
Renal failure	3	3.1	2	3.6	1	2.4
Hepatic failure	2	2.1	2	3.6	-	-
Pneumonia	2	2.1	-	-	2	4.9
Cardiac insufficiency	1	1.0	-	-	1	2.4
Cholangitis	1	1.0	1	1.8	-	-
Other	3	3.1	3	5.5	-	-
Revision laparotomy	3	3.1	2	3.6	1	2.4

Figure 1 SF-12 scores *vs* general population. A: Physical SF-12 score. Although younger patients experienced lower physical scores than did the general population, the difference between the groups decreased; B: Mental SF-12 score. Older patients experienced the same levels of mental QoL as younger patients. The younger the patients were, the more significant was the impact of surgery on their scores.

metastases, with almost 56 (48-59) compared to patients with primary liver malignancies or benign liver disease with scores of about 49 (38-56) and 50 (37-55), respectively. Physical QoL varied according to the type of liver resection. While patients who underwent major resection reached a physical score of 52 (39-54), values after minor resection were about 41 (32-51) ($P < 0.005$). Sex, age and postoperative morbidity did not have a significant impact on mental or physical QoL.

Postoperative symptoms rarely occurred (Figure 2). The median value of symptom scores was 1.75 (1.38-2.13).

Subjective impairment after surgery correlated with lower summary score ($P < 0.001$) (Table 4). Patients who were not able to manage their living alone without the help of others had lower scores for both SF-12 PCS ($P < 0.05$) and SF-12 MCS ($P < 0.001$) (Table 4). The occurrence of medical problems considered to be minor was associated with decreased QoL scores (Table 4). Patients with postoperative wound infections showed significantly

lower values for both SF-12 PCS ($P < 0.05$) and SF-12 MCS ($P < 0.05$) compared to patients without this complication and population norm.

High symptoms scores, pain and deficiencies had significant negative correlations to physical and mental SF-12 scores. Furthermore, high symptom scores correlated with high levels of pain and deficiencies in day-to-day life experienced by patients. Pain and scores of SF-12 PCS had a significant negative correlation ($r = -0.65$, $P < 0.0001$), while high degrees of deficiencies in the patients' daily routine correlated with low QoL values ($r = -0.59$ and -0.51 for PCS and MCS, respectively, $P < 0.0001$). Furthermore, high symptom scores were associated with pain and the feeling of being handicapped in life.

DISCUSSION

In mail surveys, non-responder bias has to be kept in mind. Our study provided valid data for 68% of our

Table 3 Median SF-12 scores

	n	Physical score		Mental score	
		Median	IQR	Median	IQR
Total	96	46.7	34.2-53.9	54.1	42.8-58.2
Sex					
Male	48	50.9	33.2-54.0	55.0	44.8-58.9
Female	48	44.6	36.3-53.4	52.9	40.9-57.8
Age (yr)					
< 41	10	38.5	33.4-50.4	41.8	32.0-54.1
41-50	7	38.6	35.4-46.9	54.4	37.1-62.4
51-60	16	47.4	33.3-54.9	54.6	43.6-59.8
61-70	38	49.8	36.0-54.2	55.3	43.1-58.8
> 70	25	50.8	37.4-53.8	53.4	45.0-57.9
Indication[a]					
Benign	20	43.5	33.4-54.9	49.2	37.7-56.3
Prim. malignant	22	41.6	32.9-53.1	49.6	36.5-55.1
Metastatic	54	50.6	39.0-54.2	55.9	47.5-58.8
Type of resection[b]					
Major	55	52.0	39.0-54.4	54.8	43.7-58.5
Minor	41	41.3	31.6-51.0	52.6	38.6-57.9
Morbidity					
Yes	30	44.2	29.7-53.8	53.1	43.1-57.8
No	66	50.2	37.4-54.1	54.1	42.4-58.8

Mental and physical SF-12 scores were stratified for sex, age, indication, type of resection and morbidity. Higher SF-12 scores represent better QOL. *P* values physical/mental scores: [a]*P* = 0.2966/0.0317 (metastatic *vs* primary malignant); [b]*P* = 0.0049/0.4176 (major *vs* minor resection).

Table 4 Impaired autonomy and wound healing after resection, and median physical and mental scores

	n = 96	Physical score	Mental score
Impairment			
None	35	53.8	57.9
Slight	30	43.3	54.4
Moderate	20	43.3	45.4
Heavy	9	33.7	36.9
Strongest	2	21.8	41.6
Autonomy			
Independent	90	48.4	54.6
Help needed[a]	6	28.3	41.6
Wound healing deficiency			
Yes	10	33.0	43.0
No	85	46.9	54.8
Not stated[b]	1	25.8	55.1

Questions on impairments in everyday activities, autonomy and wound healing were included in the survey. [a]*P* < 0.0008 for physical SF-12 scores, *P* < 0.0354 for mental SF-12 scores; [b]*P* = 0.0474 for physical SF-12 scores, *P* = 0.0062 for mental SF-12 scores.

Figure 2 Distribution of liver-specific symptom score. Patients were asked about fever, dyspepsia, heartburn, lack of appetite, nausea, vomiting, night sweat and exhaustion. Patients were stratified for mean levels of symptom scores ranging from 1 to 5. The mean value of 1.82 ± 0.51 indicated that postoperative symptoms rarely occurred.

patients, and data for the missing 32% could only be generated by extrapolation. Different approaches exist for the extrapolation of data from mail surveys, which have been discussed previously[37-39]. Due to the relatively high response rate - compared to a mean response rate to mail surveys published in medical journals of 30%-60% - and since there was no systematic difference as far as demographic and medical characteristics were concerned, we assumed that our data were valid in the context in which they were investigated[38].

Postoperative QoL has been assessed for many medical and surgical treatments[40-43]. Most of these studies aimed not only at providing descriptive data, but also to help physicians and surgeons to choose optimal treatment[44]. Our results describe QoL after liver resection for patients who received curative treatment. In contrast to Martin *et al*[21], we included patients with all common types of indication for liver resection. Furthermore, we ensured that only patients who were disease free at least 3 mo after liver resection were included.

The present study found that patients younger than 50 years of age had lower QoL compared to their age-matched normal population group and to older patients. The impact of surgery on younger patients' QoL might

be stronger for several reasons. Besides medical reasons (i.e. comorbidity and different indications), it has to be taken into account that, in general populations, QoL (especially physical) is usually found to decline with age, and patients might have different expectations in different age groups. Patients older than 70 years of age reported a higher physical QoL than their age-matched controls.

Patients with liver metastases reported a higher physical as well as mental QoL than those who underwent resection for primary malignant or benign liver lesions (Table 3). We assumed that most patients who underwent surgery for metastatic disease already knew from the primary surgical intervention that physical wellbeing would return some time after surgery. Furthermore, we assumed that the fulfilled hope to be cured from the metastases of an already resected primary tumor might have a great impact on QoL[45].

Surprisingly, patients who underwent major liver resection reported a higher physical QoL compared to those who underwent minor resection (Table 3). Patients might compare their QoL to their recovery period, which may be prolonged and more complicated after extensive surgery, thus leading to a subjectively improved QoL.

Neither surgical nor medical complications were more frequent after major compared to minor hepatectomy (Table 2). Furthermore, postoperative complications that are considered to be minor medical problems, such as impaired wound healing, were found to interfere with QoL (Table 4). In all patients, the symptom score, pain and deficiencies in daily routine correlated significantly with QoL.

Data presented here show that 3-36 mo after discharge from hospital, mental and physical scores tended to be close to those of the general population, as long as QoL-decreasing factors (e.g. pain, or impaired wound healing) were absent. Furthermore, we were also able to identify important determinants of QoL.

QoL is always subject to individual judgement and compared by the patients to their own experience. Thus, elderly patients, who generally experience decreased physical QoL in comparison to younger patients, might find the effect of surgery less invasive. While this is clearly speculative, this might be an explanation for the higher physical QoL scores after surgery in elderly patients.

Moreover, individual judgement of QoL offers an explanation for the counter-intuitive finding of higher QoL scores after major hepatectomy in comparison to minor resection. Patients undergoing major resection most likely experience a higher impact of the more invasive surgery. Thus, after recovery, they might experience a higher change in their physical scores.

In contemporary surgery, QoL is considered as important as disease-free survival and morbidity. Thus, we investigated important factors that potentially determine QoL in patients undergoing curative hepatic resection. This study clearly demonstrates that even after a short time following liver resection, the vast majority of patients score equal or even higher in SF-12 compared to that of the population norm.

However, there is a small number of patients whose QoL might be affected by pain, impaired wound healing and subjectively perceived deficiencies in their daily routine following liver resection. This implies that appropriate pain and wound management is needed and coping with deficiencies in daily routine needs to be addressed postoperatively.

Furthermore, clinicians should be aware that QoL might be appraised differently depending upon age and underlying disease, in order to discuss the expectations of the surgical procedure.

ACKNOWLEDGMENTS

COMMENTS

Background
Quality of life (QoL) has been appreciated in many areas of medical practice and has proven to be helpful in determining the optimal treatment. As an outcome parameter, QoL is considered as important as disease-free and overall survival. Although major and minor hepatectomy are both safe procedures, little is known about their postoperative QoL. Thus, this study was designed to assess the postoperative QoL of patients who underwent curative liver resection and to compare their QoL to that of the general German population, with a special focus on demographics and medical history.

Research frontiers
Although several studies on QoL after liver resection have been performed, to the best knowledge of the authors of this article, this is the largest study on QoL of patients who underwent curative liver resection. In the authors' opinion, this study's value is the exclusion of any bias caused by the palliative situation of the study population. It seems plausible that patients in a palliative situation might be impaired in their physical and especially mental QoL scores. Therefore, the authors decided to exclude any patients with signs of recurrence or active disease.

Innovations and breakthroughs
In contrast to other studies, the authors eliminated the palliative bias and thus were able to evaluate the effect of surgery itself on QoL. While interpretation of our findings needs to be done with due care and, as in other studies on this subject, is speculative, they were able to identify contributors to QoL, and more importantly, did not identify a negative effect of curative hepatectomy on QoL.

Applications
The authors think that their findings are helpful in defining the optimal treatment for patients who require liver resection. Thus, their data justify the indication for surgery and can help surgeons to recommend major and minor hepatectomy to patients who need this type of surgery.

Terminology
QoL is a multimodal concept and can be measured with standardized questionnaires such as the Short-Form Health Survey (SF). Both versions (SF-12 and SF-36) have been validated and provide information on both physical and mental QoL.

Peer review
This is a large study of QoL in 323 patients undergoing liver resection for various indications. QoL was assessed using standard verification protocols.

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BRIEF ARTICLE

Narrow-band imaging endoscopy to assess mucosal angiogenesis in inflammatory bowel disease: A pilot study

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Abstract

AIM: To investigate whether narrow band imaging (NBI) is a useful tool for the *in vivo* detection of angiogenesis in inflammatory bowel disease (IBD) patients.

METHODS: Conventional and NBI colonoscopy was performed in 14 patients with colonic inflammation (8 ulcerative colitis and 6 Crohn's disease). Biopsy samples were taken and CD31 expression was assayed immunohistochemically; microvascular density was assessed by vessel count.

RESULTS: In areas that were endoscopically normal but positive on NBI, there was a significant ($P < 0.05$) increase in angiogenesis (12 ± 1 vessels/field *vs* 18 ± 2 vessels/field) compared with areas negative on NBI. In addition, in areas that were inflamed on white light endoscopy and positive on NBI, there was a significant ($P < 0.01$) increase in vessel density (24 ± 7 vessels/field) compared with NBI-negative areas.

CONCLUSION: NBI may allow *in vivo* imaging of intestinal angiogenesis in IBD patients.

INTRODUCTION

Angiogenesis plays a crucial role in neoplastic and non-neoplastic chronic inflammatory disorders[1-4], including the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC)[4,5]. Potent angiogenic activity has been demonstrated in histological specimens from the mucosa of both UC and CD patients, as assessed by CD31 staining[6]. Several reports have shown that blockade of angiogenesis in preclinical models of IBD is a promising new therapeutic approach[7,8]. Narrow band imaging (NBI) is a new endoscopic technology that highlights mucosal surface structures and microcapillaries. Optical filters achieve sequential green and blue illumination, narrowing the bandwidth of spectral transmittance, and thus obtaining tissue illumination at selected narrow

wavelength bands[9,10] to achieve the greatest contrast between vascular structures and the surrounding mucosa.

The diagnostic accuracy of NBI in detecting colorectal neoplasia in patients with or without concomitant UC is superior to conventional colonoscopy and equivalent to that of chromoendoscopy[11,12]. A recent meta-analysis concluded that NBI is accurate, with high diagnostic precision for *in vivo* diagnosis of neoplasia across a range of organs (colon, esophagus, duodenal ampulla and lung)[13].

Very recently, NBI has been proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active UC[14]. In the preliminary study described herein, we investigated whether NBI colonoscopy could be a useful tool to detect *in vivo* angiogenesis in IBD patients with colonic inflammation.

MATERIALS AND METHODS

This was an open study involving patients with a diagnosis of IBD referred to our Gastrointestinal Endoscopy Unit for follow-up colonoscopy. A total of 14 patients were included (8 UC and 6 colonic CD). The extent of the disease was determined by previous colonoscopy. At the time of enrollment in the study, 3 (3/8) UC patients presented with inactive disease (Mayo score = 0) while 5 (5/8) had active disease (2 patients Mayo score = 1, 2 patients Mayo score = 2 and 1 patient Mayo score = 1); 3 (3/6) patients with CD presented with inactive disease and 3 (3/6) had active disease. For CD patients endoscopic activity was assessed by Crohn's Disease Endoscopic Index of Severity (CDEIS). After obtaining informed consent from all patients, white light colonoscopy and NBI (Olympus Medical System, Tokyo, Japan) examinations were performed.

For the white light colonoscopy, the vascular pattern was defined as normal if it did not show any irregularities, or as distorted if the pattern was tortuous. When the vascular pattern intensity was visualized with NBI, we were able to distinguish 2 different mucosal patterns: a stronger (blacker) capillary vascular pattern (NBI+), and a milder or regular capillary vascular pattern (NBI-). For this reason, in our study the vascular pattern could be classified into 4 categories: normal (with white light colonoscopy) and NBI-; distorted (with white light colonoscopy) and NBI-; normal (with white light colonoscopy) and NBI+; distorted (with white light colonoscopy) and NBI+.

For each patient, after determining the vascular pattern by NBI, biopsy specimens were obtained from 5 areas that were normal with conventional endoscopy and NBI-, 5 areas that were normal with conventional endoscopy but NBI+, and 5 areas that were endoscopically inflamed and NBI+. CD31 staining was performed by immunohistochemistry, and microvascular density was assessed by vessel count in colonic biopsies. The pathologist was blinded to the subjects.

Statistical analysis

The parametric data are expressed as the mean ± SD and non parametric data as percent. Fischer's exact probability test and the χ^2 test were used to evaluate statistical differences. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

For each of the 14 patients enrolled in the study, the mucosal vascular pattern was assessed by both conventional and NBI colonoscopy.

Mucosal areas normal with white light colonoscopy and NBI-

In the uninflamed mucosa of patients with IBD, classified as normal with white light colonoscopy and NBI-, the NBI pattern was similar to that in the mucosa from healthy control individuals. At the same time, the vascularization pattern and the microvessel density, as revealed by CD31 staining, were similar in the specimens from the uninflamed, NBI- mucosa from patients with IBD and from healthy controls. No differences were found between UC and CD patients.

Comparison of NBI+ and NBI- areas that were normal with white light colonoscopy

Compared to areas that were endoscopically normal under white light colonoscopy and NBI-, endoscopically normal but NBI+ areas displayed a significant (*P* < 0.05) increase in angiogenesis (12 ± 1 vessels/field *vs* 18 ± 2 vessels/field) (Figure 1A and B). The importance of our findings lies in the evidence that in patients with normal white light colonoscopy, areas positive on NBI showed an increased leukocyte infiltrate and a significantly increased microvessel density (*P* < 0.05) as assessed by histological analysis (Figure 1A-C). As revealed by staining for CD31, the mean microvessel diameter in IBD was 0.1 mm, a size histologically compatible with the diameter of a dot observed on the NBI image. No differences were found between UC and CD patients (not shown).

Areas inflamed on white light colonoscopy and NBI+

Lastly, in areas from IBD patients that were inflamed under white light endoscopy and were NBI+, a significant (*P* < 0.01) increase in vessel density (24 ± 7 vessels/field) was found compared with endoscopically normal, NBI- areas (Figure 2A and B), a finding compatible with a high degree of microscopical inflammation and immune-driven angiogenesis. No differences were found according to Mayo score in vessel density (not shown). No differences were found between UC and CD patients (not shown).

DISCUSSION

Angiogenesis is an integral component of non-neoplastic chronic inflammatory disorders, such as IBD[15,16]. Microscopic imaging is the approach that has thus far proven most valid for quantification of vasculature in normal and pathological tissue[17-19]. NBI has been successfully used to visualize angiogenesis and thereby to detect cancerous areas in the colon. Because of the improved mucosal contrast provided, NBI may improve the detection of colon polyps compared with standard

Figure 1 Colonic mucosa of healthy individuals and uninflamed but narrow-band imaging (NBI)+ regions from patients with inflammatory bowel disease (IBD), visualized using white light colonoscopy and NBI endoscopy. The microvasculature of histologically normal control and IBD uninflamed colonic mucosa was immunohistochemically stained for CD31 and von Willebrand/factor Ⅷ. There was increased vascularization in uninflamed IBD mucosa that presented an aberrant NBI+ pattern, in the NBI+ areas compared with controls. A: Healthy subjects. The vascular pattern was normal on both conventional colonoscopy and NBI, which was confirmed by immunohistochemical staining; B: Areas of the IBD mucosa that were not inflamed on conventional colonoscopy and showed an aberrant NBI+ pattern. Immunohistochemical staining confirmed an increased vascularization in these areas compared with NBI- areas; C: Computerized morphometric analysis of the microvasculature in control and IBD uninflamed mucosa that was NBI+. After immunohistochemical staining, sections were analyzed for the total number of vessels/field (microvascular density). [a]A statistically significant difference was found between sections from areas of uninflamed IBD colonic mucosa that were NBI+ and from controls.

white light colonoscopy. NBI has also been widely used to detect dysplasia in patients with long-standing UC, achieving good results in terms of diagnostic accuracy and detection of polyps, although if did not show any statistically significant differences compared with standard white light colonoscopy[19,20].

Figure 2 Inflamed colonic mucosa of IBD patients visualized using white light colonoscopy and NBI. The microvasculature of IBD inflamed colonic mucosa was immunohistochemically stained for CD31 and von Willebrand/factor Ⅷ. A: Areas of the IBD mucosa that were inflamed on white light colonoscopy showing an aberrant NBI+ pattern. There was increased vascularization in the inflamed IBD mucosa in areas where there was an aberrant NBI+ pattern, which was confirmed by immunohistochemical staining; B: Areas of the IBD mucosa that were not inflamed on conventional colonoscopy and showed an aberrant NBI+ pattern. Immunohistochemical staining confirmed an increased vascularization in these areas compared with NBI- areas; C: Computerized morphometric analysis of the microvasculature in the IBD inflamed mucosa. After immunohistochemical staining, sections were analyzed for the total number of vessels/field (microvascular density). [a]A statistically significant difference was found between sections from areas of inflamed IBD colonic mucosa that were NBI+ and areas of uninflamed IBD mucosa that were normal under white light colonoscopy but were NBI+ .

In a recently published study, NBI was proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active UC[12]. In our study, we found that NBI could also be used to visualize areas of abnormal mi-crovascular changes, not observed at white light colonos-copy. A statistically significant correlation exists between NBI pattern positivity (both in inflamed and normal areas at conventional endoscopic examination) and microves-

sel density, as confirmed by CD31 staining in histological specimens from the same areas. In patients who were normal under standard colonoscopy, there was increased leukocyte infiltration and microvessel density in NBI+ areas, as assessed by histological analysis.

Blockade of angiogenesis could be beneficial in patients with chronic inflammation and some drugs that have demonstrated efficacy for the treatment of IBD, such as tumor necrosis factor-α inhibitors, have potent antiangiogenic activity. Our preliminary findings suggest that NBI could be a novel tool for the *in vivo* assessment of mucosal angiogenesis. However since our study is still a preliminary study because of the limited number of patients, a larger study should be performed to define the exact role of NBI in IBD patients, and the correlation of mucosal angiogenesis with endoscopic activity.

Endoscopic imaging and monitoring of angiogenesis has the potential to be a valuable biomarker in monitoring the grade of intestinal inflammation *in vivo*, monitoring response to and optimization of available treatments, and finally in evaluating the response to new agents for the treatment of IBD in randomized clinical trials.

ACKNOWLEDGMENTS

COMMENTS

Background
Angiogenesis plays a crucial role in neoplastic and non-neoplastic chronic inflammatory disorders, including inflammatory bowel diseases (IBD).

Research frontiers
Narrow-band imaging (NBI) is a new endoscopic technology that highlights mucosal surface structures and microcapillaries. A recent meta-analysis concluded that NBI is accurate, with high diagnostic precision for *in vivo* diagnosis of neoplasia across a range of organs (colon, esophagus, duodenal ampulla and lung).

Innovations and breakthroughs
Very recently, NBI has been proposed as a tool to assess the grade of inflammation in patients with inactive or mildly active ulcerative colitis. In this preliminary study, the authors investigated whether NBI colonoscopy could be a useful tool to detect *in vivo* angiogenesis in IBD patients with colonic inflammation.

Applications
Several reports have shown that blockade of angiogenesis in preclinical models of IBD is a promising new therapeutic approach. Visualize angiogenesis *in vivo* may represent the first step for such a therapeutic approach.

Terminology
Angiogenesis: the process of new capillary formation from pre-existing vasculature in adult tissues; Narrow-band imaging: a new endoscopic technology that highlights mucosal surface structures and microcapillaries.

Peer review
The authors report that NBI may be a novel modality for imaging of intestinal angiogenesis in IBD. The results provide sufficient evidence to draw scientific conclusions. The statistical data reflect the results and are adequate for a clinical study. The discussion is well organized, and valuable conclusions are provided.

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BRIEF ARTICLE

Disagreement between symptom-reflux association analysis parameters in pediatric gastroesophageal reflux disease investigation

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Abstract

AIM: To assess the agreement within 3 commonly used symptom-reflux association analysis (SAA) parameters investigating gastroesophageal reflux disease (GERD) in infants.

