



World Journal of Gastroenterology®



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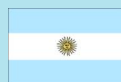
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Normal aspects of colorectal motility and abnormalities in slow transit constipation

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Abstract

Human colonic motility is a relatively difficult topic to investigate. However, the refinement of manometric techniques in recent years enabled us to study both the proximal and distal segments of the viscus. The present paper reviews our knowledge about normal aspects of colorectal motility in man and the abnormalities found in slow transit constipation (STC), one of the most frequent and difficult to treat subtypes of constipation. An internet-based search strategy of the Medline and Science Citation Index was performed using the keywords colon, colonic, colorectal, constipation, slow transit, motility, rectal, rectum in various combinations with the Boolean operators AND, OR and NOT. Only articles related to human studies were used, and manual cross-referencing was also performed. Most of colonic motor activity is represented by single nonpropagated contractions, rarely organized in bursts; this activity is maximal during the day, especially after waking and following meals. In addition, a specialized propagated activity with propulsive features is detectable, represented by high- and low-amplitude propagated contractions. In the severe form of constipation represented by the slow transit type, the above motor activity is completely deranged. In fact, both basal segmental activity (especially in response to meals) and propagated activity (especially that of high amplitude) are usually decreased, and this may represent a physiologic marker of this disorder. Human colonic motor activity is quite a complex issue, still only partly understood and investigated, due to anatomic and physiological difficulties. In recent years, however, some more data have been obtained, even in proximal segments. These data have helped in elucidating, although only in part, some pathophysiological mechanisms of chronic constipation, and especially of the STC subtype.

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INTRODUCTION

The main physiological properties of the human colon (absorption, propulsion of contents, storage and expulsion of feces) require unique motor activities, different from those of the upper gut^[1], apt to propel distally intraluminal contents, to mix them in a continuous manner, and to store and eventually expel the residuals^[2]. Since colonic contents, unlike those in the upper gut, employ hours or days to pass through the viscus, prolonged observations (24 h or more) are needed to assess the various aspects of colonic motility^[3].

Several techniques have been proposed to evaluate the motor behavior of the large bowel for prolonged periods of time^[4-6], the more diffuse being colonic manometry as both a research and (especially in the pediatric area) diagnostic tool^[7,8]. The more frequently employed technique consists of positioning a manometric catheter within the colon by the aid of a colonoscope; the catheter is then connected to a recording apparatus to detect intraluminal pressures throughout the day, and to evaluate the effects of meals, sleep, awakening, etc.^[9]. More recently, the classic water-perfused catheters were compared to solid state ones^[10] that allow prolonged ambulatory recordings of colonic motility^[11,12] and even an automated computer analysis of recordings^[13].

SEARCH STRATEGY

An internet-based search strategy of the Medline and Science Citation Index was performed using the keywords colon, colonic, colorectal, constipation, slow transit, motility, rectal, rectum in various combinations with the Boolean operators AND, OR and NOT. Only articles related to human studies were used, and manual cross-referencing was also performed. Articles published in English between January 1965 and March 2004 were selected; however, a search in non-English languages and in journals published before 1965 was also performed in our library. Letters and abstracts were quoted only when the full papers were not available.

BASIC ASPECTS OF NORMAL COLONIC MOTILITY IN HUMANS

Manometric investigations have shown that colonic motility may be grossly classified into two main types, and that these mostly agree with previous radiological observations^[14]. We

have recently proposed a simple classification of colonic contractile patterns, based on our experience with 24-h recordings in healthy humans^[9,15]. Colonic motility may therefore be divided into *segmental activity* (single contractions; bursts of contractions: rhythmic, arrhythmic) and *propagated activity* (low-amplitude propagated contractions (LAPC); high-amplitude propagated contractions (HAPC)).

Segmental activity

This kind of contractile activity constitutes mostly of the overall colonic motility, and it is usually represented by waves with amplitude ranging from 5 to 50 mmHg; occasionally, contractions of higher amplitude may be observed (Figure 1). Segmental contractions may appear singly or grouped (bursts); although the bursts appear usually as an arrhythmic array of waves, in a small percentage of time (<6% of the overall contractile daily activity) they may assume a rhythmic frequency. This rhythmic frequency is mostly comprised in the 3 cycle/min range, and it is preferentially observed in the distal (descending and sigmoid colon)^[16]. The purpose of the segmental contractile activity is to propel distally the fecal matter by means of an aborally directed pressure gradient that moves slowly the contents toward the rectum^[17,18]. This activity also allows an optimal absorption of water, electrolytes, short-chain fatty acids, and bacterial metabolites.