METHODS: Twenty three infants with suspected GERD were included in this study. Symptom index (SI), Symptom sensitivity index (SSI) and symptom association probability (SAP) related to cough and irritability were calculated after 24 h combined pH/multiple intraluminal impedance (MII) monitoring. Through defined cut-off values, SI, SSI and SAP values are differentiated in normal and abnormal, whereas abnormal values point towards gastroesophageal reflux (GER) as the origin of symptoms. We analyzed the correlation and the concordance of the diagnostic classification of these 3 SAA parameters.

RESULTS: Evaluating the GER-irritability association, SI, SSI and SAP showed non-identical classification of normal and abnormal cases in 39.2% of the infants. When irritability was taken as a symptom, there was only a poor inter-parameter association between SI and SSI, and between SI and SAP (Kendall's tau b = 0.37, $P < 0.05$; Kendall's tau b = 0.36, $P < 0.05$, respectively). Evaluating the GER-cough association, SI, SSI and SAP showed non-identical classification of normal and abnormal cases in 52.2% of the patients. When cough was taken as a symptom, only SI and SSI showed a poor inter-parameter association (Kendall's tau b = 0.33, $P < 0.05$).

CONCLUSION: In infants investigated for suspected GERD with pH/MII-monitoring, SI, SSI and SAP showed a poor inter-parameter association and important disagreements in diagnostic classification. These limitations must be taken into consideration when interpreting the results of SAA in infants.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) remains one of the most common diagnoses made by pediatric gastroenterologists. By definition, gastroesophageal reflux (GER) is the involuntary passage of gastric contents into the esophagus or oropharynx, which is a physiological process that appears in every individual particularly after meals[1]. When GER causes troublesome symptoms and/or complications, it is referred to as GERD[2]. Clinical manifestations in infants include among others regurgitation, vomiting, irritability and cough[1]. A main problem in the diagnosis of GERD is the association of symptoms with GER, especially the non-specific ones. While endoscopy can detect GER complications such as mucosal inflammation, symptom-reflux association analysis (SAA) is the only available method that can adequately identify GER as the cause of short lived symptoms[3].

SAA aims to show and quantify a temporal relation between symptoms and GER. With qualification, a significant temporal relation between symptoms and GER, thus an abnormal result of the SAA parameter, may suggest GERD. Three parameters are often used to address this temporal relationship: Symptom index (SI), symptom sensitivity index (SSI) and symptom association probability (SAP)[3-6]. A pre-condition required for SAA is a technical measure that identifies GER episodes in the individual esophagus. Two methods can be used: pH-monitoring and a combined pH/multiple intraluminal impedance (MII) monitoring.

In infants, GERD occurs frequently during the first months of life. During this period GER is often non-acid, because the frequent milk intake acts as a potent buffer of gastric acidity. pH-monitoring alone has the disadvantage of only detecting acid GER. The newer MII technique to measure GER allows the additional detection of non-acid GER and thus more GER events. This is the main difference from pH-measurement alone. It is likely that a combined pH/MII measurement will replace single pH-measurement in the future[4,7,8].

Defined cut-off values of the SAA parameters help to decide whether symptom episodes which occurred during GER monitoring are related to GER or not. Despite routine clinical use in pediatrics[6], SAA parameters have never been validated in children or in infants. The problem is the lack of an objective and reliable gold standard to which these parameters can be compared, as well as the ethical difficulty posed by obtaining data from healthy children. The correlation of these 3 parameters as well as the concordance of abnormal results within these parameters in infants is unknown. Therefore, a comparison between studies using different SAA parameters is not possible. Additional questions arise as to whether only one SAA parameter could be a substitute for the others or how to interpret different results of the various SAA parameters measured in one individual.

Condino *et al*[5] reported that in infants hospitalized for evaluation of GER, fussiness/pain and cough were the most frequent symptoms and were found to be the most frequent symptoms related to GER detected by combined pH/MII monitoring. We assessed SI, SSI and SAP for irritability and cough in a group of infants who underwent combined pH/MII monitoring for suspected GERD. In order to evaluate the agreement between these 3 parameters we analyzed the correlation and the concordance between them in normal and abnormal classification.

MATERIALS AND METHODS

In this retrospective study, combined pH/MII tracings of consecutively investigated infants performed between October 2006 and January 2009 were reviewed. All infants included had irritability and cough among other GER-like symptoms and underwent 24 h combined pH/MII monitoring for suspicion of GERD. Patients were clinically evaluated prior to combined pH/MII analysis. Various investigations such as barium swallow, esophagogastroduodenoscopy and laboratory analysis were performed based on history and clinical signs. A barium swallow was performed in infants with suspected anatomic upper gastrointestinal anomalies. Esophagogastroduodenoscopy was performed in the case of GERD resistant to therapy and to detect eosinophilic esophagitis. Diagnosis of esophagitis was based on histology of esophageal biopsies[9]. Therapy was prescribed by the referring physicians or by one of the authors (P.B.). Therapy was not discontinued during the entire observation period.

Twenty four h combined pH/MII measurement was performed using hardware and software by Sandhill Technologies (Sandhill Scientific Inc., Highlands Ranch, CO, USA). A single-use catheter (ConforTEC® pH-MII probe ZIN-BS 45) with a diameter of 2.13 mm was placed transnasally. This probe consisted of six 1.5 cm impedance recording segments and one pH-electrode, located within the distal impedance segment. The middle of each impedance segment was located at 0, 1.5, 3, 4.5, 6 and 7.5 cm above the pH-electrode. The initial positioning of the catheter was estimated using the following formula: (0.156 × height in cm) + 6.88 cm = length of pH-electrode from nose to distal esophagus[10]. Proper positioning of each catheter was confirmed by X-ray. The pH-electrode was placed 2 vertebral bodies above the diaphragm with a tolerance margin of 5 mm. The probe was connected to the portable recording device. An external reference electrode was attached posteriorly to the patient's chest.

The studies were performed on an inpatient basis. The caregivers (nurse and parents) were properly instructed by the attending physician on how to register the irritability and cough events on the portable recording device. We defined the term "irritability" as crying or whining. A child was irritable either if it cried or if it showed behavior of being unwell, expressed as making a grimace on the verge of crying. The latter was the perception of the parents. The pH/MII tracings were evaluated using the BioVIEW analysis software (Sandhill Scientific, Inc.) and each study was manually reviewed by a pediatric gastroenterologist. Patients with less than 20 h of recording were excluded from the study. A (liquid) GER episode detected by im-

pedance was defined as a retrograde drop in impedance by more than 50% of baseline in the distal 2 channels[11]. Gas only episodes were not included in the analysis. A symptom was considered related to a GER event if it occurred within a 5 min window from the GER event[12,13].

The SI was defined as the number of GER-related symptoms divided by the total number of symptoms, reflecting the percentage of symptoms related to GER episodes[12]. The SSI was defined as the number of GER-related symptoms divided by the total number of GER episodes, reflecting the percentage of GER associated with symptoms[14]. SAP was defined as the likelihood that the patients' symptoms were related to GER based on a statistical analysis (cross tabulation) of a contingency table consisting of 4 possible combinations of GER and symptoms. The SAP was calculated as $(1-P) \times 100\%$, with the P-value calculated using Fisher's exact test[15]. The SAP calculation was provided in the software package of the pH/MII monitoring device. Based on the literature, SI values of $\geq 50\%$, SSI values of $\geq 10\%$ and SAP values of $\geq 95\%$ were considered abnormal[3,5,6].

Statistical analysis

Statistical analysis was performed with SAS 9.2 (The SAS Institute, Cary, NC, USA). Continuous variables are presented as median and interquartile range (IQR) or, if an approximately normal distribution was assumed, as mean ± SD. P-values < 0.05 were considered significant. Concordance between the different SAA methods was assessed by Kendall's tau-b. Kendall's tau-b calculates a measure of concordance based on the number of concordant and discordant pairs and uses a correction for tied pairs. It ranges between -1 and +1, where +1 is perfect concordance and -1 perfect discordance. A value of zero indicates the absence of any concordance. In the case of continuous variables, rankings were compared[16].

The study was approved by the local ethics committee of the University of Fribourg, Switzerland.

RESULTS

Patient characteristics

Twenty-three infants aged 2 wk to 11 mo (median 3 mo, IQR 2.8 mo) were included in this study. 19 patients were male. One patient was under ranitidine treatment, 4 patients were under sucralfate treatment, 3 patients were under omeprazole treatment and 7 patients had thickened feeding. Mean duration of the procedure was 22 h and 44 min ± 1 h 11 min. A previous barium meal was performed in 11 patients and showed a hiatus hernia in 1 case. Two patients had undergone esophagogastroduodenoscsopy and biopsies after combined pH/MII monitoring, which showed esophagitis in both.

A total of 1692 GER episodes were detected, 395 were acid (23%) and 1297 non-acid (77%). 421 irritability episodes were reported, 165 (39%) were related to GER. There were 155 cough episodes, 73 (47%) related to GER. Table 1 summarizes the characteristics of the registered GER and symptom episodes.

Table 1 Characteristics of the registered reflux and symptom episodes

	GER episodes	Irritability episodes	Cough episodes
Min	22	2	1
Max	141	82	19
Median	67	11	5
IQR	48	18	10
Sum	1692	421	155

GER: Gastroesophageal reflux; Min: Minimal number for a single patient; Max: Maximal number for a single patient; Median: Median value for all patients; IQR: Interquartile range.

Symptom-reflux association analysis

We investigated the GER-irritability association and found the following overall values: SI 25%, IQR 37.5%; SSI 4.5%, IQR 8.1%; and SAP 54%, IQR 78%. Additionally, we investigated the GER-cough association and found the following overall values: SI 50%, IQR 75%; SSI 3.6%, IQR 4.3%; and SAP 38.4% ± 43.3%. Abnormal results for the GER-irritability association were found for 8 patients in SI (34.8%), for 5 patients in SSI (21.7%), and 3 patients in SAP (13.0%). We found abnormal results for the GER-cough association in 13 patients for SI (56.5%), 2 patients for SSI (8.7%), and in 1 patient for SAP (4.3%). Evaluation of the diagnostic classification in normal and abnormal cases for the GER-irritability association showed 60.8% of the patients with identical classification, and 39.2% with non-identical classification. Evaluation of the diagnostic classification in normal and abnormal cases for the GER-cough association showed 47.8% of the patients with identical classification, and 52.2% with non-identical classification. Table 2 summarize the data obtained.

All SAA parameters for the GER-irritability association were positive in one infant. This infant showed a notable GER on barium swallow without an anatomic anomaly. One infant had 3 positive SAA parameters for the GER-cough association. This infant had chronic esophagitis on esophagogastroduodenoscopy.

Evaluation of the diagnostic classification between the GER-irritability SAA parameters showed the following values: SI and SSI Kendall's tau b = 0.2791, P = 0.2153; SI and SAP Kendall's tau b = 0.2593, P = 0.2748; SSI and SAP Kendall's tau b = 0.4219, P = 0.1618. Evaluation of the diagnostic classification between the GER-cough SAA parameters showed the following values: SI and SSI Kendall's tau b = 0.2707, P = 0.1255; SI and SAP Kendall's tau b = 0.1870, P = 0.2987; SSI and SAP Kendall's tau b = 0.6908, P = 0.2847.

We found a poor correlation between the parameters SI and SSI in both symptom categories, irritability and cough. We also found a poor correlation between SI and SAP for the irritability symptom category (Table 3).

DISCUSSION

We investigated the agreement of different SAA parameters in infants who underwent combined pH/MII moni-

Table 2 Categorization of SI, SSI and SAP

SI	SSI	SAP	Number of infants	Percent	Cumulative frequency	Cumulative percent
Irritability/reflux association						
Abnormal	Abnormal	Abnormal	1	4.35	1	4.35
Abnormal	Abnormal	Normal	2	8.70	3	13.04
Abnormal	Normal	Abnormal	1	4.35	4	17.39
Abnormal	Normal	Normal	4	17.39	8	34.78
Normal	Abnormal	Abnormal	1	4.35	9	39.13
Normal	Abnormal	Normal	1	4.35	10	43.48
Normal	Normal	Normal	13	56.52	23	100.00
Cough/reflux association						
Abnormal	Abnormal	Abnormal	1	4.35	1	4.35
Abnormal	Abnormal	Normal	1	4.35	2	8.70
Abnormal	Normal	Normal	11	47.83	13	56.52
Normal	Normal	Normal	10	43.48	23	100.00

SI: Symptom index, abnormal if SI ≥ 50%; SSI: Symptom sensitivity index, abnormal if SSI ≥ 10%; SAP: Symptom association probability, abnormal if SAP ≥ 95%.

Table 3 Correlations between SI, SSI and SAP for the irritability/reflux association and the cough/reflux association expressed in Kendall's tau b

	Irritability			Cough		
	SI	SSI	SAP	SI	SSI	SAP
SI	-	0.37102 (*P* = 0.0147)	0.36011 (*P* = 0.0225)	-	0.32725 (*P* = 0.0362)	0.20959 (*P* = 0.2117)
SSI	0.37102 (*P* = 0.0147)	-	0.23623 (*P* = 0.1297)	0.32725 (*P* = 0.0362)	-	0.29632 (*P* = 0.0701)
SAP	0.36011 (*P* = 0.0225)	0.23623 (*P* = 0.1297)	-	0.20959 (*P* = 0.2117)	0.29632 (*P* = 0.0701)	-

toring for suspected GERD using unspecific symptoms such as irritability and cough. To the best of our knowledge this is the first study in children to compare the correlation and concordance of the diagnostic classification between SAA parameters. Taken together SI, SSI and SAP show important disagreements in the diagnostic classification of normal and abnormal symptom-GER association. Within the different SAA parameters a week correlation could be seen at most. This renders the interpretation of the SAA results and the diagnosis of GERD based on SAA parameters difficult as the detection of a pathologic symptom association depends on the SAA parameter chosen. Additionally, the comparison between different studies should be based on the same SAA parameters because our study shows that SI, SSI and SAP are not interchangeable.

Our study showed a higher frequency of non-acid GER (77%) compared to acid GER. This is comparable to the incidence of non-acid GER in infants found in the literature[17]. The high proportion of non-acid GER in our study could also be explained by the fact that some of our patients were examined under treatment. To leave the patients under treatment while presenting GER-like symptoms reflects a clinical situation common in pediatric gastroenterology, as pediatric gastroenterologists are currently increasingly asked to evaluate a patient on proton pump inhibitor treatment after an unsuccessful medical trial. Condino *et al*[5] found no relation between category of GER (acid or non-acid) and the association between GER and the symptoms irritability and cough. In that study, 49.8% of fussiness and pain episodes (comparable to irritability) were related to GER, whereas in our study only 39% of the irritability symptoms were related to GER. In addition, it was reported that 33.5% of cough episodes were related to GER, whereas our study shows that 47% of cough episodes were related to GER.

While our findings are interesting, obviously the limitations of our study must be pointed out. As with many other studies investigating the role of MII in children, the sample size is rather small[18]. Evaluation of the diagnostic classification did not show significant concordance. This is probably due to true discordance, but could also be due to a too weak study power. Furthermore, it must be noted that this study predominantly consists of male infants which reduces the possibility of generalizing the findings to female infants.

The question arises as to which of the three SAA parameters is the best for the diagnosis of GERD. To date the only study to evaluate the diagnostic accuracy of SI, SSI and SAP is a work by Taghavi *et al*[19] on an adult study group. That study validated the SAA parameters obtained on 24 h pH-monitoring against a PPI treatment-trial dividing the study group into responders and non-responders. Patients were given a high dose of omeprazole treatment and asked to complete a symptom score before and after the treatment. Those who showed an effect were considered to have GERD. SSI, with an adapted cut-off value of 1.3%, showed the highest positive and negative predictive values, followed by SAP. SI had the poorest performance

with absence of a meaningful cut-off value making it almost useless. Despite the routine clinical use of SAA parameters in pediatrics there is currently no validation study for combined pH/MII monitoring. The problem is the lack of an objective gold-standard test for the detection of GERD, independent from combined pH/MII monitoring. In an analogy to the work of Taghavi et al[19], we would need to perform a reference test in infants, with symptom scoring before and after treatment (e.g. proton pump inhibitors, prokinetics, surface agent, etc.) including combined pH/MII monitoring. This is ethically and methodologically challenging in infants.

In general use of SAA is hampered by numerous question marks concerning its accuracy. Firstly, it depends on a technique that correctly detects the GER events. It is likely that the introduction of combined pH/MII-monitoring adding the detection of non-acid GER events, compared to single pH-monitoring, makes SAA more accurate. A study of Rosen et al[6] showed that significantly more children had a positive SI using combined pH/MII-monitoring, than using pH-monitoring alone. A possible shortcoming of the 24 h-monitoring technique is the fact that the number of acid and non-acid GER events in MII varied significantly on 2 consecutive days in an adult study[20]. Additionally, investigating 48 h ambulatory pH-monitoring doubled the SI and SAP in adults with atypical GER symptoms compared to 24 h monitoring[21]. Furthermore, automatic MII analysis considers only a drop of impedance of 50% or more as a GER event; however, it is very likely that a drop of 49% could also be attributed to a GER event[4]. To date there are very few studies which evaluate the efficacy of automated analysis. A study by Roman et al[22] in adults however showed good agreement between visual analysis and the automated analysis used in our study.

Secondly, results of SAA may have some methodological weaknesses. It depends on the active participation of the observers, in our case nurse and parents[4], thus being impacted by the compliance and the variable work intensity on the ward. It has been shown that adults record only 39% of the cough episodes detected by simultaneous manometry[23]. The arbitrary choice of the time window influences the symptom association parameters. The 5 min time window used within this automatic analysis tool and in other studies may not be optimal for the evaluation of symptoms such as irritability and cough. In this context one has to keep in mind, that SAA detects timely association, which does not necessarily mean causal relation. Unspecific symptoms in infants such as irritability and cough can be evoked by a large number of factors. The temporal association of GER and an unspecific symptom can therefore be a coincidence. SI and SSI have cut-off values that were arbitrarily chosen. In addition, they do not take into account the total number of GER episodes and total number of symptoms, respectively. Therefore, the SI can be positive simply because of a high number of GER episodes, whereas the SSI can be falsely positive because of a high number of symptoms[18].

By showing poor inter-parameter association and important disagreements in diagnostic classification with the 3 established SAA parameters SI, SSI and SAP using combined pH/MII monitoring technique, we showed a common problem encountered with SAA, which is the difficulty of choosing the appropriate parameter to use. Our study cannot give an answer to this problem. Because of unanswered technological, methodological and validation questions and the limited concordance between the SAA parameters, we believe that at the moment, SAA based on combined pH/MII monitoring is a poorly reliable tool for the diagnosis of GERD. Diagnosis of GERD in infants with GERD-like symptoms therefore remains based on several elements such as the absence of more probable reasons for GERD-like symptoms, the positive response to treatment or the detection of GERD complications. However, combined pH/MII-monitoring can argue against GER as the origin of GERD-like symptoms, when showing absent or very low frequencies of GER episodes. Studies in infants are needed in order to obtain reference values and to adjust SI, SSI and SAP cut-off values for GERD, validated for the infant population.

COMMENTS

Background
Gastroesophageal reflux (GER), defined as passage of gastric contents into the esophagus is a normal process that occurs in healthy infants, children and adults. When GER causes troublesome symptoms and/or complications it is referred to as gastroesophageal reflux disease (GERD). During infancy GER is common and can manifest with specific symptoms as vomiting and non-specific symptoms as irritability and cough. Association of non-specific symptoms with GER is a main problem in the diagnosis of GERD.

Research frontiers
Combined pH/multiple intraluminal impedance (MII) detects GER episodes in the individual esophagus. The better the timely association of GER episodes and symptom episodes the more probable it is that GER is the origin of symptoms. Timely association of GER episodes and symptom episodes are expressed by symptom-reflux association analysis (SAA) parameters. Abnormal results of SAA parameters point towards GERD. Three SAA parameters are commonly used, symptom index (SI), symptom sensitivity index (SSI) and symptom association probability (SAP) but they were never validated for the diagnosis of GERD in the infant population. In this study the authors analyzed the agreement between SI, SSI and SAP.

Innovations and breakthroughs
This is the first study in infants to show an important disagreement between the 3 commonly used SAA parameters SI, SSI and SAP, which puts the accuracy of SAA into question. The study shows that the results of SI, SSI and SAP for the diagnosis of GERD often differ. In consequence, the diagnosis of GERD in infants cannot be based on a single SAA parameter as it remains unknown which SAA parameter is the most accurate for the diagnosis of GERD.

Applications
The limited agreement of these parameters must be taken into consideration when interpreting the results of SAA. The diagnosis of GERD should be based on a combination of pH/MII-monitoring, SAA results as well as on other factors such as clinical judgment, gastroscopy and follow-up under medical therapy. Validation studies to enhance the accuracy of SAA parameters and to answer the question which SAA parameter is the most accurate are needed.

Terminology
MII in combination with pH-measurement, a technique based on the fact that the passage of gastric content into the esophagus changes the impedance (electrical resistance) between esophageal segments, is more and more replacing the classic single pH-measurement as a diagnostic tool for GERD. A catheter with multiple impedance recording segments and one distal pH-electrode

connected to an exteriorly portable recording device is placed transnasally into the patient's esophagus in order to detect acid and nonacid GER events.

Peer review

This is an important topic and it has been little addressed in children.

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BRIEF ARTICLE

Alkaline phosphatase predicts relapse in chronic hepatitis C patients with end-of-treatment response

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Abstract

AIM: To investigate relapse predictors in chronic hepatitis C (CHC) patients with end-of-treatment response (ETR), after pegylated interferon-α (PegIFN-α) and ribavirin treatment.

METHODS: In a retrospective study we evaluated a spectrum of predictors of relapse after PegIFN-α and ribavirin treatment in 86 CHC patients with ETR. Viral loads were determined with real-time reverse transcription polymerase chain reaction. Hepatitis C virus genotyping was performed by sequencing analysis. Patients with genotype 1 were treated for 48 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 1000 mg/d for those under 75 kg or 1200 mg/d for those over 75 kg. Patients with genotypes 2 and 3 were treated for 24 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 800 mg/d.