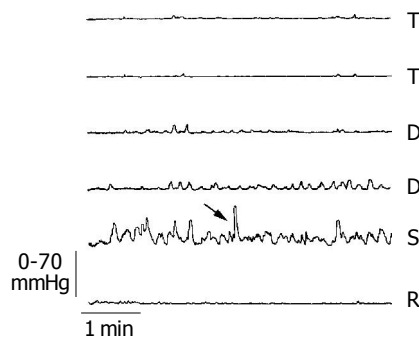


Figure 1 Representative manometric tracing of normal colonic segmental activity. Note that most contractions feature low amplitude, with sporadic ones (arrow) exceeding 50 mmHg. Recording points are 12 cm apart, and span from the transverse colon (T) to the rectum (R).

Propagated activity

To date, objective criteria (also employing automated analysis) to distinguish the various types of propagated contractions do not exist^[19]. We have classified the propagated events, on the basis of their amplitude, as LAPC and HAPC. The former (Figure 2) that feature an amplitude of less than 50 mmHg and are relatively frequent (on average, more than 100 events/d) have a probable main function of transporting fluid contents within the colon^[20,21]; LAPC have also been associated with distension of the viscus^[22] and passage of flatus^[23]. The other kind of propagated activity, represented by contractions averaging more than 100 mmHg amplitude (Figure 3) and occurring infrequently (on average, about 6 events/d), was firstly described in man by radiologists at the beginning of the last century^[24], and was subsequently demonstrated to correspond to the radiological concept of

“mass movements”, able to shift large quantities of contents for a long tract within the colon^[25,26]. More recently, HAPC have been better characterized by manometric means^[27,28], and their important role in the defecatory mechanisms established. These powerful contractions, in fact, are strongly associated with the defecation itself, and precede the expulsion of stools, so that they are considered one of the driving events of this important physiologic act^[29,30].

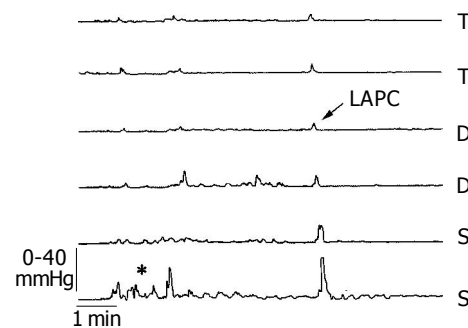


Figure 2 Representative manometric tracing showing a LAPC (arrow). Note also, in the last tracing, the presence of a burst of segmental sigmoid activity (asterisk). Recording points are 12 cm apart, and span from the transverse (T) to the sigmoid colon (S).

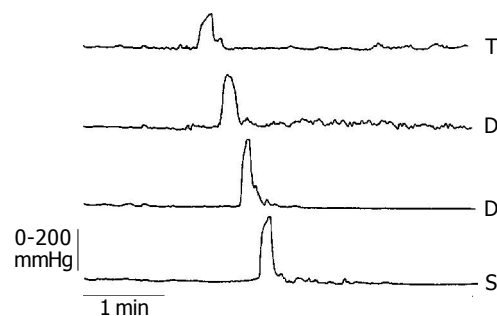


Figure 3 Representative manometric tracing of a HAPC. Note that the amplitude of the propagated sequence is well distinct with respect to the background segmental activity. Recording points are 12 cm apart, and span from the distal transverse (T) to the proximal sigmoid colon (S).

Daily organization of colonic motility and relationships with physiologic events

Unlike the upper gut, the human colon seems not to be organized in accordance to regular motor patterns that follow a cyclic timing. However, in recent years periodic motor phenomena have been documented in the rectum (“rectal motor complex”, RMC)^[31] and in the more distal colonic segments (“periodic colonic motor activity”) (Figure 4)^[32]. The physiological significance of these phenomena is still under investigation, although for the RMC a continence-preserving mechanism (especially when sleeping) has been hypothesized^[33]. No cyclic or periodic motor activity has been documented in the anal canal^[34].

More information is available concerning the fluctuations of colonic motor activity around-the-clock: in fact, prolonged manometric and electromyographic recordings have convincingly

shown that a circadian trend is recognizable, with a maximum of activity during the daylight hours, after awakening in the morning, and after meals^[4,5]. Conversely, sleep causes a profound inhibition of colonic motility, and seems to represent a major determinant of its diurnal variations^[35,36]. These variations in colonic motility are paralleled by a similar trend that may be observed in the tonic activity of the viscus' wall when measured by means of a barostat^[37].