RESULTS: In all ETR patients, binary logistic regression analysis identified absence of complete early virological response (cEVR) (OR 27.07, 95% CI: 3.09-237.26, *P* < 0.005), serum alkaline phosphatase (ALP) levels prior to therapy < 75 U/L (OR: 6.16, 95% CI: 2.1-18.03, *P* < 0.001) and body mass index > 26 kg/m^2 (OR: 8.27, 95% CI: 2.22-30.84, *P* < 0.005) as independent predictors of relapse. When cEVR patients were analyzed exclusively, ALP prior to therapy < 75 U/L remained the only predictor of relapse.

CONCLUSION: Lower levels of ALP prior to, during and after therapy seem to be associated with a higher risk of relapse in CHC patients with ETR.

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Key words: Alkaline phosphatase; Chronic hepatitis C; Pegylated interferon; Predictor; Relapse

INTRODUCTION

Chronic hepatitis C (CHC) is a major public health problem due to late complications, such as liver failure and hepatocellular carcinoma[1]. The prevalence of CHC has been estimated to be 3% worldwide[2]. Pegylated interferon-α (PegIFN-α) with ribavirin is currently the

treatment of choice for CHC[3]. Treatment response depends on patient and viral factors, the viral genotype being the most important[4]. Several studies have defined further predictive factors for response, such as hepatitis C virus (HCV)-RNA serum level, stage of liver disease, body weight, age, sex and race[5-10]. After completion of treatment, HCV may become detectable again considerably reducing sustained virological response (SVR) rates even after achievement of end-of-treatment response (ETR). The relapse rate of genotype 1 seems to be higher than in other genotypes, being as high as 30%-40%[11]. The risk of relapse was also shown to relate to treatment duration, and several efforts have been undertaken to identify those patients who would benefit from extended treatment duration[12].

In this retrospective study we investigate in a cohort of ETR patients whether further predictors of relapse may exist after treatment with PegIFN-α and ribavirin.

MATERIALS AND METHODS

Patient population

We reviewed the records of all CHC patients with an ETR who started PegIFN-α and ribavirin treatment between April 2005 and August 2007 at our clinic. Exclusion criteria were age < 18 years; any bone or bowel diseases, chronic renal failure, use of medication that could cause an elevation of liver enzymes; biliary obstruction confirmed sonographically; hepatitis B virus or human immunodeficiency virus co-infection; daily ethanol ingestion greater than 20 g for women and 40 g for men; drug abuse; history of previous antiviral treatment. Finally, 86 patients (62 men and 24 women, mean age 42.5 ± 11.3 years, range 18-67 years) were included. Genotypes 1, 2 and 3 were present in 52 (60.5%), 9 (10.5%) and 25 (29%) patients, respectively. Patients with genotype 1 were treated for 48 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 1000 mg/d for those under 75 kg or 1200 mg/d for those over 75 kg. Patients with genotypes 2 and 3 were treated for 24 wk with 180 μg PegIFN-α2a or 1.5 μg/kg PegIFN-α2b once weekly plus ribavirin at a dosage of 800 mg/d. Patients with extended treatment duration were not included in this study. A complete early virological response (cEVR) was considered present, when HCV-RNA by polymerase chain reaction (PCR) was negative 12 wk after initiation of therapy. Patients, who did not achieve a cEVR showed at least a partial early virological response (pEVR), defined as a 2-log-decline of HCV-RNA at 12 wk after the initiation of therapy compared to baseline viral load. ETR was defined as negative HCV-RNA at the end of treatment, and SVR was defined as persistent absence of HCV-RNA by PCR at 6 mo, thereafter.

Laboratory analysis

Alkaline phosphatase (ALP), alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transferase were assessed by routine colorimetric assays on the Modular Clinical Analyzer (Roche, Vienna, Austria). Diagnostic ranges were 1-1200 U/L, 4-600 U/L, 4-800 U/L and 3-1200 U/L, respectively. Normal ranges of ALP were 40-129 U/L for males and 35-104 U/L for females. The precision of the ALP assay was > 99.3%.

Viral loads were determined with the Cobas TaqMan HCV assay (Quantification range 10 to 6.9×10^7 IU/mL; Roche). HCV genotyping was performed by sequencing with the TruGene™ HCV 5'NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Bad Nauheim, Germany).

Liver biopsy was available in 33 patients. Stages of liver fibrosis and inflammatory activity were classified according to the Metavir score[7].

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (version 10, SPSS Inc., Chicago, Illinois, USA). Values are expressed as mean ± SD or as median (range). Differences between groups were compared by Student's *t*-test or Mann-Whitney *U* test where appropriate. Fisher's exact test was used to assess possible differences in categorical variables between patients with SVR and relapse. Possible correlations between variables were analyzed using the Spearman rank test. Binary logistic regression analysis was performed to identify independent predictors of relapse. Significance tests were 2-sided, and *P*-values < 0.05 were considered significant.

RESULTS

The clinical characteristics of the ETR patients are summarized in Table 1. Relapse occurred in 26 patients (30.2%), 17 patients (65.4%) with genotype 1, three patients (11.5%) with genotype 2 and 6 patients (23.1%) with genotype 3. The frequency of patients with genotype 1 was not significantly different between the relapse and the SVR group (Table 1). Median ALP prior to therapy was significantly higher, whereas mean body mass index (BMI), stage of liver fibrosis and viral load prior to therapy were significantly lower in patients with SVR compared to those with relapse. The frequency of cEVR was significantly higher in the SVR group compared to the relapse group (Table 1).

In all ETR patients, binary logistic regression identified absence of cEVR (OR 27.07, 95% CI: 3.09-237.26, *P* < 0.005), ALP prior to therapy < 75 U/L (OR: 6.16, 95% CI: 2.1-18.03, *P* < 0.001) and BMI > 26 kg/m² (OR: 8.27, 95% CI: 2.22-30.84, *P* < 0.005) as independent predictors of relapse. When only cEVR patients were analyzed, ALP prior to therapy < 75 U/L (OR: 7.79, 95% CI: 1.96-30.98, *P* < 0.005) remained the only predictor of relapse.

Prior to therapy, all ALP levels in patients with relapse were within the normal range.

During PegIFN-α and ribavirin treatment, ALP levels showed variations: in patients with SVR there was a significant decline, whereas in patients with relapse there was a slight non-significant variation (Figure 1A). Similar results were obtained when patients with genotype 1 were analyzed, only (Figure 1B).

Table 1 Clinical and biochemical characteristics of the patients prior to therapy

	SVR (*n* = 60)	Relapse (*n* = 26)	*P*-value
Age (yr)	41.3 ± 11.3	45.4 ± 10.9	NS
Male/female	43/17	19/7	NS
Body mass index (kg/m^2)	24.9 ± 4.0	28.7 ± 5.5	< 0.010
Systolic blood pressure (mmHg)	119 ± 14	119 ± 12	NS
Diastolic blood pressure (mmHg)	73 ± 10	75 ± 10	NS
Genotype 1, *n* (%)	35 (58.3)	17 (65.4)	NS
HCV-RNA prior to therapy (IU/mL)	480 500 (2430-23 000 000)	806 000 (107 000-8 400 000)	< 0.050
PegIFN α2a/PegIFN α2b	32/28	14/12	NS
cEVR, *n* (%)	58 (96.7)	17 (65.4)	< 0.001
Histological fibrosis stage	1 (0-4)	3 (1-4)	< 0.050
Histological activity	1 (1-3)	2 (1-3)	NS
Aspartate aminotransferase (U/L)	40 (15-416)	47.5 (24-156)	NS
Alanine aminotransferase (U/L)	72.5 (17-560)	70 (32-214)	NS
γ-glutamyl transferase (U/L)	39 (3-693)	48 (11-183)	NS
Alkaline phosphatase (U/L)	84 (40-232)	67 (48-129)	< 0.005
Cholinesterase (kU/L)	7.22 ± 2.18	7.50 ± 2.37	NS
Lactate dehydrogenase (U/L)	190 ± 42	193 ± 34	NS
Total bilirubin (mg/dL)	0.77 ± 0.57	0.68 ± 0.24	NS
C-reactive protein (mg/dL)	0.2 (0-1.8)	0.1 (0-1.0)	NS
Fibrinogen (mg/dL)	256 ± 52	273 ± 54	NS

SVR: Sustained virological response; HCV: Hepatitis C virus; PegIFN: Pegylated interferon; cEVR: Complete early virological response; NS: Not significant.

Figure 1 Time course of median serum levels of alkaline phosphatase (ALP) in patients with sustained virological response (SVR) and patients with relapse. A: All patients with genotypes 1-3 from week 0 to week 24. The 36 and 48 wk values exclusively represent genotype 1 patients (*n* = 52), as patients with genotypes 2 and 3 were only treated for 24 wk; B: Genotype 1 patients, only. ^aP < 0.05 *vs* SVR; ^cP < 0.005 *vs* SVR; ^eP < 0.05 *vs* baseline. p.t.: Post treatment.

DISCUSSION

The results of our study indicate that serum levels of ALP are related to the risk of relapse in CHC patients with ETR after treatment with PegIFN-α and ribavirin.

Elevated levels of ALP are found in bone, bowel and bile duct diseases. As our study did not include patients with bone or bowel diseases, the observed significant difference in pre-treatment ALP between patients with relapse and those with SVR might be caused mainly by differing amounts of ALP from the liver or the bile ducts as the primary source of disease. Bile duct inflammation has been reported in up to 95% of HCV patients and was shown to correlate with serum ALP[13]. Since immune response and inflammation play key-roles in the elimination of HCV, the higher pre-treatment ALP levels in patients with SVR may possibly reflect a higher degree of inflammation with a more sustained response to therapy. However, the histological activity score in our patients failed to show this, as there was no significant difference between patients with SVR and relapse, which could be due to our limited sample size. The observed decline in ALP during PegIFN-α and ribavirin treatment may possibly be due to a decrease of intrahepatic inflammation, which initially could have been higher in those patients with SVR. PegIFN-α and ribavirin may also influence bone metabolism and levels of bone-derived ALP, however, other studies were unable to confirm these findings[14-16]. Any pathophysiological reasoning for the association of ALP levels and risk of relapse of CHC is speculative at present.

Our study is the first to focus on ETR-patients treated with PegIFN-α and ribavirin. Most patients in our study exhibited ALP levels within the normal range with

only 5% of patients with SVR exceeding the upper limit of the normal range. The limits of normal ranges are different in males and females, but gender distribution was almost equal in both patient groups.

In previous studies, genotype 1 was associated with increased relapse rates compared to the other genotypes[11]. In our study, the relapse rate in genotype 1 patients was not significantly higher probably due to the limited number of patients studied.

The strongest predictor of relapse in our study was absence of cEVR. Nearly all patients with SVR and almost two thirds of patients with relapse exhibited a cEVR. When cEVR is achieved, treatment duration is usually not extended. As prolongation of treatment has been shown to reduce relapse rates, ALP may possibly help to identify those patients, who would benefit from extended treatment duration[12].

Limitations of our study comprise its retrospective nature, limited sample size and lack of histological examinations in all patients. As ALP is elevated in a variety of diseases, we cannot exclude that some subclinical diseases with minor ALP elevations confounded our data. Despite those limitations, median ALP was higher in our SVR patients compared to those with relapse at all time points.

In conclusion, lower levels of ALP prior to, during and after therapy with PegIFN-α and ribavirin seem to be associated with a higher risk of relapse in patients with ETR. Further studies are required to clarify whether our observation could help to identify those patients who would benefit from extended therapy despite the presence of cEVR.

COMMENTS

Background

Chronic hepatitis C (CHC) is a major public health problem due to late complications, such as liver failure and hepatocellular carcinoma. Pegylated interferon-α (PegIFN-α) together with ribavirin is currently the treatment of choice for CHC. After achievement of end-of-treatment response (ETR) in CHC patients, the occurrence of relapse reduces sustained virological response (SVR) rates.

Research frontiers

Treatment response in CHC patients depends on patient and viral factors, the viral genotype being the most important. Several studies have defined further predictive factors for response, such as hepatitis C virus (HCV)-RNA serum level, stage of liver disease, body weight, age, sex and race. In this study the authors investigated in a cohort of ETR patients, whether further predictors of relapse may exist after treatment with PegIFN-α and ribavirin.

Innovations and breakthroughs

Previous studies investigated predictors of relapse in HCV infected individuals after PegIFN-α and ribavirin therapy. Those studies examined responding as well as non-responding patients, and predictive factors for response, such as HCV-RNA serum level, stage of liver disease, body weight, age, sex and race were identified. After completion of treatment, recurrence of the virus is still a major issue and a quest has been initiated to determine whether there may be predictors of relapse, in order to identify those patients who might benefit from extended treatment duration up front. The result of this study would suggest that alkaline phosphatase (ALP) might be such a predictor in ETR patients. This study is the first to focus on ETR patients, exclusively.

Applications

The authors suggest that apart from monitoring the already known predictors of relapse, ALP monitoring should be included in the work-up of HCV infected patients in preparation for anti-HCV treatment. Furthermore, ALP as an inexpensive clinical parameter should be monitored through-out the treatment period. Their results warrant larger studies to be conducted in a prospective fashion, in order to elucidate the clinical value of ALP monitoring in HCV patients undergoing treatment.

Peer review

It's a very interesting paper and I propose it to be published. Sure AP is not the only important tool to guarantee a SVR but can be useful to speak with patient's about this possibility.

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BRIEF ARTICLE

Increased levels of homocysteine in patients with ulcerative colitis

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Abstract

AIM: To investigate serum levels of homocysteine (Hcys) and the risk that altered levels carry for thrombosis development in ulcerative colitis (UC) patients.

METHODS: 55 UC patients and 45 healthy adults were included. Hcys, vitamin B12 and folic acid levels were measured in both groups. Clinical history and thromboembolic events were investigated.

RESULTS: The average Hcys level in the UC patients was 13.3 ± 1.93 μmmol/L (range 4.60-87) and was higher than the average Hcys level of the control group which was 11.2 ± 3.58 μmmol/L (range 4.00-20.8) (*P* < 0.001). Vitamin B12 and folic acid average values were also lower in the UC group (*P* < 0.001). When multivariate regression analysis was performed, it was seen that folic acid deficiency was the only risk factor for hyperhomocysteinemia. Frequencies of thromboembolic complications were not statistically significantly different in UC and control groups. When those with and without a thrombosis history in the UC group were compared according to Hcys levels, it was seen that there were no statistically significant differences. A negative linear relationship was found between folic acid levels and Hcys.

CONCLUSION: We could not find any correlations between Hcys levels and history of prior thromboembolic events.

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic disorders of unknown etiology characterized by destructive inflammation of the gastrointestinal tract, acute exacerbations and remission periods. IBD is exemplified by two major groups of diseases. These include ulcerative colitis (UC) and Crohn's disease (CD). These two diseases are indistinguishable in 10%-15% of IBD patients. This is termed

Indeterminate Colitis (IC)[1]. Patients with IBD are at increased risk of experiencing thromboembolic complications. The incidence of arterial and venous thromboembolic diseases has been reported to be 1%-8% in UC and CD, however some autopsy studies have reported this incidence to be as high as 39%[2,3]. In the study of Bernestein *et al*[4], the risks of deep vein thrombosis and pulmonary emboli were found to be 3 times higher than that of the general population. In addition, venous thrombosis occurs earlier in IBD patients compared to the general population[5]. Thromboembolism is a multifactorial condition. Studies have indicated a state of hypercoagulability involving all the components of the coagulation system in patients with UC[6-8]. The exact etiology and pathogenesis of hypercoagulability have not been explained, however it is widely accepted that this condition is associated with acquired or genetic defects of the coagulation system and the procoagulant effect of proinflammatory cytokines[9-14]. This hypercoagulability increases the risk of thromboembolic events and has a role in the pathogenesis of UC *via* formation of microthrombi in the intestinal microcirculation[15,16].

Homocysteine (Hcys), which was first defined in 1932 by Butz and Vigneaud, is a sulphur-containing amino acid synthesized during transformation of methionine to cysteine in methionine metabolism[17]. Mild hyperhomocysteinemia occurs in about 5%-7% of the general population[18-24]. Increased Hcys levels are due to enzyme abnormalities in metabolic pathways or to nutritional deficits, including folate and vitamin B2, B6 and B12 deficiencies[25,26]. The exact relationship between hyperhomocysteinemia and induction of thrombosis is unknown, however, a state of hypercoagulability resulting from endothelial dysfunction has been blamed[26,27].

Vitamin B12 and folic acid deficiencies due to malnutrition, malabsorption and antifolate medications including methotrexate and sulphasalazine are quite common in IBD. The deficiency of these key nutrients leads to increased Hcys levels in IBD. Several recent studies have reported increased Hcys levels in IBD[28-33]. The objective of this study was to determine levels of homocysteine, folic acid and vitamin B12 in patients with UC; to compare these data with those of a healthy control group; and to investigate the relationship between homocysteine levels and disease activation, thromboembolic complications, localization of disease, and levels of folic acid and vitamin B12.

MATERIALS AND METHODS

Subjects

A total of 55 UC patients [38 males, 17 females; mean age (± SD) 47.4 ± 13.80, age range 20-78 years] were recruited in the study. The patient group consisted of patients followed up and treated for IBD at the outpatient clinic between the dates March 2006 and January 2007. Diagnosis of UC was established using clinical, endoscopic and histological criteria. All patients underwent appropriate endoscopy (colonoscopy or rectosigmoidoscopy) to determine endoscopic activity indices and localization of

disease. Biopsy samples were obtained for histopathologic examination when necessary. Rachmilewitz Endoscopic Activity Index was used to determine the degree of endoscopic activity[34]. Clinical activity index was determined according to Truelove and Witts criteria[35].

Current complaints, duration of disease, extraintestinal symptoms, smoking status, history of thromboembolic events (deep vein thrombosis, pulmonary emboli, myocardial infarction, stroke and peripheral arterial obstruction) and medications were recorded. Patients with any other systemic disorders including diabetes mellitus, hyperthyroidism, chronic liver and renal disease, and history of cancer, as well as patients on vitamin B12, folic acid and oral multivitamin supplements, were excluded from the study.

A detailed clinical history was obtained from all patients regarding any previous events of arterial or venous thrombosis. A total of 3 subjects (5.5%) had history of arterial and venous thromboembolic events. Two of these (3.63%) were peripheral deep vein thrombosis, and 1 (1.81%) was myocardial infarction (deep vein thrombosis was diagnosed using Doppler USG, and myocardial infarction using ECG and cardiac enzymes).

A healthy control group was composed of patients with similar age and sex distribution, and without any metabolic, neoplastic or inflammatory diseases or any history of thromboembolic diseases. The healthy control group consisted of a total of 45 healthy individuals [31 males, 14 females; mean age (± SD) 46.4 ± 13.89, age range 20-77 years].

Blood samples

Blood samples were obtained from all patients following 12-h fasting and these samples were used for complete blood count, sedimentation rate, C reactive protein (CRP), routine biochemical analysis (including total cholesterol, triglycerides, LDL cholesterol, VLDL cholesterol), thyroid function tests, folic acid, vitamin B12, homocysteine level examinations. Biochemical parameters were measured using standard methods. Serum levels of folic acid and vitamin B12 were measured using chemiluminescent microparticle immunoassay (CMIA) (Abbott System). Serum homocysteine levels were measured with high pressure liquid chromatography (HPLC) (Betamed, Agilent 1100 series, Chromosystems Reagent Kit).

Statistical analysis

Data were analyzed using SPSS 11.5 package software. Definitive statistics were indicated as mean ± SD or median (minimum-maximum) in continuous variables, whereas categorical data were expressed as number of observations (%). Presence of any significant differences between case and control groups in terms of measurements was evaluated using Mann Whitney *U* test. In contrast, Kruskal Wallis was used to determine any significant differences between treatment types, clinical activity index and localization groups in terms of Hcys levels. Spearman correlation test was used to determine the asso-

Table 1 Demographic features of UC and control groups			
	Patients with UC (*n* = 55)	Healthy controls (*n* = 45)	*P*
Mean age (yr ± SD)	47.4 ± 13.80	46.4 ± 13.89	0.484
Gender (male/female)	38/17	31/14	0.983
Smoking status (%)	17 (30.9)	15 (33.3)	0.796
Localization (%)			
Rectum/sigmoid	34 (61.8)		
Left colitis	12 (21.8)		
Pancolitis	9 (26.4)		
Activity of UC (%)			
Active	28 (50.9)		
Inactive	27 (49.1)		
Medical treatment (%)			
5-ASA	41 (74.5)		
Steroids	3 (5.5)		
AZA	11 (20.0)		

UC: Ulcerative colitis; ASA: Aminosalicylic-acid; AZA: Azathioprine.

Table 2 Hcys, folic acid and vitamin B12 levels in UC and control groups (95% CI)			
	Patients (*n* = 55)	Controls (*n* = 45)	*P*
Hcy (μmol/L)	13.3 ± 1.93	11.3 ± 3.58	< 0.001
Folic acid (ng/mL)	5.1 ± 2.19	6.3 ± 0.87	< 0.001
Vitamin B12 (pg/mL)	250.4 ± 82.49	327.4 ± 73.90	< 0.001
Thromboemboli	3 (5.5%)	0 (0%)	0.250

Table 3 Correlation between Hcys and other variables among patients with UC		
	Relationship coefficient	*P*
Disease duration	0.105	0.447
Folic acid	-0.311	0.021
Vitamin B12	-0.146	0.288
CRP	0.244	0.073
Clinical activity index	0.115	0.403

ciation of Hcys levels and duration of disease, clinical activity index, vitamin B12, folic acid and CRP. χ^2 or Fisher's exact test was used for categorical comparisons. A *P* level of < 0.05 was accepted for statistical significance.

RESULTS

Fifty-five patients with UC were recruited into the study. Patients consisted of 38 (69.1%) males, 17 (30.9%) females and mean age was 47.4 ± 13.80 years with a range between 20-78 years. Forty-five healthy individuals were recruited in the control group, consisting of 31 (68.9%) males, 14 (31.1%) females, with a mean age of 46.4 ± 13.89 years, range between 20-77 years. Groups did not differ statistically significantly in terms of age, sex and smoking status (*P* = 0.484, *P* = 0.983, *P* = 0.796, respectively).

Analysis of distribution of disease localizations in the UC group indicated that 34 (61.8%) patients had distal involvement, 12 (21.8%) patients had left colitis and 9 (16.4%) had pancolitis. Clinical activity indices of patients in the UC group showed that mild, moderate and severe cases were 28 (50.9%), 21 (38.2%) and 6 (10.9%) in number. Analysis of current treatment indicated that 41 patients (74.5%) received 5-ASA, 11 (20%) received azothioprine and 3 (5.5%) received corticosteroid treatment (Table 1).