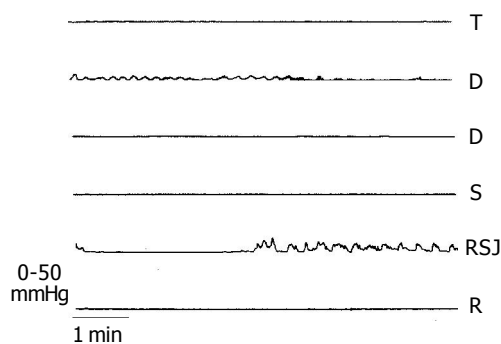


Figure 4 Representative manometric tracing showing two episodes of periodic colonic motor activity (asterisks) occurring independently at different colonic levels (proximal descending colon and rectosigmoid junction, respectively). Recording points are 12 cm apart, and span from the transverse colon (T) to the rectum (R).

The ingestion of food is a major physiological stimulus for colonic motor activity^[38], and it is represented by an increase (of at least 2-h duration) in the overall motility following the first mouthful of food (Figure 5)^[39]; this response, mostly represented by segmental contractions, is also accompanied by an increased smooth muscle tone^[40]. The colonic motor response to eating is driven by a dual component, cerebral and gastric, in that it may be evoked by both sham feeding and gastric distension^[41]. Meal composition influences colonic motor response: in fact, although carbohydrate meals are able to elicit motor activity, the effects are short lived when compared with fat meals^[42].

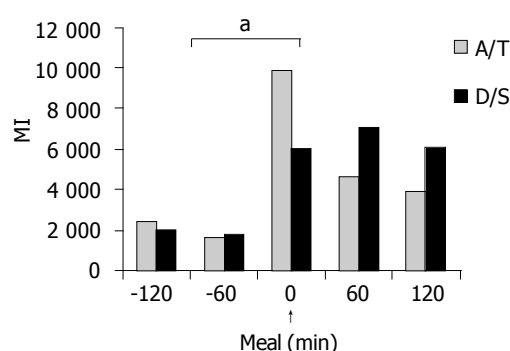


Figure 5 Colonic motor response to eating in normal subjects. All post-meal values (asterisk) are significantly ($P < 0.05$) different with respect to basal values in both proximal (A/T) and distal (D/S) segments. MI, motility index.

SLOW TRANSIT CONSTIPATION: DEFINITION

Constipation is a frequent clinical symptom, and its

diagnostic criteria have been recently revised in the Rome II classification^[43] as the presence in the last year, for at least 12 wk (which need not be consecutive) and in more than 1/4 of defecations, of two or more of the following: straining, lumpy or hard stools, sensation of incomplete evacuation, sensation of anorectal obstruction/blockade, manual maneuvers (digital evacuation, support of the pelvic floor, *etc.*) to facilitate defecation, and/or less than three defecations per week. Loose stool are not present and there are insufficient criteria for irritable bowel syndrome.

Two major pathophysiologic subtypes of constipation may be identified, with a third being the coexistence of both in the same patient^[44]. The most frequent subtype is represented by the pelvic floor dysfunction (also known as "outlet obstruction", "obstructed defecation", "dyschezia", "anismus", "pelvic floor dyssynergia"), which features normal or slightly slowed colonic transit with residual storage in the rectum or rectosigmoid area^[45]. The main pathophysiologic mechanism is due to an inability to evacuate adequately contents from the rectum, and it will not be discussed further, since it has been recently reviewed extensively elsewhere^[46]. The other subtype of constipation is the so-called slow transit constipation (STC), a clinical syndrome usually affecting women and characterized by often intractable constipation and heavily delayed colonic transit^[47]; this condition is usually attributed to disordered colonic motor function^[48]. In the last years, STC had been the subject of several investigations, aimed at evaluating the motility abnormalities of the large bowel as responsible for the patients' complaints. These pathophysiological aspects of STC will be discussed below. We feel that many of the available data are quite significant because (and differently than those available for other subtypes of constipation or other functional diseases of the large bowel) they have often been obtained in relatively homogenous cohorts of patients.

COLONIC MOTOR ABNORMALITIES IN STC

Although the etiology of STC remains elusive, there is ample evidence that motility of the large bowel is profoundly deranged in this condition, and this derangement may be observed for almost all the physiologic variables described above.

Segmental activity

Most of the few studies available were limited to the anorectal-sigmoid area (thereby justifying in part the heterogeneity of findings). These studies showed either overall reduced rectosigmoid motility^[49] and impaired response to intrarectal bisacodyl^[50], or normal or increased rectosigmoid motor activity^[51]. More recently, investigations on more proximal segments of the colon have demonstrated that in these patients the segmental contractile response to cholinergic stimulation is heavily impaired^[52] and that the regular contractile frequency activity is reduced to about half (i.e., <3%) of that recorded in normal subjects^[53].

Propagated activity

Data on LAPC in STC are still scarce; we have recently shown that this activity may be preserved in such patients,

thereby acting as a sort of alternative mechanism able to vicariate, at least in part, the severely impaired main propulsive force (see below)^[54]. Conversely, there are several observations showing that HAPC activity is usually deranged, up to complete absence of such events, in severely constipated patients, and especially in those with STC^[55-59]; this impairment consists of both a decreased average daily number of HAPC (Figure 6) and decreased amplitude and duration. The latter finding is consistent with the report that HAPC amplitude is a major factor for the propulsion of colonic contents to the rectum^[18]. In extreme cases, such an activity is completely absent, and it is also accompanied by almost complete disappearance of the segmental contractile activity: these are the patients with true “colonic inertia”, in whom no medical therapy is successful, and surgical intervention is often the only choice^[60,61].

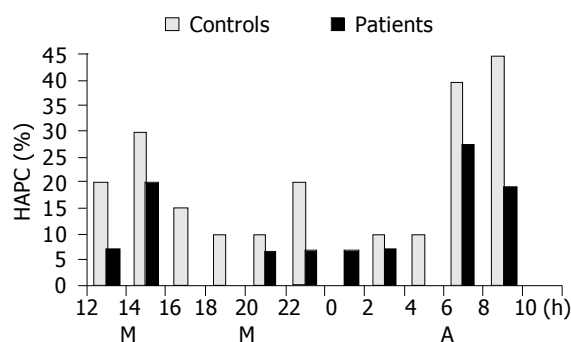


Figure 6 Percentage and distribution of HAPC/24 h in control subjects and severely constipated patients. M, meal, A, awakening. Adapted from Ref.^[65].

Daily organization of colonic motility and relationships with physiologic events

Concerning RMC and periodic colonic motor activity, there is evidence that this kind of activity may be abnormal in STC, with a decreased periodicity and reduced frequency^[59,62,63]. A recent investigation, however, reported that these patients may display excessive and un-coordinated RMC activity that, added to the reduced propulsive activity discussed above, may further delay the flow of colonic contents into the rectum^[64]. Looking at the daily motor activity, a study showed an overall decrease of colonic contractile activity^[65], although the day-night cycle seemed to be maintained, suggesting that normal circadian trend may be preserved, as also shown by a normal contractile response following sudden awakening from sleep^[66].

Some of the main physiological reflexes appear to be deranged in STC. In fact, an abnormal motor response to eating seems to be a constant feature in these patients, as shown by both electromyographic and manometric studies consistently demonstrating an overall scarce response to meal ingestion^[67-69]. This response is also characterized by a significant impairment of both segmental and propagated activity that follows the meals with respect to that observed in healthy controls^[70,71].

CONCLUSIONS

Human colonic motor activity is quite a complex issue, still

only partly understood and investigated, due to anatomic and physiological difficulties. In recent years, however, some more data have been obtained, even in proximal segments. These data have helped in elucidating, although only in part, some pathophysiological mechanisms of chronic constipation, and especially of the STC subtype. Unfortunately, the heterogeneity of the populations under study (that however are probably best selected with respect to other subtypes of constipation) accounts for certain discrepancies in the interpretation of the pathophysiological results. In addition, we are still lacking the “why”, i.e. we need more knowledge about the basic abnormalities of this disorder (nerve and/or smooth muscle involvement), which are still elusive and controversial^[72-75], in order to gain a more oriented therapeutic approach to this disease process.

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• ESOPHAGEAL CANCER •

Free radicals and antioxidant systems in reflux esophagitis and Barrett's esophagus

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be a therapeutic target in the treatment of patients with esophagitis and Barrett's esophagus.

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Abstract

AIM: Experimental studies suggest that free radicals are involved in acid and pepsin-induced damage of esophageal mucosa. The profile and balance between free radicals and antioxidant systems in human esophagitis are unknown.

METHODS: Superoxide anion and its powerful oxidant reaction with nitric oxide (peroxynitrite) generation were determined in esophageal mucosal biopsies from 101 patients with different gastro-esophageal reflux diseases and 28 controls. Activity of both superoxide dismutase (SOD) and catalase, and reduced glutathione (GSH) levels, were also assessed. Expression of Cu,ZnSOD, MnSOD and tyrosine-nitrated MnSOD were analyzed by Western blot and/or immunohistochemistry.

RESULTS: The highest levels of superoxide anion generation were found in patients with severe lesions of esophagitis. Peroxynitrite generation was intense in Barrett's biopsies, weaker in esophagitis and absent/weak in normal mucosa. Expression of Cu,ZnSOD and MnSOD isoforms were present in normal mucosa and increased according to the severity of the lesion, reaching the highest level in Barrett's esophagus. However, SOD mucosal activity significantly decreased in patients with esophagitis and Barrett's esophagus, which was, at least in part, due to nitration of its tyrosine residues. Catalase activity and GSH levels were significantly increased in mucosal specimens from patients with esophagitis and/or Barrett's esophagus.