Mean homocysteine level was 13.3 ± 1.93 μmol/L (range 4.60-87) in the UC group and 11.2 ± 3.58 μmol/L (range 4.00-20.8) in the control group (*P* < 0.001). Mean serum folic acid level was 5.1 ± 2.19 ng/mL (range 1.90-10.90) in the UC group and 6.3 ± 0.87 ng/mL (range 5-10) in the control group (*P* < 0.001). Mean level of vitamin B12 was 250.4 ± 82.49 pg/mL (range 96-505) in the UC group and 327.4 ± 73.90 pg/mL (range 200-482) in the control group (*P* < 0.001).

Thromboembolic events had similar prevalence in UC and control groups and did not differ statistically significantly in this respect (*P* = 0.250) (Table 2). It was determined that a total of 3 (5.5%) subjects had a history of arterial and venous thromboembolic events. Two of these (3.63%) were peripheral deep vein thrombosis and 1 (1.81%) was myocardial infarction (diagnoses were established using Doppler USG for deep vein thrombosis and ECG plus cardiac enzymes for myocardial infarction). The UC group did not differ from control to a statistically significant degree (*P* = 0.250). These three patients had normal Hcys levels.

No directly proportional relationship was determined in the UC group between homocysteine levels and disease duration, vitamin B12, CRP and clinical activity index; however, there was a negatively proportional relationship found between folic acid and homocysteine levels (*r* = -0.311 and *P* = 0.021) (Table 3).

There were no statistically significant differences within the disease localization groups of UC in terms of homocysteine levels (*P* = 0.096). Additionally, there were no statistically significant differences within the clinical activity index groups of UC in terms of homocysteine levels (*P* = 0.698). Similarly, there were no statistically significant differences within the treatment groups of UC in terms of homocysteine levels (*P* = 0.695).

DISCUSSION

Studies have shown that the risks of deep vein thrombosis and pulmonary emboli are three times higher in patients with IBD compared to the general population[4]. The exact pathogenesis of thromboembolism is not known and considered to be multifactorial[36-38]. Moderate hyperhomocysteinemia (hHcy) has been shown to be one of the independent risk factors associated with development of arterial and venous thrombosis[24,39-44].

Elevated Hcys might be due to genetic factors (MTH-FR mutation), nutritional factors (deficiencies of folic acid, vitamin B6, vitamin B12) or medications (salazopyrin, methotrexate, corticosteroids)[28,45]. The most common cause of hHcy has been found to be folic acid deficiency in patients with UC[31,46,47].

Several previous studies also reported higher Hcys levels in patients with UC[28-33,45,48,49]. In our study, serum Hcys levels were higher in patients with UC than in healthy controls. Increased Hcys levels were negatively correlated with lower vitamin B12 and folic acid levels. However, multivariate regression analysis demonstrated that decreased folic acid levels were the most significant parameter in determining increased Hcys levels. In a similar study by Mahmut *et al*[28], increased Hcys levels were found to be associated particularly with folic acid deficiency. Hcys levels were observed to decrease following folate replacement and the authors recommended that folic acid levels should be determined and prophylactic folate treatment should be initiated in all patients with IBD. Zezoz *et al*[50] performed a similar study and found that the prevalence of increased Hcys was higher in the UC group compared to the control group (prevalence of Hcys was 30% in UC patients and 10% in control group). Statistical analysis showed that male sex, decreased folic acid and vitamin B12 levels were indicators of hHcy in patients with UC and that decreased folic acid levels were the most important factor. In our study also, decreased folic acid level was found to be the most important indicator of hHcy.

Folic acid deficiency might be associated with several factors in patients with IBD. These include inadequate intake, increased consumption or folate malabsorption in patients using medications, including particularly sulphasalazine (SASP)[30]. In the study of Zezos *et al*[50], one patient who was receiving SASP was found to have increased Hcys levels. An antifolate effect of SASP has also been demonstrated in other studies[28,29]. Only one patient was receiving SASP in our study and this patient had low folic acid and high Hcys levels.

Vitamin B12 levels were found to be decreased in our UC group compared to our control group; however this finding did not attain statistical significance. Studies have shown normal vitamin B12 levels among patients with increased Hcys levels[32,47]. The study of Romagnuolo *et al*[32] performed with regard to this issue has indicated that the prevalence of hHcy was 15.4% in IBD patients and that 80% of these patients had normal vitamin B12 levels. These authors suggested that there could be significant deficiency of vitamin depots or subclinical vitamin B12 deficiency in IBD patients with increased homocysteine and normal serum vitamin levels. In the study by Lambert *et al*[47], vitamin B12, B6 and folate deficiencies were determined as the most sensitive indicators of hHcy. These authors also suggested that subclinical vitamin deficiencies or intracellular vitamin deficiencies heralded vitamin deficiencies in circulation and that multivitamin (including folate) supplements were effective in decreasing Hcys levels. Similar to several previous studies, we found that hHcy did not correlate with disease activation, localization and medications[28,29,31,33]. However, some studies have shown a statistically significant relationship between disease activation and hHcys and reported that Hcys levels were markedly higher in patients with active disease compared to those with inactive disease[49,51].

Two recent studies have investigated Hcys levels in colon mucosa and aimed to discover the role of Hcys in the pathogenesis of UC[52,53]. Morgenstern *et al*[52] obtained biopsy samples from transverse colon and sigmoid or descending colon mucosa of 11 UC and 5 CD patients and examined these samples using high performance liquid chromatography (HPLC). Concentrations of Hcys were found to be higher in the UC and CD patients compared to healthy individuals. This study is the first to demonstrate elevated Hcys levels in human colon mucosa. No statistically significant differences were determined between Hcys levels, and disease activation or current treatment (particularly those receiving and not receiving sulphasalazine) in IBD patients. Interestingly, patients with other chronic inflammatory bowel diseases had normal Hcys levels; and patients with history of colorectal cancer had higher Hcys levels in colon mucosa compared to the normal[52]. The incidence of colon cancer among patients with UC is about 8 times higher than sporadic colon cancer. Hcys has mitogenic and proliferative features[54]. Elevated Hcys levels have been reported to be a risk factor for carcinogenesis[52].

In their study, Danese *et al*[53] measured both plasma and colon mucosal Hcys levels concomitantly in patients with IBD. They reported that Hcys levels were increased in both plasma and colonic mucosa of patients with UC and CD and suggested that Hcys could have a proinflammatory role in IBD. Similar to our study, this investigation did not determine any correlation between Hcys levels and disease activation or medications. A negative correlation was determined between circulatory and mucosal Hcys levels and folic acid levels, emphasizing the necessity of folic acid supplementation in patients with IBD[53].

Large retrospective studies have shown the rate of thromboembolic complications in patients with IBD to be between 1.3%-6.4%[55,56]. Oldenburg *et al*[29] reported that the rate of complications in a total of 231 patients with IBD was 5.7% in those with CD, and 9.0% in those with UC. Comparison of patients with and without venous thrombosis did not yield any statistically significant difference with regard to Hcys concentration. They suggested that hHcy did not influence the prevalence of thrombosis in patients with IBD; but that thrombosis developed secondary to multifactorial phenomena. These authors also suggested that hHcy could contribute to the pathogenesis of thromboembolic events in some of these patients[29].

Papa *et al*[33] reported the rate of thromboembolic complications to be 9.4% (6 patients) in 39 UC and 25 CD patients. There was hHcy in 2 of these patients. There was no statistically significant difference compared to the control group. Additionally, no statistically significant differences were found in Hcys levels in the within group comparison of IBD patients with regard to history of previous thromboembolic events[33]. In our study there were 3 patients with history of thromboembolic events and no statistically significant differences were present compared to the control group. We concluded that hHcy is not the major factor contributing to the development of thromboembolic complications; and that multifactorial etiology was more relevant.

In conclusion, hyperhomocysteinemia is a common

phenomenon in patients with IBD. Vitamin deficiencies should be determined in all patients with IBD and folate and vitamin B complex supplementations should be included in their treatment. In our study, we could not find any correlation between Hcys levels and history of arterial and venous thrombosis. Further studies should be performed to investigate the multifactorial etiology in the development of thromboembolic events in patients with IBD.

COMMENTS

Background
Patients with inflammatory bowel diseases (IBD) are at increased risk of experiencing thromboembolic complications. Studies have indicated a state of hypercoagulability involving all the components of the coagulation system in patients with ulcerative colitis (UC). The exact etiology and pathogenesis of hypercoagulability have not been explained, however, it is widely accepted that this condition is associated with acquired or genetic defects of the coagulation system and with the procoagulant effect of proinflammatory cytokines.

Research frontiers
In their study, Danese *et al* measured both plasma and colon mucosal homocysteine (Hcys) levels concomitantly in patients with IBD. They reported that Hcys levels were increased in both plasma and colonic mucosa of patients with UC and Crohn's disease (CD) and suggested that Hcys could have a proinflammatory role in IBD.

Innovations and breakthroughs
The results of studies investigating the influence of hyperhomocysteinemia on thrombosis in patients with IBD are controversial. Some studies suggest that it could contribute to the pathogenesis of thrombosis whereas other authors suggest that thrombosis develops in IBD patients secondary to multifactorial phenomena.

Applications
In this study, the authors found that the average Hcys level in UC patients was significantly higher than the average Hcys level of the control group. They could not find any correlation between Hcys levels and history of arterial and venous thrombosis. No directly proportional relationship was determined in the UC group between homocysteine levels and disease duration, vitamin B12, CRP level and clinical activity index; however there was a negatively proportional relationship found between folic acid and homocysteine levels. Vitamin B12 and folic acid average values were also lower in the UC group ($P < 0.001$). When multivariate regression analysis was performed, it was seen that folic acid deficiency was the only risk factor for hyperhomocysteinemia. For this reason the authors think that vitamin deficiencies should be determined in all patients with IBD and folate and vitamin B complex supplementation should be included in their treatment.

Terminology
Homocysteinemia is defined as elevation of homocysteine (a sulphur-containing amino acid) level in blood. It is an established risk factor for cardiovascular diseases and premature atherosclerosis.

Peer review
The study demonstrates increased levels of Hcys related to a decrease of folic acid and B12 in patients affected by UC. These data are not associated with an increase of thromboembolic events. The manuscript is well structured.

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BRIEF ARTICLE

eGFR is a reliable preoperative renal function parameter in patients with gastric cancer

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Abstract

AIM: To evaluate the validity of the estimated glomerular filtration rate (eGFR) as a preoperative renal function parameter in patients with gastric cancer.

METHODS: A retrospective study was conducted in 147 patients with gastric cancer. Preoperative creatinine clearance (Ccr), eGFR, and pre- and postoperative serum creatinine (sCr) data were examined. Preoperative Ccr and eGFR were then compared for their reliability in predicting postoperative renal dysfunction.

RESULTS: Among 110 patients with normal preoperative Ccr values, 7 (6.3%) had abnormal postoperative sCr values, and among 112 patients with normal preoperative eGFR values, postoperative sCr was abnormal in 5 (4.5%) ($P = 0.53$). Among 37 patients with abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and of 35 patients with abnormal preoperative eGFR values, postoperative sCr was normal in 25 (71.4%) ($P = 0.34$). Preoperative Ccr was significantly correlated with eGFR ($r = 0.514$), and postoperative sCr was significantly correlated with preoperative Ccr ($r = -0.334$) and eGFR ($r = -0.02$).

CONCLUSION: Preoperative eGFR is as effective as Ccr for predicting postoperative renal dysfunction. eGFR should therefore be used as an indicator of preoperative renal function in place of Ccr since it is a cheaper and easier to perform test.

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INTRODUCTION

The creatinine clearance (Ccr) test has been used as a gold standard for evaluation of preoperative renal function in patients with gastric cancer[1,2]. However, it is known that Ccr is not strictly equivalent to glomerular filtration rate (GFR) because of the intrinsic secretion of creatinine into urine from tubuli[3]. Furthermore, in order to measure Ccr, it is necessary to collect 2-h or

24-h urine samples, which is inconvenient in older patients and relatively expensive to implement.

Chronic kidney disease (CKD) is defined as a person having GFR less than 60 mL/min, and the frequency of CKD in the Japanese population is around 10%[4]. Therefore, in patients with gastric cancer, the detection of CKD before operation is a crucial matter to prevent postoperative renal complications. If there are other tests which reflect GFR more accurately and that can be performed easily and cheaply, this would be valuable in a clinical setting[5,6].

Although inulin clearance can be used for measuring GFR, it is not a standard preoperative renal function test because of its cumbersome methodology, and alternatively, the Ccr test has been used. In 1999, Levey *et al*[7] reported an equation for the estimation of GFR. The Modification of Diet in Renal Disease (MDRD) equation includes age, sex, race, serum creatinine (sCr) value, serum blood urea nitrogen value, and serum albumin value. It was suggested that the MDRD equation precisely reflects the GFR in Caucasians but not in Asians, and therefore an equation for estimating the GFR for Japanese individuals (eGFR) was postulated[8,9]. eGFR is calculated using three parameters: sex, age, and sCr. If eGFR is at least as precise as Ccr for estimation of postoperative renal dysfunction, it would be valuable as a preoperative renal function parameter for patients with gastric cancer, as eGFR is easier and cheaper to measure. To clarify this issue, therefore, we analyzed Ccr and eGFR retrospectively in a series of gastric cancer patients.

MATERIALS AND METHODS

One hundred and forty-seven patients diagnosed histologically as having gastric cancer who underwent surgery between January 2003 and December 2005, and for whom preoperative Ccr data were available, were enrolled in the present study. The patients' backgrounds are summarized in Table 1. Gastrectomy was performed based on the guidelines of the Japan Gastric Cancer Study Group[10]. Basically, patients took meals until lunch on the day before operation, and no hyperalimentation was performed. As antibiotics, 2 g/d of cephalosporin was administered from the day of the operation for 3 d.

Measurement of sCr was performed by standard enzymatic method. Ccr was measured by 24-h urine collection, and a value of less than 50 mL/min was regarded as abnormal. eGFR was calculated using the following equation[9]: eGFR (mL/min per 1.73 m^2) = $194Cr^{-1.094}$ × Age$^{-0.287}$ (× 0.739; for female patients). eGFR of less than 60 mL/min was regarded as abnormal. The upper normal limits of sCr for male and female patients were 1.1 mg/dL and 0.8 mg/dL, respectively.

The reliability of preoperative Ccr and eGFR for detecting postoperative renal dysfunction was analyzed.

Statistical analysis

Data were expressed as actual number of patients or mean ± SD. χ^2 test was used for two-group comparisons.

Table 1 Patients' backgrounds

Age (yr)	68.3 ± 9.8
Sex (M/F)	107/40
Pre-sCr (mg/dL)	0.74 ± 0.20
Pre-Ccr (mL/min)	71.5 ± 24.5
Pre-eGFR (mL/min)	71.1 ± 20.5
Post-max-sCr (mg/dL)	0.82 ± 0.30
Method of operation	
Distal gastrectomy	79
Total gastrectomy	59
Others	9

Pre-sCr: Preoperative serum creatinine; Pre-Ccr: Preoperative creatinine clearance; Pre-eGFR: Preoperative estimated glomerular filtration rate; Post-max-sCr: Maximum value of postoperative sCr.

Table 2 Preoperative Ccr and eGFR

	Normal	Abnormal	*P*-value
Pre-Ccr	37	110	
Pre-eGFR	35	112	0.89

There were no differences between Ccr and eGFR, with regard to abnormalities in preoperative tests.

Table 3 Distribution of postoperative *s*Cr in patients with preoperative Ccr and eGFR abnormality

	Abnormal pre-Ccr	Abnormal pre-eGFR	*P*-value
Post-sCr			
Normal	8	5	
Abnormal	9	12	0.29

Postoperatively, 17 patients had abnormal sCr values. Of these 17 patients, 9 (52.9%) had abnormal preoperative Ccr values and 12 (70.6%) had abnormal preoperative eGFR values.

Statistical analyses were performed by using Graphpad software version 4.0. Significance of correlations was calculated by the Spearman correlation coefficient test. Differences at *P* < 0.05 were considered significant.

RESULTS

Preoperative Ccr was abnormal in 37 patients (25.2%) and preoperative eGFR was abnormal in 35 patients (23.8%) (Table 2, *P* = 0.79). Postoperatively, 17 patients had abnormal sCr values. Of these 17 patients, 9 (52.9%) had abnormal preoperative Ccr values and 12 (70.6%) had abnormal preoperative eGFR values (Table 3, *P* = 0.29).

Among the 110 patients who had normal preoperative Ccr values, 7 (6.3%) had postoperative sCr abnormality, and among the 112 patients who had normal preoperative eGFR values, 5 (4.5%) had postoperative sCr abnormality (Table 4, *P* = 0.53).

Among the 37 patients who had abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and among the 35 patients who had abnormal preoperative eGFR values, 25 (71.4%) had normal postoperative sCr values (Table 5, *P* = 0.34).

Table 4 Distribution of postoperative sCr in patients with preoperative Ccr and eGFR normality

	Post-sCr		*P*-value
	Normal	Abnormal	
Normal pre-Ccr	103	7	0.53
Normal pre-eGFR	107	5	

Among the 110 patients who had normal preoperative Ccr values, 7 (6.3%) had postoperative sCr abnormality, and among the 112 patients who had normal preoperative eGFR values, 5 (4.5%) had postoperative sCr abnormality.

Table 5 Distribution of postoperative sCr in patients with preoperative Ccr and eGFR abnormality

	Post-sCr		*P*-value
	Abnormal	Normal	
Abnormal pre-Ccr	7	30	0.34
Abnormal pre-eGFR	10	25	

Among the 37 patients who had abnormal preoperative Ccr values, 30 (81.1%) had normal postoperative sCr values, and among the 35 patients who had abnormal preoperative eGFR values, 25 (71.4%) had normal postoperative sCr values.

Figure 1 Correlation between preoperative creatinine clearance (Ccr) and estimated glomerular filtration rate (eGFR). Preoperative Ccr and eGFR were significantly correlated ($r = 0.514$, $P < 0.01$).

Next, we evaluated the correlation between preoperative Ccr, eGFR, and the maximum postoperative sCr value. Preoperative Ccr and eGFR showed a significant correlation ($r = 0.514$, $P < 0.01$, Figure 1). Preoperative Ccr was correlated with the maximum postoperative sCr value ($r = -0.334$, $P < 0.01$, Figure 2A). Also, preoperative eGFR was correlated with the maximum postoperative sCr value ($r = -0.188$, $P = 0.02$, Figure 2B).

There were no severe postoperative complications, such as anastomotic leakage, bleeding, or infection in the patients who had abnormal preoperative Ccr or eGFR values. None of the patients required postoperative hemodialysis.

DISCUSSION

The present study showed that eGFR was as equally reli-

Figure 2 Correlation between preoperative Ccr and eGFR, and maximum postoperative serum creatinine (sCr). The maximum value of sCr was correlated with preoperative Ccr ($r = -0.334$, $P < 0.01$) (A) and eGFR ($r = -0.188$, $P = 0.02$) (B).

able as Ccr for assessment of preoperative renal function in patients with gastric cancer.

To date, Ccr has been used as a preoperative renal function parameter in patients with gastric cancer. Ccr is measured by 24-h or 2-h urine collection, and it has been suggested that either method can sometimes be inappropriate because of the difficulty involved in implementation, especially in older patients. Furthermore, there is a dissociation between Ccr and GFR in individuals with a low GFR[1]. Although sCr is a simply determined parameter for estimating renal function, it is not a reliable indicator of preoperative renal function because it is easily influenced by sex, muscle volume, and exercise. Also, sCr usually remains normal until GFR decreases to 50 mL/min[11].

GFR has not been used as a gold standard of preoperative renal function because measurement of inulin clearance is more labor-intensive than measurement of Ccr. However, the establishment of the MDRD equation by Levey *et al*[7] in 1999, and its modification postulated by the Japan Kidney Association, has made it possible to estimate GFR[8,9]. Therefore it is expected that eGFR will replace Ccr in various clinical settings.

In this study, we set the cut-off values of Ccr and eGFR at 50 mL/min and 60 mL/min, respectively. The validity of these cut-off values was endorsed by our finding that there was no significant difference in the number of patients who had abnormal postoperative sCr values between patients having preoperative Ccr ab-

normality and preoperative eGFR abnormality (Table 3).

There was no difference in the number of patients who had abnormal sCr values between patients who had normal preoperative Ccr values and normal preoperative eGFR values (Table 4), and also no difference in the number of patients who had normal sCr values between patients with preoperative Ccr abnormality and preoperative eGFR abnormality (Table 5). These results indicate that there is no significant difference in reliability between preoperative Ccr and eGFR for detecting postoperative renal dysfunction.

Preoperative Ccr and eGFR values showed a significant correlation (Figure 1), and Ccr and eGFR were correlated with the maximum postoperative sCr value (Figure 2). Thus, either Ccr or eGFR can be used to predict the maximum postoperative sCr value.

Cystatin C is a cysteine protease and a member of the cystatin super family. Cystatin C is excreted from the glomerulus and reabsorbed by the proximal tubuli, and has been used as a marker for GFR[12,13]. sCr is influenced by prerenal factors, such as muscle volume; on the other hand, cystatin C is not influenced by sex, age, and other prerenal factors. In this retrospective study, cystatin C was not included as a parameter; it would be valuable to evaluate the relationship between eGFR and cystatin C as a preoperative renal function test of gastric cancer.

We conclude that eGFR is as equally valuable as Ccr as an indicator of preoperative renal function in patients with gastric cancer, and that eGFR may now be used in place of Ccr in view of the former's clear medical and socioeconomic advantages.

COMMENTS

Background
Creatinine clearance (Ccr) is not strictly equal to glomerular filtration rate (GFR). It has been accepted that estimated GFR (eGFR) is equal to measured GFR in chronic kidney disease. However, there have been no previous studies regarding the reliability of eGFR as a preoperative renal function test in gastric cancer patients.

Research frontiers
If eGFR is as good as Ccr as a preoperative renal function test, eGFR may replace Ccr because of its simplicity of measurement.

Innovations and breakthroughs
eGFR is useful as a preoperative renal function parameter in patients undergoing gastrectomy. Ccr is no longer recommended as a first-choice preoperative renal function test.

Applications
eGFR should replace Ccr as a routine preoperative renal function test in various surgical fields.

Terminology
eGFR is estimated GFR which is calculated from age, sex, serum creatinine

value (eGFR3), or by adding serum albumin concentration and blood urea nitrogen value (eGFR5).