CONCLUSION: A decrease in SOD antioxidant activity leading to increased mucosal levels of superoxide anion and peroxynitrite radicals may contribute to the development of esophageal damage and Barrett's esophagus in patients with gastroesophageal reflux. Administration of SOD may

INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most important and frequent gastrointestinal disorders of the Western world, and may lead to esophageal cancer^[1]. Although reflux of gastric acid into the esophagus is an important pathogenic factor in the development of esophagitis and Barrett's esophagus, the severity of reflux esophagitis cannot be predicted only on the basis of acid exposure, which suggests that other factors are involved. Recent studies have shown that oxygen free radicals are involved in esophageal mucosal damage from reflux esophagitis in both experimental animal models and humans^[2-9].

Reactive oxygen species (ROS) are highly reactive molecules produced during many normal biological processes. However, their synthesis is markedly increased as part of the inflammatory response, and can cause oxidative injury to cells by damaging DNA, proteins and cell membranes. Under normal conditions cells are protected by antioxidant defense systems from the toxic effects of ROS during cellular metabolism. Thus, when oxidative stress arises as a consequence of a pathological event, a defense system promotes the regulation and expression of enzyme antioxidants^[10] such as superoxide dismutase (SOD), catalase and glutathione peroxidase, which constitute the principal cellular defense mechanisms against ROS.

In the esophagus, the generation of the superoxide anion as the main free radical implicated in the mucosal damage has been well established in different experimental models of acute and chronic esophagitis^[2,4]. In human studies, a positive association between mucosal free radicals levels and the grade of esophagitis has been described^[7,8]. However, it must be noted that the efficiency of endogenous antioxidant defense mechanism may be of critical importance in protecting against the development of esophageal mucosal injury, and although administration of various free radical scavengers

has been found to prevent esophageal mucosal damage in animals^[2,6], studies on the expression, activity and relevance of endogenous enzymatic antioxidant systems in the human esophageal mucosal defense are very limited^[8,9]. Therefore, the aim of this study was to evaluate the profile and balance between free radicals and antioxidant systems in the spectrum of lesions associated with gastroesophageal reflux diseases, including esophagitis and Barrett's esophagus, which we regard as the first step in order to identify future therapeutic targets.

MATERIALS AND METHODS

Materials

All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO) except liquid scintillation counting medium, which was purchased from Packard (Groningen, The Netherlands).

Patients

Three to five distal esophageal biopsies were taken from 3 cm above the gastroesophageal junction from consecutive patients attending the Endoscopy Unit of the Service of Digestive Diseases of the University Hospital of Zaragoza (Video Endoscope Olympus Exera). The study protocol was approved by the Institutional Review Board Committee of the University Hospital of Zaragoza and written informed consent was obtained from all patients. Patients were stratified in four groups. Group 1 (control patients): subjects with no evidence of clinical symptoms of GERD or endoscopic esophagitis who underwent upper gastrointestinal endoscopy as part of clinical evaluation for different conditions such as abdominal pain, anemia, *etc.* Group 2 (GERD symptoms): patients with clinical symptoms of GERD, and/or erythema but no ulcerative lesions were included in this group. Symptoms were either heartburn or regurgitation. Group 3 (esophagitis): patients with esophagitis II-IV according to the Savary-Miller classification^[11]. Symptoms were either heartburn or regurgitation or chest pain. Group 4 (Barrett's esophagus): patients with Barrett's esophagus (mucosal length ≥ 3 cm). The presence of both endoscopic and histological examination of the esophageal mucosa was required to establish the diagnosis of Barrett's esophagus^[12]. In order to evaluate whether oxidative stress levels in Barrett's epithelium was specific and associated with gastroesophageal reflux or they were intrinsic to the columnar epithelium, additional duodenal biopsies were taken in patients with/without Barrett's esophagus.

Samples were immediately stored in Krebs-Ringer solution at 4 °C for determination of free radicals, in 10% formaldehyde for histological studies and in liquid nitrogen for Western blot analysis.

Measurement of superoxide anion

The presence of superoxide anion was evaluated by chemiluminescence according to a method described by Olyaei^[7] with modifications. Biopsies were suspended in 3 mL of oxygenated Krebs-Ringer and then lucigenin (N-methylacridinium nitrate) was added at a final concentration of 0.25 mmol/L and placed in the chemiluminescence spectrophotometer where light production was measured for 2 min (1900 TR, Packard Instruments Company, Meriden,

CT). At this point, SOD (200 U/mL) was added to the incubation medium and chemiluminescence levels were measured again for 2 min. Data were expressed as counts per minute (cpm) per milligram of protein. Proteins were determined by a colorimetric assay similar to Lowry assay (Bio-Rad, Hercules, CA)^[13].

Measurement of SOD

Total SOD activity was measured according to a method previously described by Thomas *et al*^[14]. The assay is based on the inhibition of nitro blue tetrazolium (NBT) mediated by superoxide anion. Thus, esophageal biopsies were sonicated in Tris buffer (0.1 mol/L pH 8.1 at 4 °C) and centrifuged at 12 000 *g* for 10 min at 4 °C. Afterwards, 200 μ L of supernatant were incubated with 1.8 mL of buffer containing Na₂CO₃ (0.05 mol/L), xanthine (10⁻⁴ mol/L), EDTA (10⁻⁴ mol/L), and NBT (2.5 $\times$ 10⁻⁵ mol/L). Superoxide radical was generated by adding xanthine oxidase (200 U/L) to the reaction cuvettes and measurements were recorded at 560 nm (Shimadzu spectrophotometer, Tokyo, Japan). Results are expressed as units per milligram of protein.

Measurement of reduced glutathione

Reduced glutathione (GSH) concentration was determined according to the method described by Toth *et al*^[15], with modifications. Samples were sonicated in 1 mL of phosphate buffered saline (PBS) and centrifuged at 12 000 *g* for 10 min at 4 °C. Ten percent metaphosphoric acid was added to 300 μ L of supernatant and centrifuged. Afterwards, the supernatant was incubated with 75 μ L of DNTB (5,5'-dithiobis-(2-nitrobenzoic acid)) and 0.3 mol/L Na₂HPO₄ (600 μ L). The change of absorbance was monitored at a wavelength of 412 nm. Results are expressed as micromole per milligram of protein.

Measurement of catalase

Catalase was determined according to the method described by Aebi^[16]. Esophageal mucosal biopsies were sonicated in phosphate buffer (KH₂PO₄, 25 mmol/L) and centrifuged at 12 000 *g* for 10 min at 4 °C. One milliliter of hydrogen peroxide (10 mmol/L) was added to 300 μ L of supernatant. Absorbance decrease was read at 240 nm for 1 min. Results are expressed as units per milligram of protein.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples were sectioned and applied to slides. Slides were subsequently deparaffinized with xylene for 15 min, hydrated gradually in a graded series of ethanol (100%, 96%, 70%, twice for 3 min in each) and washed in deionized water. Then, slides were treated with a 0.3% H₂O₂-methanol solution for 30 min to quench endogenous peroxidase activity and incubated for 30 min in 1.5% normal blocking serum in PBS. Immunohistochemistry was performed using the ABC staining system. The sections were incubated with the primary antibody for 30 min at room temperature. The rabbit anti-nitrotyrosine polyclonal antibody (Chemicon International, Temecula, CA) was diluted 1:2 000 and the rabbit Cu,ZnSOD polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted 1:1 000. Slides were incubated with the secondary

antibody for 30 min and then, washed with three changes of PBS for 5 min each. The chromogen used was 3,3'-diaminobenzidine tetrahydrochloride. Finally, the sections were washed in deionized H₂O, counterstained with Harri's hematoxylin solution and dehydrated through ethanol (65% and 100%, twice each) and xylene (thrice). The intensity of immunohistochemistry staining was determined by semiquantitative analysis and was graded blinded according to the follo-wing scale: 0: absent, 1: weak, 2: moderate, and 3: intense.

Nitrotyrosine immunoprecipitation

Esophageal biopsies were sonicated in PBS containing 0.1 mmol/L phenylmethyl sulfonyl fluoride, 5 µg/mL aprotinin and 1 mmol/L sodium orthovanadate. Supernatants were collected and protein concentrations were determined using a protein assay based on the Bradford method (Protein Assay Dye Reagent, Bio Rad Laboratories, München, Germany). Solubilized proteins were precleared (3 h at room temperature) with 5 µL of protein Gagarose (Roche Applied Science, Mannheim, Germany). After a brief centrifugation, the supernatants were collected in order to determine Cu, ZnSOD and MnSOD expression and the presence of tyrosine-nitrated MnSOD (22 µg/lane). In order to purify those proteins containing tyrosine-nitrated residues, we performed immunoprecipitation using a monoclonal anti-nitrotyrosine antibody. For this purpose, the supernatants were incubated (1 h at room temperature) with 10 µL of monoclonal anti-nitrotyrosine antibody conjugated to protein A agarose (Alexis Biochemicals, Lausen, Switzerland). After incubation, beads were collected by brief centrifugation, and the supernatant was carefully pipetted off. Then the beads were washed thrice with PBS. After the last wash, the beads were resuspended in 25 µL of 1× Laemmli sample buffer and boiled for 5 min. The sample was briefly centrifuged to pellet the beads, and the supernatants were immediately subjected to electrophoresis in 12% SDS polyacrylamide gel^[17,18].