Peer review
I think the conslusion should state "may be as good" not be as categorical as eGFR should be used instead of Ccr. This study based on a 147 patient is a good proof of principle but certainly does not have the power to change the standard of care.

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BRIEF ARTICLE

EZH2 and STAT6 expression profiles are correlated with colorectal cancer stage and prognosis

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Abstract

AIM: To investigate the role of enhancer of zeste homologue 2 (EZH2) and STAT6 immunohistochemistry in the evaluation of clinical stages and prognosis of colorectal cancer (CRC).

METHODS: The expression patterns were examined by immunohistochemistry in both tumor and adjacent non-neoplastic tissues of 119 CRC patients who underwent operation during the time period from 2002 to 2004.

RESULTS: The positive rates of EZH2 and STAT6 in CRC cases were 69.7% (83 of 119) and 60.5% (72 of 119), respectively, and there was significant difference when compared with tumor adjacent non-neoplastic tissues ($P < 0.05$). In all CRC cases, patients with EZH2-positive, or STAT6-positive expression had lower survival rates than those with EZH2-negative or STAT6-negative expression ($P = 0.002$ and $P = 0.005$, respectively). Co-expression of EZH2 and STAT6 showed significantly higher levels in CRC cases of high clinical TNM stages ($P = 0.001$), and the expression of STAT6 was also correlated with lymph node metastasis and distant metastasis ($P = 0.001$ and $P = 0.016$, respectively). Multivariate analysis revealed that EZH2 expression was an independent prognostic indicator of CRC ($P = 0.039$).

CONCLUSION: EZH2 and STAT6 expressions have significant values in distinguishing clinical stages of CRC and predicting the prognosis of the patients.

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INTRODUCTION

Colorectal cancer is the third most common cancer, and the second leading cause of death from cancers in the United States[1]. Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced an increase of 2-4 times in the incidence of CRC during the

past few decades[2]. To reduce the mortality and improve treatment, a number of studies have been performed to search for tumor biomarkers, especially for those highly correlated with both staging and prognosis that can help not only predict patients' survival, but also lead to optimal therapeutic project.

EZH2 is a member of the polycomb group of genes (PcG), which is important for transcriptional regulation through nucleosome modification, chromatin remodeling, and interaction with other transcription factors[3]. EZH2, also called histone lysine methyltransferase (HKMT), has the function of methylating lysine 9 and 27 of histone H3, which consequently leads to the repression of target gene expression. Furthermore, dysregulation of this gene silencing mechanism may lead to cancer[4,5]. Recently, EZH2 has been investigated as a potential molecular biomarker for the diagnosis and prognosis of prostate cancer by Varambally *et al*[6], using cDNA microarray analysis. Overexpression of EZH2 has been observed in the most aggressive forms of prostate cancer, exhibiting a correlation with poor clinical outcome, thus acting as a novel marker of aggressiveness and unfavorable prognosis in this type of cancer. A causal effect between EZH2 up-regulation and poor prognosis was also observed in human gastric cancers[7]. However, there have been few reports of EZH2 expression in CRC, and its relationship with clinicopathological characteristics or prognostic value is yet unclear.

Like other members of the signal STAT (transcriptional activation by signal transducer and activator of transcription) family of proteins, STAT6 has a dual role as a signaling molecule and transcription factor. STAT6 is activated in response to interleukin (IL)-4 and IL-13 stimulation, and plays a key role in Th2 polarization of the immune system[8]. Recently, the STAT6 signaling pathway was found to be highly activated in some tumors, such as prostate cancer, mammary carcinoma and lymphoma[9-11]. In addition, a close correlation between IL-4/STAT6 activities and apoptosis and metastasis of colon cancer was reported by Li *et al*[12] in a study using STAT6-high and STAT 6-null colon cancer cell lines to analyze anti-apoptotic and pro-metastatic genes expression. Nevertheless, there have been no studies investigating the clinicopathological features and prognosis of CRC in relation to STAT6 expression. In this study, we analyzed the expression of EZH2 and STAT6 in biopsied CRC tissues to determine their relationship with clinicopathological features and clinical outcome of CRC patients.

MATERIALS AND METHODS

Patient and follow-up
A group of 119 consecutive patients with CRC were studied. All patients were diagnosed and treated in the Beijing People's Hospital between December 2002 and June 2004. There were 74 men and 45 women with a mean age of 62.2 ± 12.8 years (range 25-89 years). No patient had received preoperative chemotherapy or radiotherapy. All of the patients were followed up by direct evaluation or phone interview until death or December 2008. This study was approved by the Ethics Committee of People's Hospital, Peking University.

Tissue samples and tissue array
Core tissue biopsies (2 mm in diameter) were taken from Formalin fixed paraffin-embedded tumor samples and adjacent non-neoplastic tissues. These blocks were arranged in a new recipient paraffin block (tissue array block) using a commercially available micro-array instrument (Beecher Instruments, Micro-Array Technologies, Silver Spring, MD). Two cores were sampled from each case. From the 119 tumor samples and adjacent non-neoplastic tissues, 11 tissue array blocks were prepared, each containing 10-12 tumor and adjacent non-neoplastic sample cores. To qualify for this study, a case needed to have a tumor occupying more than 10% of the core tissue area. Clinical and pathological information for this group of patients was obtained by review of histopathological reports and medical records.

Immunohistochemistry
Eleven paraffin-embedded blocks of tissue arrays were cut into 4-mm sections. The sections were put in the oven at 59℃ for 1h, deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with 3% hydrogen peroxide solution for 10 min. Antigen retrieval was done by microwaving the tissue in 10 mmol/L citric acid buffer for 10 min, then cooling to room temperature for 1 h. The sections were incubated with an anti-EZH2 monoclonal antibody (1:500, Invitrogen), and an anti-STAT6 monoclonal antibody (1:100, Abcam) overnight at 4℃. Primary antibodies were detected using the Powervision two-step histostaining reagent (Zhongshan, Beijing), with PV-6001 as the secondary antibody and detection was performed by diaminobenzidine (DAB) chromogenic reaction. Tissues were counterstained with hematoxylin, 1% hydrochloric acid of alcohol, dehydrated with graded ethanols, and mounted. Positive and negative immunohistochemical controls were included. Two experienced pathologists independently examined EZH2 and STAT6 staining while blinded to the clinicopathological data and clinical outcomes of the patients. Cases with > 30% positive tumor cells in a section were recorded as having positive expression. As negative controls, samples were stained with only secondary antibody. A total of 119 cases were used for data analysis.

Statistical analysis
All data were analyzed using SPSS 11.0 software. The association of EZH2 and STAT6 expression with various clinicopathological features was analyzed using χ^2 test. Cumulative survival was estimated by the Kaplan-Meier method and differences between survival curves were analyzed by log-rank test. The influence of each variable

Table 1 Relationship between EZH2 or STAT6 and clinicopathological features

	Cases	EZH2 expression		P	STAT6 expression		P
		EZH2+	EZH2-		STAT6+	STAT6-	
Tissue							
Non-neoplastic samples		22	97	0.000	50	69	0.004
Tumor samples		83	36		72	47	
Gender							
Female	47	30	17	0.334	24	23	0.089
Male	72	52	20		48	24	
Location							
Colon	90	62	28	0.719	57	33	0.268
Rectum	29	21	8		15	14	
Differentiation							
Poor	21	16	5	0.479	16	5	0.101
Moderate or well	98	67	31		56	42	
TNM classification							
I	11	4	7	0.049	3	8	0.004
II	42	29	13		20	22	
III	44	35	9		32	12	
IV	22	15	7		17	5	
Depth of wall invasion							
T1	1	1	0	0.014	1	0	0.188
T2	19	9	10		8	11	
T3	93	71	22		58	35	
T4	6	2	4		5	1	
Lymph node metastasis							
No	55	34	21	0.081	25	30	0.001
Yes	64	49	15		47	16	
Distant metastasis							
M0	97	66	31	0.668	54	43	0.024
M1	22	16	6		18	4	
Recurrence							
No	86	61	25	0.088	53	33	0.775
Yes	11	6	5		4	7	

on survival was analyzed by multivariate analysis using the Cox proportional hazard model. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

EZH2 and STAT6 expression

Positive and negative staining of EZH2 and STAT6 are illustrated in Table 1. The positive rates of EZH2 and STAT6 were 69.7% (83 of 119) and 60.5% (72 of 119), respectively in CRC cases, and there was an obvious difference when compared with tumor adjacent non-neoplastic tissues ($P < 0.05$). We observed that EZH2 expression was mainly present in the nuclei in most of the cases, and EZH2 immunoreactivity was found not only in the nuclei but also in the cytoplasm in a few CRC samples (8 of 110). STAT6 expression was mostly detected in the cytoplasm, and at cell membranes in some cases (Figure 1).

The expression of EZH2 in CRC samples was significantly correlated with more malignant phenotypes, including TNM classification ($P = 0.049$), and tumor invasion depth ($P = 0.014$). The expression of EZH2 had nothing to do with gender, tumor location, differentiation, lymph node metastasis, or distant metastasis. There was no significant correlation between cytoplasmic

EZH2 immunoreactivity and clinicopathological features in the CRC carcinoma tissue samples (data not shown).

We found a positive correlation between STAT6 expression and TNM classification ($P = 0.004$), and expression of STAT6 was significantly associated with lymph node metastasis ($P = 0.001$) and distant metastasis ($P = 0.024$). As shown in Table 1, expression of STAT6 was not correlated with other clinicopathological features.

Prognostic implications of EZH2 and STAT6 expression in CRC

Tissue biopsies with EZH2-positive cells had a significantly lower post-operative survival rate than tissue biopsies with EZH2-negative cells ($P = 0.02$, Figure 2A). The STAT6-positive group also had significantly lower survival rates than the STAT6-negative group ($P = 0.005$, Figure 2B).

On the basis of the expression profiles of EZH2 and STAT6, the 119 patients were categorized into three groups: Group A: EZH2-/STAT6- ($n = 30$); Group B: EZH2+/STAT6- or EZH2-/STAT6+ ($n = 40$); and Group C: EZH2+/STAT6+ ($n = 59$). There were significant differences in survival rates between group A and one of the other groups ($P = 0.006$, Figure 2C). The survival rate of group A was significantly higher than that of group B ($P = 0.008$), the survival rate of group B was not

Figure 1 Expression of EZH2 (A, C, E) and nuclear STAT6 (B, D, F) in adjacent non-neoplastic tissues and CRC tissues visualized by DAB. A: Non-neoplastic tissues stained with EZH2; B: Non-neoplastic tissues stained with STAT6; C, E: Tumor tissues stained with EZH2; D, F: Tumor tissues from the same case stained with STAT6. Original magnifications: × 100 (A-D); × 400 (E, F).

Figure 2 Kaplan-Meier survival curves for 119 CRC patients categorized by EZH2 and/or STAT6 expression. A: Survival was significantly lower in patients with positive EZH2 expression than in those with negative expression ($P = 0.02$); B: Survival was significantly lower in patients with positive STAT6 expression than in those with negative expression ($P = 0.005$); C: Patients with EZH2+/STAT6+ expression profiles had worse survival outcome ($P = 0.006$).

Table 2 Multivariate analysis of prognostic factors in 119 cases using the Cox proportional hazard model

	B	P	RR	95% CI for RR	
				Lower	Upper
Metastasis	3.075	0.000	21.648	8.212	57.070
Lymph node metastasis	1.149	0.018	3.157	1.218	8.178
Grading	-0.675	0.051	0.509	0.258	1.003
Recurrence	0.510	0.173	1.665	0.799	3.469
EZH2	1.167	0.039	3.214	1.061	9.732

B: Regression coefficient.

Table 3 Relationship between EZH2 and STAT6 expressions

EZH2	STAT6		Total
	+	-	
+	59	24	83
-	13	23	36
Total	72	47	119

r_s = 0.329, P = 0.00.

significantly higher than that of group C (P = 0.333), and the survival rate of group A was significantly higher than that of group C (P = 0.001, Figure 2C).

Using Cox regression analysis for the 119 patient samples, positive EZH2 expression (P = 0.039), distant metastasis (P = 0.000), and lymph node metastasis (P = 0.018) seemed to be independent prognostic indicators (Table 2).

Combined analysis of EZH2 and STAT6 expression in distinguishing TNM classification

A significant association was found between EZH2 and STAT6 expression by Spearman's correlation analysis (r_s = 0.329, P = 0.00; Table 3), when EZH2 and STAT6 expressions were evaluated. Table 4 shows the relationship between the expression profile of EZH2 and STAT6 and TNM classification assignment for 119 CRC samples. Co-expression of EZH2 and STAT6 was significantly correlated with high clinical TNM classification (P = 0.003). Furthermore, the expression levels of co-expression of EZH2 and STAT6 tended to rise as the TMN staging increased (18%, 40%, 59% and 72% in stages Ⅰ-Ⅳ, respectively).

DISCUSSION

In the present study, we have found that EZH2 and STAT6 are sensitive and potential biomarkers that can be used for the prediction of clinical TNM classification and prognosis of CRC. An association between EZH2 overexpression and the biological malignancy of tumors has been reported in many cancers, including breast cancer, lymphoma, bladder carcinoma and hepatocellular carcinoma. Those studies suggested that EZH2 may play a role in carcinoma progression and indicate

Table 4 Relationship between EZH2 and STAT6 co-expression and TNM classification

TNM classification	EZH2 and STAT6 expressions				EZH2 + and STAT6 + expression in different stages (%)
	EZH2 + and STAT6 +	EZH2- or STAT6-	EZH2- and STAT6-	Total	
Ⅰ	2	3	6	11	18
Ⅱ	15	19	8	42	40
Ⅲ	26	10	8	44	59
Ⅳ	16	5	1	22	72
Total	59	37	23	119	49

P = 0.003.

a worse clinical outcome[13-16]. Fluge *et al*[17] investigated the expression of EZH2 in colorectal cancer by IHC. The authors concluded that a strong EZH2 expression indicated an improved relapse-free survival. Notably, this finding was only seen in colonic cancer, but not in rectal cancer. Interestingly, this result is conflicted with Mimori' study[18], who believes that EZH2 may be an oncogene and a prognostic marker in either colonic or rectal cancer. In our study, no relationship was found between the expression of EZH2 and colorectal tumor location. Furthermore, those CRC cases with higher levels of EZH2 expression were of more malignant phenotypes and have poorer prognosis.

The underlying mechanism is related, most probably, to tumor suppressor gene silencing by EZH2 mediated histone methylation and DNA methylation[19]. We found a positive correlation between EZH2 expression and TNM staging. Multivariate analysis revealed that EZH2 represents an independent prognostic indicator. These findings suggest the potential clinical utility of incorporating EZH2 into clinical consideration to help determine the clinical TNM staging and predict the outcome.

Our data showed that STAT6 expression was positively related to clinical TNM staging, lymph node metastasis and distant metastasis, suggesting that STAT6 may promote the progression of cancer. STAT6 is a member of STAT family of latent transcription factors known to be activated constitutively in cancers[20]. STAT6 is activated in response to interleukin (IL)-4 and IL-13 stimulation, STAT6-/-mice were deficient in responsiveness to IL-4 and IL-13, hence preferentially producing CD4_Th1 cells rather than CD4_Th2[21]. This observation may indicate that STAT6-deficient mice might have heightened immuno-surveillance against primary and metastatic tumors, because the development of Th1 cells optimized CD8-mediated tumor immunity[22]. Our data support these mechanism studies about STAT6, and suggest that STAT6 plays a key role in progression and metastasis of CRC.

In this study, STAT6 expression was also found to be associated with a poorer prognosis and TNM classification, which was consistent with EZH2 expression. Consequently, STAT6 enhanced the predictive efficiency of EZH2 expression, and patients with positive expres-

sion of EZH2 and STAT6 had the worse outcome and higher TNM staging than patients with negative expression of EZH2 or STAT6. These results suggest that combined analysis of EZH2 and STAT6 may be a powerful biological marker to predict clinical TNM staging and prognosis. According to the NCCN guidelines, patients with different clinical stages should receive correspondingly optimal treatment protocols. Therefore, it is critical to find effective biomarkers which can predict clinical staging in order to enable clinicians to make a more precise prejudgment. Our data suggest that combined EZH2 and STAT6 analysis may have a potential ability for this purpose.

In conclusion, the prognosis of CRC patients with EZH2-positive or STAT6-positive expression is significantly worse than those CRC patients with EZH2-negative or STAT6-negtive expressions. In addition, combined analysis of EZH2 and STAT6 expressions could enable clinicians to prejudge a more accurate clinical TNM staging before operation.

COMMENTS

Background
Enhancer of zeste homologue 2 (EZH2) is a member of the polycomb group of genes that is involved in epigenetic silencing and cell cycle regulation. Recently, EZH2, as well as STAT6 signaling pathway, were found to be highly activated in some tumors. However, the clinical significance of these proteins has not yet been determined in colorectal cancer (CRC).

Research frontiers
Recently, EZH2 has been under investigation as a potential molecular biomarker for the diagnosis and prognosis in prostate cancer. Overexpression of EZH2 has been observed in the most aggressive forms of prostate cancer, exhibiting a correlation with poor clinical outcome, thus acting as a novel marker of aggressiveness and unfavorable prognosis in this type of cancer. A causal effect between EZH2 up-regulation and poor prognosis was also observed in human gastric cancer. STAT6 has a dual role as a signaling molecule and transcription factor. STAT6 signaling pathway was found to be highly activated in some tumors, such as prostate cancer, mammary carcinoma and lymphoma. In addition, a close correlation between IL-4/STAT6 activities and apoptosis and metastasis of colon cancer was reported.

Innovations and breakthroughs
The authors found that patients with EZH2-positive expression had lower survival rates than those with EZH2-negative expression in both colonic and rectal cancers. Furthermore, the data suggest that combined analysis of EZH2 and STAT6 expression can be of significant value in distinguishing clinical stages of CRC and predicting prognosis.

Applications
The combined analysis of EZH2 and STAT6 may be a powerful biological marker, which could enable clinicians to prejudge a more accurate clinical TNM staging and prognosis of CRC patients before operation.

Terminology
EZH2 is a member of the polycomb group of genes (PcG), which is important for transcriptional regulation through nucleosome modification, chromatin remodeling, and interaction with other transcription factors. EZH2, also called histone lysine methyltransferase (HKMT), has the function of methylating lysine 9 and 27 of histone H3, which consequently leads to the repression of target gene expression.

Peer review
Generally, the paper is well written and designed.

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BRIEF ARTICLE

Expression of calreticulin is associated with infiltration of T-cells in stage ⅢB colon cancer

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Abstract

AIM: To investigate the correlation between expression of calreticulin and infiltration of lymphocytes in stage ⅢB colon cancer.

METHODS: Sixty-eight pathologically-confirmed specimens were obtained from stage ⅢB (T3N1M0) colon cancer patients who underwent radical resection between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China. Immunohistochemical analysis was performed to show infiltration of lymphocytes and expression of calreticulin in colon cancer. Association between calreticulin expression, infiltration of lymphocytes, and 5-year survival rate of patients was assessed.

RESULTS: The expression level of calreticulin was lower in cancer nest than in its adjacent normal epithelium since 61.8% (42/68) of the samples were stained with calreticulin in colon cancer. The expression of calreticulin in colon cancer was associated with the infiltration of CD45RO+ cells rather than with that of CD3+ cells. In addition, the stronger expression of calreticulin and the higher infiltration of CD3+ and CD45RO+ cells in colon cancer were associated with the higher 5-year survival rate of patients.

CONCLUSION: Expression of calreticulin is associated with infiltration of T-cells, which implies that a low expression level of molecular marker may represent a new mechanism underlying immune escape in colon cancer.

ed with infiltration of T-cells in stage ⅢB colon cancer. *World J Gastroenterol* 2010; 16(19): 2428-2434 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i19/2428.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i19.2428

INTRODUCTION

Mounting evidence indicates that colorectal cancer is immunogenic[1]. For example, when immunologic effector cells, such as CD3+ T-cells, CD45RO+ T-cells and macrophages, infiltrate colorectal cancer tissue, tumor progression is decreased[2-7]. However, colorectal cancer cells have developed multiple immune escape mechanisms, such as reduced expression of HLA-I molecules, expression of immune inhibitors including TGF-β and indoleamine 2,3-dioxygenase (IDO), and induction of Treg cells[8-11]. More importantly, targeting the immune system can stimulate an immune response in colorectal cancer[12-16].

The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. Recently, evidence indicates that a subset of damage-associated molecular patterns (DAMPs) is involved in the induction of immune response. During the apoptosis of cancer cells, translocation of calreticulin (CRT) from endoplasmic reticulum (ER) to membrane (ecto-CRT exposure), expression of heat-shock proteins, release of HMGB1 from cells, and expression of NKG2D may serve as danger signals, thereby inducing an immune response[17,18]. CRT is a highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, such as chaperone activity, regulation of Ca^{2+} homeostasis, adhesion signaling, involving gastrointestinal mucin synthesis at the stage of folding and oligomerization in ER, inhibiting angiogenesis and tumor growth[19-23]. Within ER, CRT interacts with various molecules like ERp57 and calnexin (CNX) to aid in proper folding of proteins[24]. Furthermore, CRT plays an important role both in the assembly of MHC class Ⅰ molecule and in the loading of antigen peptides onto the MHC-Ⅰ molecule within ER[25]. Besides, it has been shown that nuclear CRT can regulate nuclear protein transport and influence signaling *via* nuclear steroid receptors and integrins[26,27]. When cells are treated with anthracyclines, oxaliplatin, radiation and hypoxia, ecto-CRT exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response[28-30].

Altered expression of calreticulin has been detected in melanoma, and in liver, bladder, prostate, lung, pancreatic and breast cancers[31-37]. However, the clinical significance of CRT expression remains poorly understood and controversial. It has been reported that overexpression of CRT is related to the promotion of breast cancer and decreases the progression of prostate cancer[34,36]. Additionally, interaction between calnexin and calreticulin contributes to metastasis of melanoma[31]. Since the clinical evidence supporting the immune regulation of CRT is scant, this study examined the expression of CRT in stage ⅢB colon cancer patients to evaluate whether the expression of CRT is associated with the immunogenicity of colon cancer.