Western blot analysis

Western blot analysis of Cu,ZnSOD and MnSOD was performed. MnSOD was performed in both immunoprecipitated and non-immunoprecipitated tyrosine-nitrated protein fractions. After separation by SDS/PAGE, proteins were transferred electrophoretically (100 V, 1.5 h) to PDVF membranes (Bio Rad Laboratories, München, Germany), which were blocked (4 °C, overnight) with 5% blocking agent in 50 mmol/L Tris-HCl, pH 7.4/150 mmol/L NaCl/0.05% Tween 20 (TBST). For detection of MnSOD, blots were incubated (1 h, room temperature) with a rabbit polyclonal anti-MnSOD antibody (1:1 000 dilution) (Upstate,

Charlottesville, VA, USA). For detection of Cu,ZnSOD, blots were incubated (1 h, room temperature) with a rabbit polyclonal anti-Cu,ZnSOD antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After incubation with primary antibodies, membranes were washed in TBS/T, then the immunocomplexed membranes were probed (1 h, room temperature) with 1:3 000 dilution of peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK) in TBS with 0.1% of blocking agent. Probed membranes were washed in TBS/T and immunoreactive proteins were detected using enhanced chemiluminescence (ECL Western blotting analysis system, Amersham Biosciences).

Statistical analysis

Results from biochemical assays were expressed as the mean±SE. Values obtained from biopsies were compared between the groups by nonparametric methods using Kruskal-Wallis and Mann-Whitney tests. Descriptive statistics were used for immunohistochemical data, analyzing median, minimum, maximum and percentage. Significance between the intensity of immunohistochemistry staining and grade of esophagitis was analyzed using the Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics are shown in Table 1. Age and sex of patients and controls were homogenous across the different groups. Table 1 also includes the type of treatment received by patients 30 d before the endoscopic procedure.

Determination of reactive oxygen species

ROS were significantly increased in mucosal biopsy specimens obtained from patients with erosive esophagitis (grade II-IV) and Barrett's esophagus. Duodenal biopsies showed similar ROS levels than esophageal biopsies of control patients (Figure 1). The addition of exogenous SOD inhibited chemiluminescence in all groups, indicating that the increase in mucosal reactive species was due to the presence of superoxide anion (data not shown).

Determination of reactive nitrogen species

Peroxynitrite generation in the esophageal mucosa was investigated by measuring nitrotyrosine formation in esophageal biopsies by immunohistochemistry. Nitrotyrosine was detected in both the squamous and Barrett's epithelium. Nitrotyrosine staining was intense in Barrett's biopsies, weaker in esophagitis and absent/weak in normal esophageal mucosa (Figure 2 and Table 2).

Table 1 Characteristics of patients with different gastroesophageal reflux diseases that were included in the study

Group	n	Sex (M/F)	Mean age (yr) (Range)	Treatment ¹				
				H2-RA	PPI	Antacids	Other ²	No Rx
Control (non- GERD)	28	20/8	59 (20-81)	1	3	3	2	19
GERD symptoms	50	32/18	52 (18-80)	10	10	10	3	17
Erosive esophagitis	23	18/5	59 (25-90)	4	2	12	1	4
Barrett's esophagus	28	20/8	58 (21-88)	7	11	5	1	4

¹Treatment taken by patients within 30 d before endoscopy. ²Other includes prokinetic agents (cisapride, clebopride, cinitapride, domperidone).

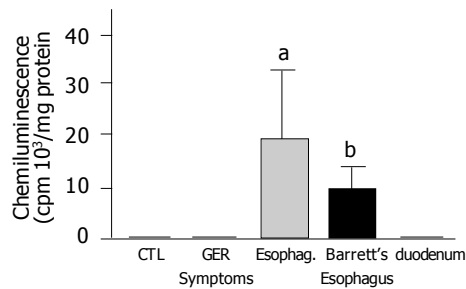


Figure 1 Anion superoxide generation measured by chemiluminescence in human esophageal biopsies with different grades of damage and in normal esophageal and duodenal epithelium. Chemiluminescence was significantly higher in patients with erosive esophagitis (grade II-IV) and Barrett's esophagus compared to control patients. ($n = 10-28$ ^a $P < 0.05$, ^b $P < 0.01$ vs normal esophageal mucosa).

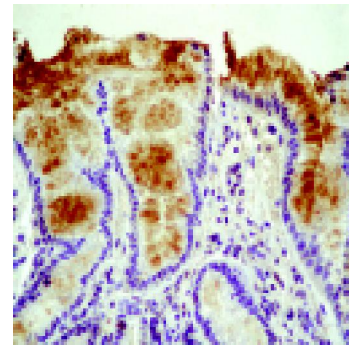


Figure 2 Microphotograph showing nitrotyrosine immunohistochemical staining in a patient with Barrett's esophagus. Nitrotyrosine presence was visualized using a specific antibody.