MATERIALS AND METHODS

Materials

Sixty-eight pathologically-confirmed specimens were obtained from patients with stage ⅢB (T3N1M0; AJCC, 2002) colon cancer between January 1999 and May 2002 at the Cancer Center of Sun Yat-Sen University, Guangzhou, China (Table 1). All the patients underwent radical resection and 5-FU-based adjuvant chemotherapy after operation for 6 mo. The patients were evaluated every 3 mo during the first year, every 6 mo in the second year, and once every year thereafter for a total of 5 years. If a recurrence or a metastasis occurred, 5-FU-based chemotherapy was given according to the national comprehensive cancer network (NCCN) guidelines. No patients received preoperative blood transfusion or non-steroidal anti-inflammatory drugs. Overall survival was defined as the time from surgery to death. Data analysis was done on the last known day when the patient was alive.

Immunohistochemical assay and scoring systems

Formalin-fixed, paraffin-embedded tissue was cut into 4-μm thick sections. The size of each tissue section was about 1 cm × 1 cm. Then, the sections were dewaxed, rehydrated, and blocked with hydrogen peroxide. Antigens were retrieved in 10 mmol/L citrate buffer (pH 6.0) for 10 min and cooled to room temperature. After blocked with sheep serum, the sections were incubated overnight at 4℃ with either rabbit polyclonal antibody against human calreticulin at a dilution of 1:2000 (Abcam, Cambridge, MA, USA) or mouse monoclonal antibody against human CD3 and CD45RO (Zymed, San Diego, CA, USA), both of which were diluted to 1:100. Subsequently, biotinylated secondary antibodies and streptavidin-biotinylated horseradish peroxidase complexes were used. The sections were developed with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Negative controls in which primary antibody was replaced with a phosphate buffered solution (PBS), were employed.

Infiltration of lymphocytes in the tumor was scored with Hussein's method[38] and expression of calreticulin in colon cancer was interpreted *via* immunoreactivity using the 0-4 semi-quantitative system derived from Remmele and Stegner for both the intensity of staining and the percentage of positive cells (labeling frequency percentage)[39]. The cells were counted in at least 10 different fields for each section, and the size of each high-power field (× 400) was about 300 μm × 300 μm. The cells were counted in tumor stroma. The highest infiltration areas of lymphocytes were chosen. Necrotic areas were avoided. Two observers counted the cells at the same time and in the same field under a multiple-lens microscope. The results were expressed as mean ± SE. Cytoplasm staining

Table 1 Parameters of patients ($n = 68$)

Parameters	n (%)
Age (yr)	
< 60	30 (44.1)
≥ 60	38 (55.9)
Gender	
Male	38 (55.9)
Female	30 (44.1)
Tumor site	
Left hemicolon	45 (66.2)
Right hemicolon	23 (33.8)
Pathological grade	
G1	10 (14.7)
G2	51 (75.0)
G3	7 (10.3)
Survival time (mo)	
≥ 60	52 (76.5)
< 60	16 (23.5)

Table 2 Relation between expression of calreticulin, infiltration of lymphocytes and parameters of patients ($n = 68$)

Parameters	Calreticulin expression		P
	(-)	(+)-(+++)	
Gender			0.813
Male	15	23	
Female	11	19	
Age (yr)			0.204
≥ 60	12	26	
< 60	14	16	
Tumor site			0.245
Left hemicolon	15	30	
Right hemicolon	11	12	
Pathological grade			0.643
G1	5	5	
G2	19	32	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.387
> 18	18	33	
≤ 18	8	9	
Infiltration of CD45RO+ cells per high-power field			0.010
> 26	15	36	
≤ 26	11	6	

was divided into no staining/background of negative controls (score = 0), weak staining above background (score = 1), moderate staining (score = 2), and intense staining (score = 3). The labeling frequency was scored as 0 (≤ 1%), 1 (1%-24%), 2 (25%-49%), 3 (50%-74%), and 4 (≥ 75%), respectively. The product index was obtained by multiplying the intensity and percentage and scored as (-), (+), (++), and (+++), which indicate the cross-indices of 0-2, 3-5, 6-8, and 9-12, respectively. (-) was defined as no or negative expression, and (+)-(+++) as positive expression. Each section was independently scored by two pathologists. If an inconsistency occurred, a third pathologist was consulted to achieve a consensus.

Statistical analysis

Correlation between calreticulin expression, or infiltration of lymphocytes and parameters of patients was analyzed by χ^2 test or Fisher's exact test. Factors, including gender and age of the patients, pathologic grade, tumor site, infiltration of CD3+ cells and CD45RO+ cells, and calreticulin expression level in colon cancer, were assessed by univariate and multivariate analysis to determine their influence on the overall survival rate of patients. Kaplan-Meier curve and log-rank test were used to estimate the distribution of variables in relation to survival. Cox regression model was used to correlate the assigned variables with the overall survival rate. All statistical analyses were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of CRT and infiltration of lymphocytes in stage ⅢB colon cancer

CRT was stained in cytoplasm rather than in nuclei of cancer cells and normal epithelium. The expression level of CRT was lower in colon cancer than in its adjacent normal epithelium. Of the stained samples, 61.8% (42/68) were positive for CRT in cancer nest (Figure 1A and B,

Table 2). CD3+ and CD45RO+ cells were observed in all samples from tumor stroma and its adjacent normal mucosa. Positively stained antigens were found on cell membrane (Figure 1C-F).

Relation between expression of CRT and infiltration of CD3+ or CD45RO+ cells

The cut-off value for infiltration of lymphocytes in colon cancer was 75%. Infiltration of 18 CD3+ cells and 26 CD45RO+ cells was observed in per high-power field, and was thus recorded as high and low infiltration. Log-rank test was used to analyze the relation between expression of CRT and infiltration of CD3+ and CD45RO+ cells in colon cancer, showing that positive expression of CRT (+-+++) was associated with high infiltration of CD45RO+ cells ($P = 0.010$, Table 2). No correlation was observed between expression of CRT and infiltration of CD3+cells or other parameters of the patients, such as age, gender, tumor site.

Univariate and multivariate survival analysis

By the end of a 5-year follow-up period, 52 patients were alive with a 5-year survival rate of 76.5%. Kaplan-Meier survival analysis indicated that positive expression of CRT was associated with a higher 5-year survival rate. The 5-year survival rate of patients with positive and negative expression of CRT was 85.5% (36/42) and 61.5% (16/26), respectively ($P = 0.022$, Table 3). However, the survival curves for patients with positive and negative expression of CRT were crossed at 18 mo (Figure 2). In addition, high infiltration of CD3+ and CD45RO+ cells in colon cancer was associated with a higher 5-year survival rate ($P = 0.000$, Figure 3).

Figure 1 Expression level of CRT in cancer nest (A) and in its adjacent normal epithelium (B) [× 100 in A, × 200 in B, normal epithelium (red arrow), atypical hyperplasia (black arrow) and tumor tissue (blue arrow), respectively], high infiltration of CD3+ cells (C) and CD45RO+ cells (E) in colon cancer stroma (× 400), low infiltration of CD3+ cells (D) and CD45RO+ cells (F) in colon cancer stroma (× 400).

Table 3 Univariate survival analysis (*n* = 68)

Parameters	Survival time (mo)		*P*
	< 60	≥ 60	
Gender			0.542
Male	10	28	
Female	6	24	
Age (yr)			0.973
≥ 60	9	29	
< 60	7	23	
Tumor sites			0.722
Left hemicolon	10	35	
Right hemicolon	6	17	
Pathological grade			0.919
G1	2	8	
G2	12	39	
G3	2	5	
Infiltration of CD3+ cells per high-power field			0.000
> 18	6	45	
≤ 18	10	7	
Infiltration of CD45RO+ cells per high-power field			0.000
> 26	5	46	
≤ 26	11	6	
Calreticulin expression			
(-)	10	16	
(+)-(+++)	6	36	0.022

Figure 2 Relation between CRT expression and 5-year survival rate.

Cox regression model revealed that neither expression of CRT nor infiltration of CD3+ cells or CD45RO+ cells in colon cancer had an independent prognostic value (Table 4).

DISCUSSION

In this study, the expression level of CRT was lower in co-lon cancer than in its adjacent normal epithelium, indicating that expression of CRT is associated with infiltration of CD45RO+ cells rather than with CD3+ cells and may serve as a mechanism underlying immune escape in colon cancer, although it is not an independent prognostic indicator.

A few studies have examined the role of CRT in the progression of colon cancer[40,41]. Toquet *et al*[40] reported that the expression of CRT is decreased in non-mucinous colonic adenocarcinoma while Vougas reported that the expression of CRT is increased in poorly-differentiated co-lon cancer and advanced tumor[41]. In this study, however, neither the pathological classification nor the differentia-tion grade was associated with the expression level of CRT.

It is still unknown whether CRT is involved in the mechanism underlying immune escape in colon cancer. Colorectal cancer with microsatellite instability (MSI-H) is immunogenic due to infiltration of a large number of lym-

Table 4 Multivariate survival analysis (*n* = 68)

Parameters	B	SE	Wald	df	Sig.	Exp(B)	95% CI for Exp (B)	
							Lower	Upper
Pathological grade	0.172	0.261	0.435	1	0.510	1.188	0.712	1.979
Gender	-0.027	0.247	0.012	1	0.912	0.973	0.599	1.580
Age	0.079	0.255	0.095	1	0.758	1.082	0.656	1.784
Tumor site	0.085	0.276	0.094	1	0.759	0.919	0.535	1.579
Infiltration of CD3+ cells	-0.057	0.511	0.013	1	0.911	0.944	0.347	2.537
Infiltration of CD45RO+ cells	-0.743	0.552	1.809	1	0.179	0.476	0.161	1.404
Expression level of calreticulin	0.133	0.279	0.226	1	0.634	0.876	0.507	1.512

B: Regression coefficient; SE: Standard error; df: degree of freedom; Sig.: Significant; Exp (B): Odds ratio; CI: Confidence interval.

Figure 3 Correlation between high infiltration of CD3+ cells (A) and CD45RO+ cells (B) in colon cancer and 5-year survival rate of patients.

phocytes, resulting in a favorable prognosis[42,43]. Microarray analysis showed that CRT expression was up-regulated in 27 cases of colorectal cancer with MSI-H, while quantitative RT-PCR analysis failed to confirm it in another 26 cases of colore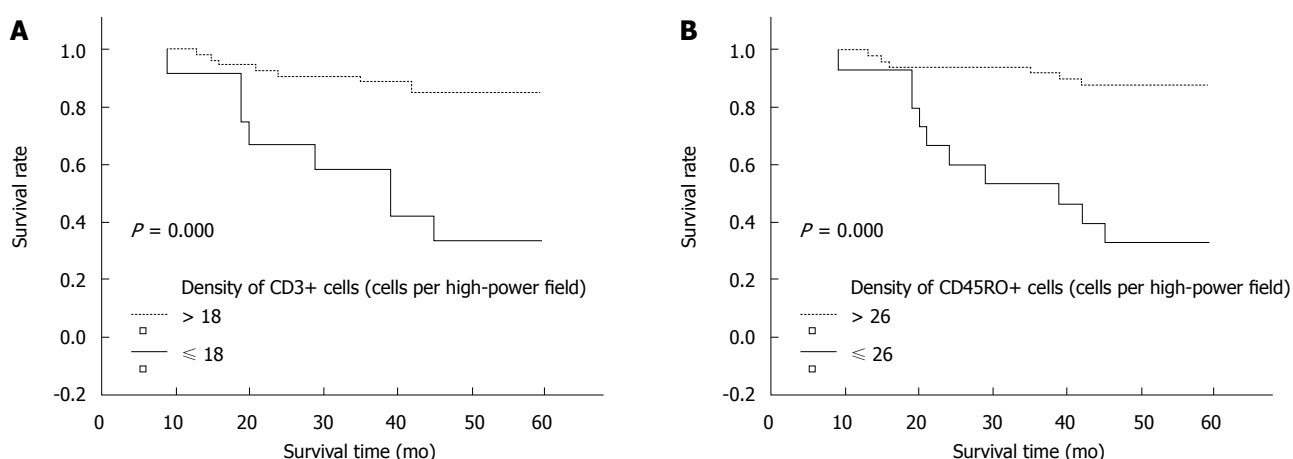ctal cancer with MSI-H[44]. In this study, the relation between expression level of CRT and infiltration of lymphocytes was assessed, which indicates that expression of CRT is related with infiltration of CD45RO+ cells in colon cancer. Since CD45RO+ cells contribute to a favorable prognosis of colon cancer patients, it is reasonable to infer that CRT expression is involved in immune response that occurs in colon cancer. However, this study failed to show that either CRT expression or infiltration of CD45RO+ cells was associated with the 5-year survival rate in a Cox statistical model. Therefore, further study is needed to confirm the role of CRT expression in the progression of colon cancer.

In conclusion, a low expression level of endogenous "danger signals", such as CRT, may represent a new mechanism underlying immune escape in colon cancer.

COMMENTS

Background

There is evidence that colorectal cancer is immunogenic. The mechanisms by which colorectal cancer cells induce an immunologic response remain unknown. A subset of damage-associated molecular patterns (DAMPs) has been recently found to be involved in the induction of immune response. Calreticulin

is one of the most important DAMPs. Whether calreticulin is associated with the immunogenicity of colon cancer remains controversial.

Research frontiers

Calreticulin is a multifunctional chaperone protein which mainly locates in the lumen of endoplasmic reticulum (ER). When exposed on the cell surface, calreticulin works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response. Whether the expression of calreticulin in colon cancer is involved in induction of immune response is a hot topic on the role of calreticulin in pathogenesis of colon cancer.

Innovations and breakthroughs

Studies indicate that the expression of calreticulin is decreased in non-mucinous colonic adenocarcinoma while is increased in poorly -differentiated colon cancer and advanced tumor. Other studies have shown that one of the calreticulin fragments inhibits angiogenesis and growth of colon cancer cells. However, the correlation between expression of calreticulin and survival of colon cancer patients remains unknown. In this study, the expression of calreticulin in stage ⅢB colon cancer was associated with the lower infiltration of CD45RO+ lymphocytes which was associated with a shorter 5-year survival time, suggesting that reduced expression of CRT may serve as a mechanism underlying immune escape in colon cancer.

Applications

In this study, reduced expression of endogenous "danger signals", such as calreticulin, was found to be a new mechanism underlying immune escape in colon cancer, which is important for a better understanding of the immunogenicity of colon cancer, thus providing a new immunotherapy for colon cancer.

Terminology

Calreticulin: A highly conserved 46 kDa Ca^{2+}-binding protein, which is mainly located in the lumen of ER and has various versatile functions, like chaperone activity, regulation of Ca^{2+} homeostasis, and adhesion signaling. When cells were treated with anthracyclines, oxaliplatin, radiation and hypoxia, calreticulin exposure works as an "eat me" signal for dendritic cells and macrophages, initiating an immune response.

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BRIEF ARTICLE

Early mortality of alcoholic hepatitis: A review of data from placebo-controlled clinical trials

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Abstract

AIM: To investigate the early mortality of placebo-treated alcoholic hepatitis patients.

METHODS: Mortality data about alcoholic hepatitis patients who participated in randomized placebo-controlled trials were searched from PubMed, EMBASE, and Cochrane Library, extracted and analyzed.

RESULTS: A total of 661 placebo-treated patients in 19 trials were included. The overall mortality rate was 34.19% with a median observation time of 160 d (range 21-720 d). Hepatic failure, gastrointestinal bleeding and infection were the three main causes of death, accounting for 55.47%, 21.17% and 7.30% of all deaths, respectively. One-month mortality data about 324 placebo-treated alcoholic hepatitis patients in 10 trials were reported with a pooled mortality rate of 20.37%. The one-month mortality rate of patients with moderate to severe alcoholic hepatitis tended to be higher than that of general patients (22.69% *vs* 10.93%, *P* < 0.05), whereas no significant difference was observed between the patients from North America or Europe (22.43% *vs* 18.45%, *P* > 0.05), neither any difference was found between the studies published before and after 1990 (18.18% *vs* 21.88%, *P* > 0.05).

CONCLUSION: Alcoholic hepatitis is a severe liver disease with a high mortality rate, and hepatic failure, gastrointestinal bleeding and infection are the three main causes of death.

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INTRODUCTION

Alcohol abuse causes a variety of liver diseases including alcoholic steatosis, alcoholic hepatitis, liver fibrosis and cirrhosis[1,2]. Generally, alcoholic steatosis is a benign lesion with a favorable prognosis if the patient abstains from alcohol use, whereas alcoholic hepatitis, which is observed in approximately 20% of heavy drinkers, is much more serious and requires treatment[3]. With increasing alcohol consumption worldwide, alcoholic liver disease has become a significant global health concern[4,5].

Alcoholic hepatitis is characterized by the development of hepatocellular necrosis and inflammation in

alcoholic patients[6]. Its pathogenesis is a multifactorial process involving metabolism of alcohol to toxic products, Kupffer cell stimulation by endotoxin and nutritional impairment lead to liver injury and inflammation[7]. The treatment of alcoholic hepatitis is still mostly symptomatic or empirical at best. Abstinence and supportive care are the two critical principles for the treatment of alcoholic hepatitis[7]. Several clinical trials have shown that corticosteroids can improve the short-term survival of patients with severe alcoholic hepatitis[8]. Recent evidence also suggests that inhibiting tumor necrosis factor-alpha release is beneficial for alcoholic hepatitis[9].

Since most studies on alcoholic hepatitis have focused either on its pathogenesis or on its potential therapies[7-9], its natural history has not been clearly defined. One possible explanation for this is that many alcoholic hepatitis patients are treated with available therapies immediately upon its diagnosis. Active therapies may significantly influence its natural progression and it is inappropriate to withdraw the treatment for studying its natural history. An understanding of the progression of alcoholic hepatitis would contribute to its prevention and treatment.

Randomized placebo-controlled clinical trials are considered the gold standard for evaluating the efficacy of medical interventions on the disease[10]. Data from placebo-controlled trials may also provide valuable information about its natural history. Since the use of placebo has very little effect on the progression of alcoholic hepatitis[11], placebo-treated patients may be the most suitable subjects for studying its natural history. Since a number of randomized placebo-controlled clinical trials are available on evaluating the treatment of alcoholic hepatitis worldwide[9], it has become feasible to study its natural history by extracting data from these studies.

In this study, we conducted a pooled analysis of data about alcoholic hepatitis patients who were treated with placebo in the eligible randomized placebo-controlled trials, in an attempt to evaluate the early mortality of such patients.

MATERIALS AND METHODS

Systematic literature search
English literature on alcoholic hepatitis patients in randomized placebo-controlled trials was searched from PubMed (1966 - March 2009), EMbase (1974 - March 2009), and Cochrane Library (2009, Issue 1) by two independent investigators using the search terms "alcoholic hepatitis", "alcoholic steatohepatitis", "alcoholic liver disease", "placebo" and "randomized controlled trial". Editorial or letter or comment or review was excluded. Reference lists of the retrieved relevant articles were also searched for additional trials.

Study selection
Two investigators independently performed the study selection. Search findings were screened for potentially eligible trials and full-text articles were obtained for their detail evaluation. Disagreements between the two investigators regarding studies included were solved by consensus.

Studies fulfilling the following criteria were included: studies of a randomized placebo-controlled clinical trial in alcoholic hepatitis patients, studies with enrollment, treatment and analysis of patients well-defined, and studies with more than 15 patients involved in the placebo arm, since we believed that they would not generate enough meaningful data to allow quality assessment. Trials were excluded if relevant data were not extractable or if the case-mix included unclassified alcoholic liver disease patients. Alcoholic hepatitis patients in the placebo arms that received basic active treatment with drugs such as steroids were also excluded.

Data extraction and quality assessment
Data extracted from the studies included name of the first author, publication year, study design, location(s), sample size, randomized method, and gender, mean age and disease severity of patients, description of active therapy, duration of therapy, duration of follow-up, mortality data and causes of death. Data extraction was performed by two independent reviewers. Sections of METHODS and RESULTS were coded to blind reviewers to the above information. Primary investigators were contacted if data were incomplete. The methodological quality of studies included was assessed using a validated quality checklist[12] with a maximum score of 32. A score of 12 (38%) or greater was considered to have acceptable quality[13].

Statistical analysis
Pooled estimate of each variable of interest was calculated and presented as a mean. The cumulative rate of each outcome of interest was calculated in the placebo arm of eligible studies. χ^2 test was used to compare qualitative variables. $P < 0.05$ (2-tailed test) was considered statistically significant.

RESULTS

Description of trials
We identified 892 potentially relevant articles and excluded 845 articles describing studies that obviously did not fulfill the inclusion criteria in this study by reviewing their titles and abstracts. Of the 47 studies selected for full-text review, 19 were excluded because they were not designed as placebo-controlled trials, 3 were excluded because the patients received other active medications[14-16], 4 were excluded because less than 15 patients received placebo[17-20], 1 was excluded because detailed survival data were not available[21], and 1[22] sharing the same placebo with a previous patient[23] was also excluded. Thus, only 19 studies fulfilled the inclusion criteria in this study (Figure 1).

Of these 19 clinical trials, 9 were single center studies, 10 were multi-center studies. A total of 661 patients with a mean age of 46.4 years served as placebo controls. Detailed information and summarized information about the studies included are shown in Tables 1 and 2, respectively.

Pooled mortality
Of the 661 patients, 226 (34.19%) died during a median

Table 1 Description of studies included in pooled analysis

Study	Country	Study type	Severity of alcoholic hepatitis	Placebo size	Mean age	Gender (M/F)	Active therapy	Therapeutic time	Follow-up time	Death (1-mo/overall)
Helman *et al*[24], 1971	US	Single center	Unclassified	17	47.7	NA	Prednisolone	4 wk	3 mo	NA/6
Blitzer *et al*[25], 1977	US	Single center	Unclassified	16	48.4	NA	Prednisolone	26 d	9 wk	NA/5
Maddrey *et al*[26], 1978	US	Single center	Moderate to severe	31	42.3	23/8	Prednisolone	28-32 d	4 wk	6/6
Baker *et al*[27], 1981	US	Single center	Unclassified	25	41.0	13/12	Insulin/glucagon	3 wk	3 wk	NA/6
Hallé *et al*[28], 1982	US	Single center	Severe	36	38.9	32/4	Propylthiouracil	6 wk	8 wk	7/7
Mendenhall *et al*[29], 1984	US	Multi-center	Moderate to severe	88	50.4	NA	Oxandrolone and prednisolone	1 mo	2 yr	NA/50
Fehér *et al*[30], 1987	Hungary and Spain	Multi-center	Unclassified	33	46.0	18/15	Insulin/glucagon	3 wk	3 wk	NA/14
Carithers *et al*[31], 1989	US	Multi-center	Severe	31	44.4	21/10	Methylprednisolone	4 wk	4 wk	11/11
Trinchet *et al*[32], 1989	Belgique	Multi-center	Unclassified	34	52.0	17/17	Colchicine	6 mo	6 mo	0/0
Akriviadis *et al*[33], 1990	US	Single center	Severe	36	40.8	25/11	Colchicine	1 mo	4 mo	6/8
Panos *et al*[34], 1990	UK	Single center	Unclassified	51	49.3	22/29	Polyunsaturated phosphatidyl choline	2 yr	2 yr	NA/20
Bird *et al*[35], 1991	UK	Single center	Severe	43	51.0	16/14	Insulin/glucagon	3 wk	6 mo	14/15
Mezey *et al*[36], 1991	Spain	Multi-center	Severe	26	43.7	12/14	Amino acid suppl.	3 wk	23 mo	5/16
Ramond *et al*[23], 1992	France	Multi-center	Severe	29	48.2	9/20	Prednisolone	4 wk	6 mo	NA/16
Trinchet *et al*[37], 1992	France	Multi-center	Severe	35	48.0	17/18	Insulin/glucagon	3 wk	1 mo	5/5
Bird *et al*[38], 1998	UK	Multi-center	Unclassified	30	51.0	16/14	Amlodipine	4 wk	4 wk	7/7
Akriviadis *et al*[9], 2000	US	Single center	Severe	52	40.8	40/17	Pentoxifylline	4 wk	160 d	NA/24
Mezey *et al*[39], 2004	US and Spain	Multi-center	Unclassified	26	49.0	16/10	Vitamin E	3 mo	1 yr	NA/5
Boetticher *et al*[40], 2008	US	Multi-center	Moderate to severe	22	49.1	17/5	Etanercept	3 wk	6 mo	5/5

NA: Not available.

Potentially relevant studies screened from literature search (*n* = 892)

Articles excluded after initial screening of titles and abstracts (*n* = 845)

Articles retrieved for more detailed evaluation and full paper review (*n* = 47)

Articles excluded after detailed screening (*n* = 28)
Not placebo-controlled trials (*n* = 19)
Placebo patients also received active therapies (*n* = 3)
Placebo size smaller than fifteen (*n* = 4)
Detailed survival data not available (*n* = 1)
Studies with duplicated placebo patients (*n* = 1)
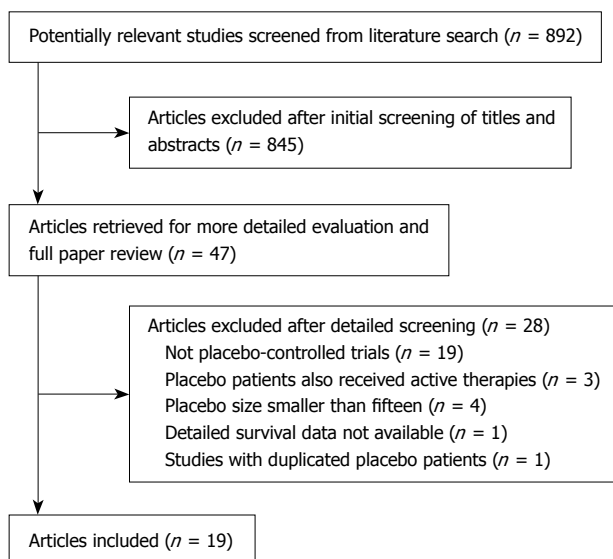
Articles included (*n* = 19)

Figure 1 Schema for literature search and study inclusion.

observation time of 160 d (range 21-720 d). Detailed causes of 137 deaths were available from 13 of the studies. Hepatic failure, gastrointestinal bleeding and sepsis were the three main causes of death, accounting for 55.47%, 21.17% and 7.30% of all deaths, respectively. One-month mortality data were available in 10 studies and the pooled mortality rate for these patients was 20.37% (66/324).

Subgroup analysis
Since the observation time was different among the

studies included, we did not directly compare the overall mortality rate of alcoholic patients. In subgroup analysis, we compared the one-month mortality rate of alcoholic patients based on the data extracted from 10 studies.

Of the 10 studies selected for comparison, 2 were performed in moderate to severe alcoholic hepatitis patients, 6 in severe alcoholic hepatitis patients, 2 in unclassified alcoholic hepatitis patients. The pooled one-month mortality rate of such patients tended to increase with increasing severity of the disease. The pooled mortality rate of unclassified, moderate to severe and severe alcoholic hepatitis patients was 10.94% (7/64), 20.75% (11/53), and 23.19% (48/207), respectively (Table 2).

Of the 10 studies, 5 were conducted in the United States and 5 in Europe (UK, France, Belgium and Spain) showing a pooled mortality rate of 22.44% (35/156) and 18.45% (31/168), respectively. No significant difference was found in pooled mortality rate between the patients from North America or Europe ($\chi^2 = 0.791$, $P = 0.409$) (Table 2).

Of the 10 studies, 5 were published before December 31, 1990, showing a pooled total mortality rate of 17.86% (30/168), 5 were published after January 1, 1991, showing a pooled mortality rate of 23.08% (36/156). No significant difference was observed in pooled mortality rate between the publication time ($\chi^2 = 1.359$, $P = 0.244$) (Table 2).

DISCUSSION

In this study, we extracted data about alcoholic hepatitis patients in randomized placebo-controlled trials and

Table 2 Comparison of pooled one-month mortality by different variables

	Studies (*n*)	Placebo-treated patients (*n*)	Gender (M/F)	One-month death (*n*)	One-month mortality rate (%)	χ^2	*P* value
Severity of disease							
Unclassified	2	64	33/31	7	10.94	4.375	0.038
Moderate to severe	8	260	171/89	59	22.69		
Geographical area(s)							
US	5	156	119/37	35	22.44	0.791	0.409
Europe	5	168	85/83	31	18.45		
Publication year							
Before 1990	5	168	118/50	30	17.86	1.359	0.271
After 1991	5	156	86/70	36	23.08		

analyzed their mortality, showing that the mortality rate of alcoholic hepatitis patients is positively correlated with the severity of the disease. Our pooled analysis also showed that hepatic failure, gastrointestinal bleeding and infection were the three main causes of early death of alcoholic hepatitis patients, which may be of great importance in clinical practice.

Unlike nonalcoholic hepatitis, a chronic form of liver disease associated with obesity, alcoholic hepatitis is an acute and potentially life-threatening form of liver disease[41]. The cause of its early mortality is not clear since it is difficult to evaluate a sufficient number of such patients without prompt treatment upon diagnosis. In this study, the data about 661 placebo-treated patients extracted from 19 placebo-controlled trials were evaluated, showing that the mortality rate of alcohol hepatitis patients is 34.19% with a pooled one-month mortality rate of 20.37%, which increases with increasing disease severity. These findings suggest that alcoholic hepatitis patients should be treated promptly upon diagnosis.

Recognizing the causes of death in alcoholic hepatitis patients contributes to the treatment of alcoholic hepatitis. Hepatic failure, gastrointestinal bleeding and infection are the three main causes of early death in alcoholic hepatitis patients. Hepatic failure can be prevented by avoiding exposure to certain hepatotoxic drugs, such as acetaminophen, carbon tetrachloride and galactosamine. Corticosteroid therapy is effective against alcoholic hepatitis[8], but it may result in complications such as gastrointestinal bleeding. It is, therefore, necessary to evaluate the risks and benefits of corticosteroid therapy for alcoholic hepatitis before it is used.

Due to the limited available data, there are some limitations in this study. First, we did not analyze the factors predicating the early mortality rate of alcoholic hepatitis patients since necessary data could not be extracted from the studies included. However, our pooled analysis provided the main causes of death, which may be of importance in clinical practice. Second, we did not clarify whether the mortality rate of alcoholic hepatitis patients varies between males and females. Since females are reported to be more sensitive to alcohol abuse[7], it would be of interest to compare mortality rates of both genders. Third, we did not analyze the potential heterogeneity due to the relative small number of placebo patients. We grouped all the patients irrespective of their age, gender, ethnic distinction,

or severity of disease. The pooled analysis of all these cases provided a clearer general picture of early mortality rate in a relatively large sample of alcoholic hepatitis patients.

In summary, alcoholic hepatitis is a severe liver disease with a high early mortality, especially among those with moderate to severe alcoholic hepatitis. Hepatic failure, gastrointestinal bleeding and infection are the three main causes of early death in alcoholic hepatitis patients.

COMMENTS

Background
Alcoholic hepatitis is a potentially life-threatening complication of alcohol use, but its natural history has not been clarified so far.

Research frontiers
This study investigated the early mortality of placebo-treated alcoholic hepatitis patients.

Innovations and breakthroughs
This study has confirmed that alcoholic hepatitis is a severe liver disease with a high early mortality, especially among those with moderate to severe disease.

Applications
The results of this study suggest that hepatic failure, gastrointestinal bleeding and infection should be treated in order to prevent early death of alcoholic hepatitis patients.

Terminology
Alcoholic hepatitis is a disease resulting from hepatocellular necrosis and inflammation in alcoholic patients. Its pathogenesis is a multifactorial process involving metabolism of alcohol to toxic products. Kupffer cell stimulation by endotoxin and nutritional impairment lead to liver injury and inflammation, *etc*.

Peer review
This is an interesting review of data concerning the early mortality of alcoholic hepatitis. The authors critically discussed the limitation of their survey.

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CASE REPORT

Acute respiratory distress syndrome associated with severe ulcerative colitis

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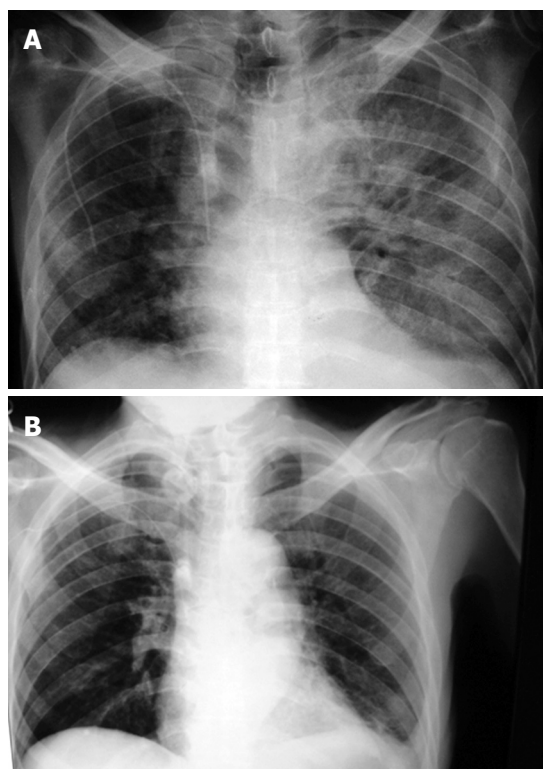Telephone: +81-18-8321601 Fax: +81-18-8321603

Abstract

Various extraintestinal manifestations including pulmonary abnormalities have been reported in patients with ulcerative colitis. Acute respiratory distress syndrome (ARDS) is a serious and fatal pulmonary manifestation. We have experienced a 67-year-old male patient with ARDS associated with a severe type of ulcerative colitis (UC). Severe dyspnea symptoms occurred during the treatment of UC in a previous hospital and the patient was transferred to our hospital on June 27, 2007. Both blood and sputa cultures for bacteria and fungi were negative. Cytomegalovirus antigenemia was also not detected. From the clinical and radiological [Chest X-ray, computed tomography (CT)] findings, the patient was diagnosed with ARDS on the basis of the definition of ARDS developed by the European-American Consensus Conference on ARDS. Both colonic inflammations and ARDS symptoms of the patient were resistant to any medical treatment including corticosteroids and antibiotics. However, ARDS symptoms were dramatically improved after surgical colectomy. We believe that severe colonic inflammation from UC was closely associated with the onset of ARDS of the patient. Our case report suggests that a severe type of ulcerative colitis might be taken into consideration as one of the predisposing factors of ARDS.

INTRODUCTION

Ulcerative colitis (UC) is a chronic intractable colitis with unknown etiology. Various extraintestinal manifestations, including hepatobiliary, musculoskeletal, dermatologic, ocular and pulmonary symptoms, have been reported in patients with UC[1-3]. Acute respiratory distress syndrome (ARDS) is a serious and fatal pulmonary manifestation and overall mortality from ARDS has been typically reported to be greater than 50%[4]. Here, we showed an ARDS patient associated with severe UC. ARDS symptoms of the patient were resistant to any medical treatment but dramatically improved after surgical colectomy. Our case suggests that severe colonic inflammation from UC was closely associated with the onset of ARDS.

CASE REPORT

A 67-year-old male patient visited a previous hospital due to bloody diarrhea on March 1, 2007. He was diagnosed as having UC based on clinical, endoscopic and histological criteria. He was admitted to the hospital and underwent treatment with mesalamine, prednisone (40 mg), and aza-thioprine (50 mg). Leukocytapheresis therapy was also performed. However, his abdominal symptoms and endoscopic findings got worse and an intensive intravenous steroid regimen with 60 mg prednisone was started. During the treatment, he complained of severe dyspnea and a chest-X ray revealed pulmonary edema. Treatment with azathioprine and mesalamine was discontinued after he complained of severe dyspnea. Then, the patient was transferred to Akita University Hospital for intensive treatment on June 27, 2007. On physical examinations, he had tachycardia (134 bpm) and a fever (38.1℃), and coarse crackles were audible in both lung fields. Tenderness was observed in the lower abdomen but no symptoms suggesting peritonitis were found. Blood chemistry values on June 28 were as follows: hemoglobin (Hb) 8.9 g/dL, hematocrit (Ht) 27.9%, white blood cell count (WBC) 3100/L, C-reactive protein (CRP) 2.92 mg/dL, total protein (TP) 4.4 g/dL, albumin 2.4 g/dL, L-aspartate 2-oxoglutarate aminotransferase (AST) 74 U/L, L-alanine 2-oxoglutarate aminotransferase (ALT) 73 U/L, alkaline phosphatase (ALP) 785 U/L, blood urea nitrogen (BUN) 31.9 mg/dL, creatinine (Cr) 0.38 mg/dL. Anemia, inflammatory changes, hypoproteinemia, and liver dysfunction were observed. Chest X-ray showed pulmonary edema without enlargement of the heart (Figure 1A). Arterial blood gas values were PaO_2 39 mmHg; $PaCO_2$ 35.7 mmHg; pH 7.518; and HCO_3 28.4 mEq/L, showing impaired oxygenation. Ultrasound cardiography showed normal heart function, with no enlargement of the right atrium and ventricle. From these clinical and radiological findings, the patient was diagnosed as having ARDS. Chest computed tomography (CT) scan images of the patient were also compatible with ARDS (Figure 2). Both blood and sputum cultures for bacteria and fungi taken in the hospital were negative. Low levels of Candida antigen and a slight increase of β-D glucan were found in the patient's blood. However, serum Candida antigen promptly decreased after treatment with an antifungal drug (fosfluconazole). Cytomegalovirus antigenemia was not detected. Dyspnea of the patient was severe and not improved by nasal oxygen supplementation. Then, the patient was admitted to the intensive care unit (ICU) with intubation and positive pressure ventilation. However, despite intensive medical treatment including prednisone, antibiotics (cefazolin sodium, imipenem/cilastatin sodium), and sivelestat sodium hydrate, pulmonary condition and abdominal symptoms of the patient were not improved. Colonoscopic examination on July 2nd also showed severe colonic inflammation with multiple deep ulcers (Figure 3). However, cytomegalovirus antigenemia was not detected. Both fecal culture and fecal Clostridium difficile toxins were negative. Bronchoscopy on July 6th showed

Figure 1 Chest X-ray. A: Chest X-ray taken at time of admission showing Acute respiratory distress syndrome (ARDS); B: Chest X-ray after surgical colectomy showing a significant improvement of ARDS.

Figure 2 Chest computed tomography (CT) scan image taken before surgical treatment showing compatibility with ARDS.

wet bronchial mucosa with a little inflammatory change, and no bacteria and fungi were detected by culturing the sputa collected by bronchoscopy. Colonoscopic findings together with his clinical course strongly suggested that the predisposing factor of ARDS of the patient might be severe inflammation of the colon. Thus, we thought that surgical colectomy was essential to rescue the patient from ARDS, and subtotal colectomy was performed on July 11. Postoperatively, gas exchange was dramatically improved, and he was weaned off the ventilator on July 19. A chest X-ray taken August 11 (Figure 1B) was also significantly improved. After an additional 55 d recovery, he was discharged from the hospital with no respiratory symptoms.
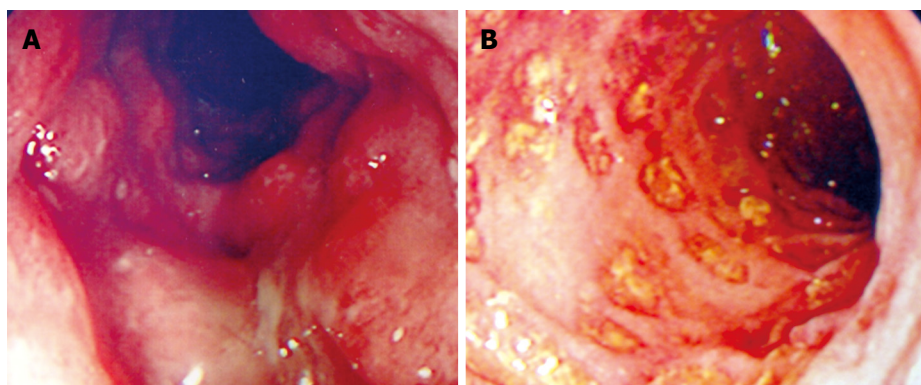
Figure 3 Colonoscopic images showing deep ulcer with severe mucosal edema (A) and multiple discrete ulcers (B).

DISCUSSION

Both UC and Crohn's disease are chronic intractable inflammatory bowel diseases (IBD). It has been shown that 30%-50% of IBD patients have pulmonary abnormalities[1]. In addition, drug-induced lung injuries have also been reported in IBD patients[5,6]. ARDS is a complex response of the lung to direct and indirect insults and numerous predisposing factors causing ARDS, such as toxic inhalation, diffuse infection, sepsis, pancreatitis, *etc.*, have been reported[4]. With regard to an association between UC and ARDS, only one ARDS patient accompanied by UC has been reported to date[7]. In this report, we diagnosed the patient as having ARDS based on the definition of ARDS developed by the European-American Consensus Conference on ARDS[4], namely, $PaO_2/FiO_2 < 200$, the detection of bilateral pulmonary infiltrates on frontal chest radiography, and no clinical evidence of elevated left atrial pressure. With regard to the predisposing factors of ARDS, we could not detect particular factor that clearly caused ARDS in our patient. Although very low levels of Candida antigen was detected in the blood, Candida was not detected by sputa cultures and ARDS symptoms of the patient were not improved after the decrease in the Candida antigen. Thus, we did not think that Candida was the predisposing factor of ARDS in our patient. Other fungi as well as Candida were not detected by sputa cultures, which were examined several times. Although severe inflammation with deep ulcers was endoscopically observed in the colonic mucosa, Cytomegalovirus antigenemia was not detected in the patient during the admission.

Nagy *et al*[6] recently reported a case of interstitial pneumonitis, which was suggested to be ARDS, in a patient with UC treated with azathioprine (150 mg/d, 10 years). They demonstrated that the pulmonary condition of this patient was gradually improved by the discontinuation of azathioprine and treatment with corticosteroids. Our patient was also treated with azathioprine. However, the dose of azathioprine of our patient (50 mg/d, 2 mo) was much smaller than that of Nagy *et al*'s case[6]. Although an association between azathioprine and the onset of ARDS of our patient was not completely excluded, we consider that ARDS symptoms of our patient were not due to administration of azathioprine but due to severe inflammation of ulcerative colitis on the basis of the following findings. First, ARDS symptoms were not improved but worsened after the discontinuation of azathioprine. In addition, ARDS symptoms were not improved by the intravenous corticosteroid treatment. Second, ARDS symptoms of the patient appeared to be correlated with the severity of UC and were dramatically improved after surgical colectomy. From these findings, we believe that severe colonic inflammation from UC was closely associated with the onset and the persistence of ARDS of our patient. It is suggested that pathogenic factors that caused ARDS in our patient were probably produced in the inflamed colon. ARDS is a serious and fatal pulmonary manifestation caused by a systemic inflammatory status. Our case report suggests that a severe type of UC might be taken into consideration as one of the predisposing factors of ARDS.

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CASE REPORT

Filiform polyposis in the sigmoid colon: A case series

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Abstract

Filiform polyposis is a rare condition of uncertain pathogenesis that is usually found in association with Crohn's disease, ulcerative colitis, intestinal tuberculosis or histiocytosis X. We report seven interesting cases of polyposis with various pathologic components, mainly located in the left side of the colon with no associated inflammatory bowel disease, intestinal tuberculosis or histiocytosis X. Multiple finger-like polypoid lesions with the appearance of stalactites were noted on the left side of the colon, especially in the sigmoid area, at the time of colonoscopy. The polyps had a variety of sizes and shapes and were shown to have various histopathologic components among the different patients. Although filiform polyposis localized in the sigmoid colon appears not to have high oncogenic potential, periodic follow-up seems to be needed.

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Key words: Filiform polyposis; Sigmoid colon; Inflammatory bowel disease

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INTRODUCTION

Filiform polyposis is a rare form of pseudopolyposis associated with ulcerative colitis, Crohn's disease, or granulomatous disease, which is formed by non-specific mucosal and submucosal reactions to previous severe inflammation[1-5]. The transverse and descending colon are the most common locations, although the polyps can be seen in any portion of the large bowel[3-6]. However, reports regarding filiform polyposis confined to the sigmoid colon, and the natural history or management of incidentally detected filiform polyposis in patients without the definite history of ulcerative colitis, Crohn's disease, or intestinal tuberculosis are inadequate[7,8].

We report seven interesting cases of filiform polyposis with mixed histologic components, including mainly inflammatory and hyperplastic or adenomatous components, located in the sigmoid area, without a definite history of severe inflammation such as inflammatory bowel disease (IBD) or intestinal tuberculosis.

CASE REPORT

Case 1

A 38-year-old male patient had sudden onset abdominal pain, diarrhea, and hematochezia for 2 d and had been diagnosed as having infectious colitis. He did not have a medical history of tuberculosis, IBD, or infectious colitis. Colonoscopic examination showed multiple, variable-sized polyps that were found in the sigmoid colon (Figure 1A). These polyps were histopathologically proven to be inflam-

matory and hyperplastic. Up to 44 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 2

A 64-year-old healthy male patient had no medical history of IBD or lower gastrointestinal symptoms. Colonoscopy was performed for screening purposes. On the colonoscopic examination, multiple long worm-like polyps were noted at the sigmoid colon (Figure 1B). These growths were histopathologically proven to be inflammatory polyps. Two large polyps were also found in the ascending colon and snare polypectomy was performed. These polyps were adenomas by pathologic evaluation.

On the follow-up colonoscopic examination 6 mo later, multiple finger-like polypoid lesions with the appearance of stalactites persisted on the left side of the colon, especially in the sigmoid area (Figure 1C). Large polyps with a hyperemic mucosa were removed by snare. These polyps were hyperplastic, tubular adenomas and filiform polyps with lymphoid hyperplasia. Up to 43 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 3

A 37-year-old male patient had frequent intermittent diarrhea after abdominal pain and was diagnosed as having irritable bowel syndrome. Colonoscopy was performed and multiple polyps were seen, which were densely packed, forming a roof in the sigmoid colon (Figure 1D). A representative polyp was removed by snare and shown to be a filiform or hyperplastic polyp on pathologic assessment. Up to 46 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 4

A 67-year-old asymptomatic male patient stated that colonofiberscopy had been performed at another hospital 2 years previously and the colonoscopic findings were lesions suspicious for intestinal tuberculosis. Colonofiberscopy was conducted again and multiple polypoid lesions with variable shape and size were found at the sigmoid colon (Figure 1E). To establish the exact pathologic diagnosis, snare polypectomy was performed. The pathologic results revealed that the polyps were mainly filiform and hyperplastic. Up to 46 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 5

A 77-year-old male patient generally had no lower gastrointestinal symptoms. The colonoscopic findings showed multiple polyps forming a densely packed bridge in the sigmoid colon (Figure 1F). These polyps were pathologically diagnosed as being mainly filiform and hyperplastic. Up to 34 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.

Case 6

An 81-year-old female patient was referred to our hospital for evaluation of sigmoid polyposis. Colonoscopy was performed and worm-like polyps located in the sigmoid colon were shown (Figure 1G). In addition, one polyp was reported as chronic non-specific inflammation with crypt cell hyperplasia and another was a hyperplastic polyp with focal atypical glands, favoring a reactive hyperplasia. Up to 38 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination.
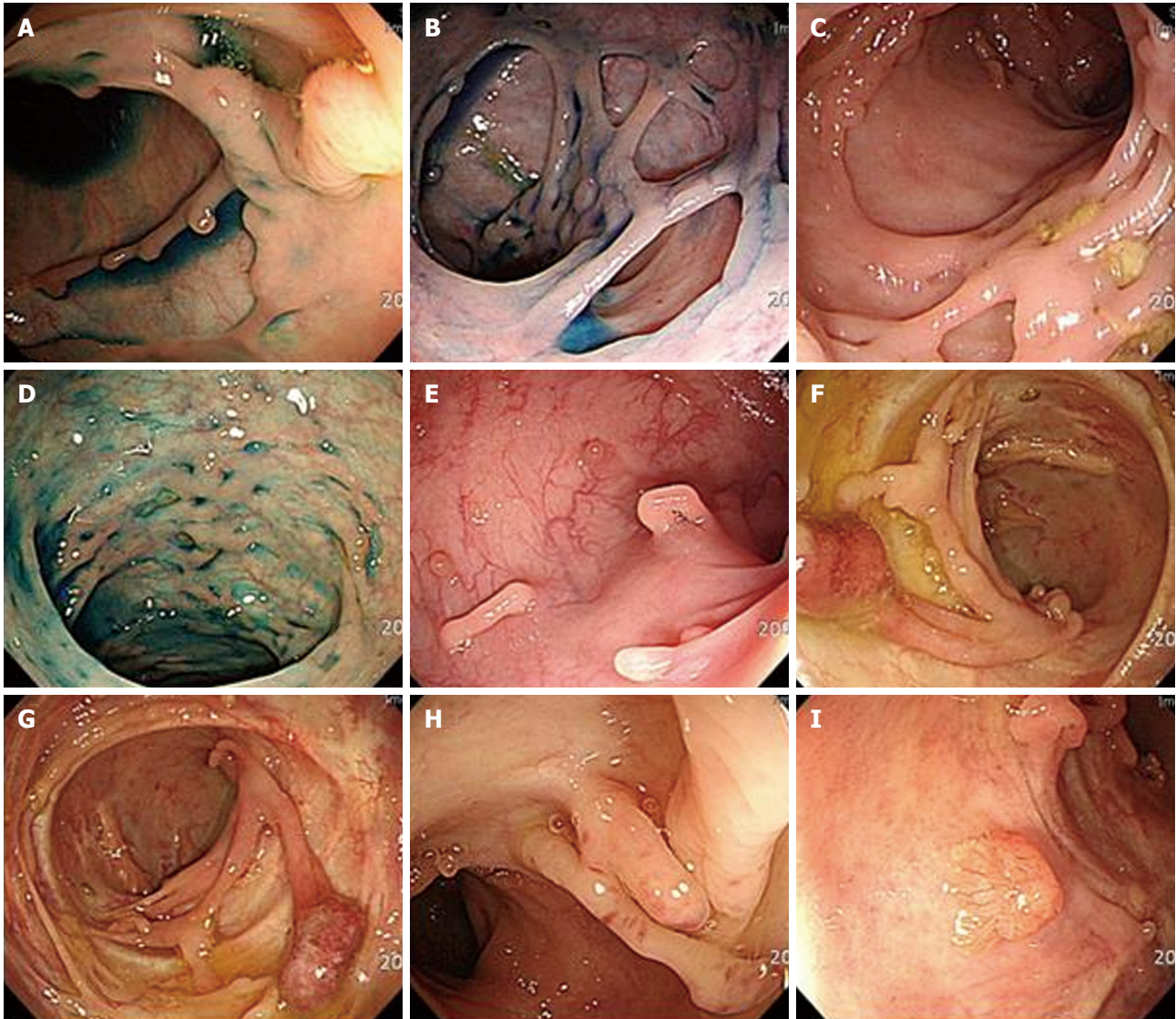
Case 7

A 63-year-old female had a history of diarrhea, abdominal pain, and weight loss for several months. The patient was referred to our hospital for further evaluation of multiple colon polyps. On colonoscopy, multiple variable polyps were noted in the cecum, ascending colon, and mainly in the sigmoid colon. Multiple finger- or worm-like polypoid lesions were noted in the sigmoid colon (Figure 1H and I). The polyps were of various size and shape. The tips of the polyps adhered to other parts of the mucosa, forming loops or bridges between opposing walls. Large polyps were removed by snare. All these polyps, except one, were shown to be hyperplastic or filiform (inflammatory) based on pathologic evaluation (Figure 2A and B). Only one polyp was shown to be a villous adenoma (Figure 2C). Up to 36 mo later, there was no evidence of malignant change in the polyposis on follow-up colonoscopy examination. We recommended periodic colonoscopy for cancer surveillance.
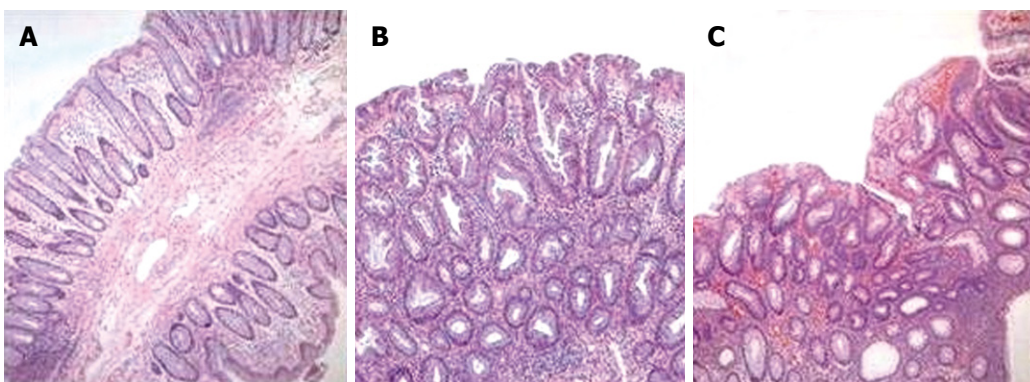
DISCUSSION

We report here seven cases of previously undiscovered polyposis in the sigmoid colon without a definite history of severe chronic inflammation including IBD or intestinal tuberculosis. This report is intended to characterize the clinical and pathologic features of filiform polyposis mainly located in the sigmoid colon. The clinicopathologic characteristics of filiform polyposis in the sigmoid colon of all the seven cases are shown in Table 1.

Filiform polyposis is an uncommon entity that is most often encountered in the colon of patients with a history of IBD[2]. Filiform polyposis is characterized by a large number of worm-like polyps lined by histologically normal colonic mucosa[5,8]. Filiform polyps usually have a thin, straight shape resembling the stalks of polyps without the heads[4]. The polyps can range in size from 1.5-3.0 cm in length and up to 0.5 cm in diameter[2,3]. The projections can occur as solitary polyps or as diffuse polyposis distributed over large areas of the colonic mucosa[8]. Long-term inflammation of the colonic mucosa during chronic IBD with alternating periods of ulceration and healing may lead to the formation of finger-like projections, these so-called filiform polyps[2-4]. Only rare cases without a history or evidence of IBD have been reported[3]. Additionally, in rare cases, several

Figure 1 Colonoscopic findings show multiple worm-like or finger-like polypoid lesions, with a stalactite appearance, in the left-sided colon, especially in the sigmoid area (A-I).

Figure 2 Pathologic features of inflammatory polyp, hyperplastic polyp and tubular adenoma (HE, × 100). A: Inflammatory polyp showing chronic inflammatory cells in lamina propria without hyperplastic or adenomatous epithelial change; B: Hyperplastic polyp showing hyperplastic epithelia with stellate lumen; C: Tubular adenoma showing low grade dysplasia.

filiform polyps form large tumor masses, termed giant filiform polyposis[5]. Histologically, the polyps are fili-form, with a central core, containing vessels and smooth muscle fibers[6]. Clinicopathologic and immunopheno-

Table 1 Clinicopathologic characteristics of filiform polyposis in the sigmoid colon

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Age (yr)	38	64	37	67	77	81	63
Sex	M	M	M	M	M	F	F
Location	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon	Sigmoid colon
Tuberculosis history	None	None	None	None	None	None	None
IBD	None	None	None	None	None	None	None
Chest PA	None	Normal	Normal	Normal	Normal	Normal	Normal
Pinch biopsy pathologic findings	Inflammatory Hyperplastic	Inflammatory Hyperplastic Tubular adenoma	Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic	Inflammatory Hyperplastic
Polypectomy pathologic findings	Filiform Hyperplastic	Filiform Hyperplastic Villotubular adenoma	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Hyperplastic	Filiform Villous adenoma
Diarrhea	Yes	None	Yes	None	None	Yes	Yes
Hematochezia	Yes	None	None	None	None	None	Yes
Abdominal pain	Yes	None	Yes	None	None	Yes	Yes
Weight loss	None	None	None	None	None	Yes	Yes
Follow-up period (mo)	44	43	46	46	34	38	36

IBD: Inflammatory bowel disease.

typic studies regarding filiform polyposis without IBD demonstrate that there is generalized polyposis, generally considered to be an asymptomatic sequela of ulcerative colitis[2]. Generally, there is no definite evidence that filiform polyposis itself represents a pre-cancerous condition[2]. Filiform polyposis may resemble villous adenomas on colonoscopy[4]. Therefore, biopsy should be recommended in all cases of filiform polyposis if it is necessary[3,8,9]. Filiform polyposis alone is not an indication for surgical resection, but complications, such as acute massive hemorrhage or intestinal obstruction, may necessitate surgical intervention[10,11].

In our case series it cannot be completely excluded that filiform polyps may have emerged as asymptomatic sequelae of intestinal tuberculosis, IBD, other infectious colitis, ischemic colitis, or histiocytosis X.

Sporadic hyperplastic polyps are generally thought to be non-malignant and malignant transformation into adenocarcinoma has been rarely reported[12]. However, contrary to single sporadic hyperplastic polyps, hyperplastic polyposis is considered to be a pre-cancerous lesion[12-17]. Our case series is distinct from hyperplastic polyposis syndrome. The diagnostic criteria for hyperplastic polyposis generally include the presence of (1) at least 5 histologically diagnosed hyperplastic polyps proximal to the sigmoid colon, of which two are > 10 mm in diameter, or (2) any number of hyperplastic polyps proximal to the sigmoid colon in an individual who has a first degree relative with hyperplastic polyposis, or (3) > 30 hyperplastic polyps of any size that are distributed throughout the colon[12-15]. Our case series did not satisfy these diagnostic criteria.

Diffuse colonic mucosal ulceration is a possible finding in histiocytosis X[18]. Histiocytosis X may have gastrointestinal symptoms such as diarrhea, malabsorption, or gastrointestinal bleeding[18]. However, these gastrointestinal symptoms are rare[18]. Diagnosis of histiocytosis X is based on mucosal biopsy and immunopathologic findings which demonstrate the presence of a disseminated proliferation of histiocytes[18]. Our case series did not satisfy this pathologic finding.

We report seven patients with filiform polyposis mainly localized in the sigmoid colon. Although none of our cases showed progression into adenocarcinoma, various pathologic findings, including mainly filiform polyps, hyperplastic polyps or adenomas, were found. None of the patients had a definite history of IBD, intestinal tuberculosis, infectious or ischemic colitis, but the filiform polyposis seemed to be a sequela of asymptomatic chronic colonic inflammation. Although definite evidence is insufficient, filiform polyposis localized in the sigmoid colon appears not to have high oncogenic potential. However, periodic follow-up seems to be necessary because large polyps were intermittently proven to be adenoma in our case series.

We have reported seven cases of filiform polyposis involving the sigmoid colon in Koreans who had no definite history of IBD or intestinal tuberculosis. Up to the present, studies regarding filiform polyposis syndrome located in the left side of the colon are insufficient. Continuous close follow-up of these seven patients and collection of large numbers of cases should be performed in order to understand the natural history and observe the possibility of progression into cancer.

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Herbal extracts as hepatoprotectants against acetaminophen hepatotoxicity

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Abstract

Many plant-derived natural products have the potential to be hepatoprotective and therefore can be used to treat acute and chronic liver diseases. The challenge is to identify the most promising compounds and evaluate their protective mechanism. In a recently published article, Wang *et al* evaluated extracts of the plant *Gentiana manshurica* Kitagawa (GM) in a model of acetaminophen hepatotoxicity. The authors concluded that GM is hepatoprotective against acetaminophen-induced liver injury due to its antioxidant properties and anti-apoptotic capacity. We would like to discuss the limitations of this experimental approach and question the conclusion based on the data presented in this manuscript and the published literature.

Peer reviewers: Dr. Christoph Reichel, Priv.-Doz., Head of the Gastroenterological Rehabilitation Center Bad Brückenau, Clinic Hartwald, German Pension Insurance Federal Office, Schlüchterner Str. 4, 97769 Bad Brückenau, Germany; Dr. Vandana Panda, Pharmacology and Toxicology, Prin. K. M. Kundnani College of Pharmacy, Jote Joy Building, Rambhau Salgaonkar Marg, Cuffe Parade, Colaba, Mumbai 400 005, India

TO THE EDITOR

We read with interest a recent paper published in *World Journal of Gastroenterology* by Wang *et al*[1], who showed that 2 h pretreatment with an extract of the plant *Gentiana manshurica* Kitagawa (GM) could protect mice against acetaminophen (APAP) hepatotoxicity (300 mg/kg). The authors concluded that GM is hepatoprotective against acetaminophen-induced liver injury due to its antioxidant properties and anti-apoptotic capacity. A comparison of GM to the well-established clinically used antidote *N*-acetyl-*L*-cysteine (NAC) resulted in similar beneficial effects. According to the authors, their data suggest that GM is a potent hepatoprotective agent with a comparable mechanism to NAC. However, some of these mechanistic conclusions are clearly not justified by the data and whether or not GM is actually hepatoprotective under clinically relevant conditions has not been established.

The first concern is the authors' conclusion that GM has an anti-apoptotic effect. Apoptotic cell death is characterized by cell shrinkage and nuclear condensation[2,3]. However, none of these morphological features was evident from the liver sections shown by Wang *et al*[1]. In contrast, extensive centrilobular necrosis was shown, which correlates with the extensive release of liver enzymes into the plasma. The cells that are stained positive for DNA strand breaks with the transferase-mediated

dUTP nick end-labeling (TUNEL) assay are not apoptotic. These cells show the characteristic nuclear and cytosolic staining of oncotic cells, which has been extensively described for APAP hepatotoxicity[2-4]. The nuclear fragmentation is caused by the mitochondrial release of endonuclease G and apoptosis-inducing factor[4,5], rather than by caspase-activated DNase, which is responsible for DNA fragmentation during apoptosis[6]. Although the authors claimed that caspase-3 was activated based on the appearance of a 17 kD cleavage product, no other bands (pro-caspase-3) have been shown and no positive controls as reference points were included. Most importantly, a dramatic increase in procaspase-3 cleavage should go along with a dramatic increase in caspase-3 enzyme activity, which has never been shown in APAP-induced liver injury[2,7-9]. Consistent with these observations, caspase inhibitors do not protect against APAP hepatotoxicity arguing against a relevant role of caspase activation in the pathophysiology[8,9]. Furthermore, in both primary cultured mouse[10] and human hepatocytes[11], APAP induces necrotic cell death. Taken together, oncotic necrosis is the predominant mode of cell death (> 95%) during APAP hepatotoxicity in animal models *in vivo* and in humans[2]. The only exception is APAP-induced cell death in metabolically incompetent hepatoma cell lines. However, these mechanisms have no relevance to the *in vivo* hepatotoxicity of this drug[7].

A second major concern is related to the extensive mechanistic conclusion including the hypothesis that GM acts as an antioxidant. There is direct evidence that reactive oxygen species and peroxynitrite are formed in mitochondria during APAP hepatotoxicity and play a critical role in cell death[4,12,13]. However, the fact that at 12 h after APAP administration, there was less lipid peroxidation, together with other evidence for reduced tissue injury, does not prove that GM acts as an antioxidant. The same results would be obtained if GM protected and improved cell viability through other mechanisms with the consequence of less oxidant stress. In fact, one of the most likely mechanisms of protection, i.e. that one or several compounds in this plant extract may have inhibited cytochrome P450 activities or may have competed with APAP for metabolism, was not investigated. Toxicity of APAP is entirely dependent on its metabolic activation[14], which means that any interference with its reactive metabolite formation will substantially reduce or even eliminate toxicity. In the absence of clear evidence that this extract does not affect reactive metabolite formation, any conclusion regarding more distal mechanisms is not justified.

The third concern is the conclusion that GM acts as a hepatoprotectant similar to NAC. However, in clinically relevant situations of drug overdose, the antidote has to be effective when administered after the insult not as a pretreatment. The effectiveness of GM against APAP hepatotoxicity when treated after drug overdose has not been investigated. Furthermore, the comparison to NAC is not justified. NAC given as pretreatment to fasted animals will support glutathione (GSH) synthesis in the liver resulting in much higher GSH levels than the respective controls 2 h later[15]. These elevated GSH levels will more effectively scavenge the reactive metabolite of APAP and therefore prevent initiation of liver injury[16]. This protection mechanism of NAC is independent of the antioxidant effect of GSH mainly because no oxidant stress is generated at this point. However, if NAC is administered several hours after APAP, i.e. at a time when hepatic GSH is depleted and mitochondria have already generated an oxidant stress, GSH synthesized at this time is used to scavenge reactive oxygen species and peroxynitrite[13,17]. In addition, some of the excess NAC will also be used to support the impaired mitochondrial energy metabolism[17]. Both mechanisms contribute to the late protection against APAP hepatotoxicity[13,17]. Thus, NAC can have 3 different mechanisms of action depending on the time of administration relative to APAP. The pretreatment with NAC as used by Wang *et al*[1] will mainly scavenge the reactive metabolite of APAP, an effect that is unlikely to be relevant to GM.

Taken together, the protective effect of GM against APAP hepatotoxicity is an interesting observation. However, GM as a hepatoprotectant against drug toxicity under clinically relevant conditions has not been demonstrated. In addition, the actual protection mechanism of GM remains unclear. More mechanistic studies considering clinically relevant conditions are needed to evaluate the potential of this plant extract as an antidote against drug hepatotoxicity.
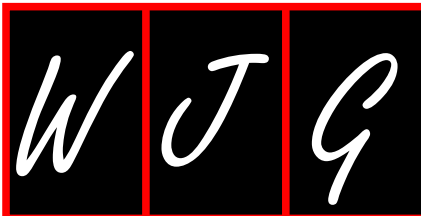
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Acknowledgments to reviewers of *World Journal of Gastroenterology*

Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical
Negligence and Litigation in Medical
Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology
Conference

January 28-30
Hong Kong, China
The 1st International Congress on
Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
Gastroenterology & Endoscopy
Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian

Pacific Association for the Study of
the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in
Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology
and Hepatology Conference, EGHC
2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic
Surgery

April 14-18
Vienna, Austria
The International Liver Congress™
2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress
of surgery and the 5th Croatian
Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual
Meeting

May 06-08
Munich, Germany
The Power of Programming:
International Conference on
Developmental Origins of Health
and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and
Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical
Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on
Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in
the Research of Probiotics and
Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver
Transplantation Society ILTS Annual
International Congress

June 20-23
Mannheim, Germany
16th World Congress for
Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific
Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on
Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's
Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory
Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference
on Antimicrobial Agents and
Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on
Controversies in Gastroenterology &
Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International
Symposium of Society of
Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of
Gastroenterology Annual Scienitfic
Meeting

October 23-27
Barcelona, Spain
18th United European
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October 29-November 02
Boston, Massachusetts, United States
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Management of Inflammatory Bowel
Disease

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The Medical Management of HIV/
AIDS

Instructions to authors

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1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224   DOI: 10.3748/wjg.13.6356]

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2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462   PMCID:2516377   DOI:10.1161/01.HYP.0000035706.28494.09]

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]

No author given

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]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325   DOI:10.1046/j.1526-4610.42.s2.7.x]

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8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900   DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

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/eid.htm

Patent (list all authors)

16   **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, ρ (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, ρ (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc*. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, *etc*.
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