Table 2 Quantification of nitrotyrosine expression in Barrett's esophagus, esophagitis and control patients

Group	mean±SE	Median (range)	Intensity ¹ /Percent(%) ²	
Control	0.70 ± 0.15	1.00 (0.00-1.00)	0	30
			1	70
Esophagitis	1.27 ± 0.14	1.00 (1.00-2.00)	1	72.7
			2	27.3
Barrett's esophagus	2.41 ± 0.14 ^a	2.00 (2.00-3.00)	2	58.3
			3	41.7

^a $P < 0.05$ vs control ($n = 10-12$ cases per group). ¹The intensity of immunohistochemistry was graded blinded according to the following scale: 0: absent, 1: weak, 2: moderate and 3: intense. ²Percentage of samples according to degree of staining.

Determination of antioxidant status

SOD activity showed a significant decrease in both biopsies taken from patients with erosive esophagitis and Barrett's esophagus when compared to normal esophageal epithelium. The lowest SOD activity was found in biopsies with erosive esophagitis. SOD activity was significantly lower in duodenal mucosa than in normal esophagus samples (Figure 3A). GSH levels increased with esophageal mucosal damage. Significant differences were found between patients with gastroesophageal reflux as compared to normal esophagus. In duodenal mucosal specimens, values were comparable to those of Barrett's epithelium, which were significantly higher than in normal esophageal mucosa (Figure 3B).

Catalase activity was significantly higher in patients with

erosive esophagitis and Barrett's esophagus compared to normal squamous epithelium. Duodenal mucosa also had higher values of catalase activity than the squamous epithelium (Figure 3C).

SOD expression

To investigate the mechanisms leading to a decrease of total SOD activity in damaged esophageal mucosa, MnSOD and Cu,ZnSOD protein expression were evaluated by immunohistochemistry and/or Western blot analysis. Furthermore, nitration of MnSOD was evaluated by Western blot. By immunohistochemistry, semiquantitative evaluation revealed that Cu,ZnSOD was present in control subjects and patients with esophagitis and Barrett's esophagus, but its expression increased according to the severity of lesion, reaching the highest level in mucosal specimens from patients with Barrett's esophagus (Table 3 and Figure 4). In addition, Western blot analysis of Cu,ZnSOD expression in esophageal biopsies (Figure 5A) demonstrated an increase of Cu,ZnSOD expression in Barrett's esophagus biopsies (lanes 3-5) compared to control subjects (lanes 1 and 2) and patients with esophagitis (lanes 6 and 7).

The expression of MnSOD protein was examined by Western blot. Protein extracts from biopsies were immunoblotted with a polyclonal antibody anti-MnSOD. A band at 24 ku corresponding to monomeric MnSOD was observed in all groups evaluated. Increased staining for MnSOD was observed in Barrett's esophagus and esophagitis compared to control

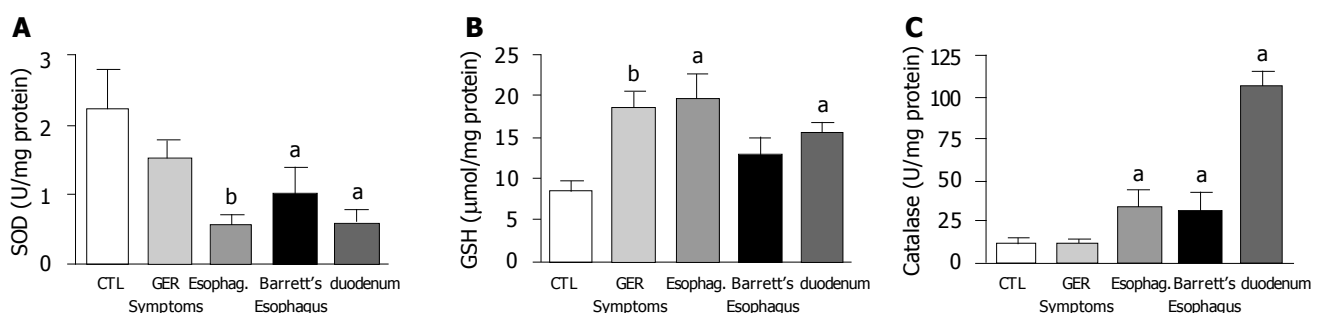


Figure 3 A: SOD activity decreased with damage. Significant differences were found between patients with esophagitis (grade II-IV) or Barrett's esophagus and the control group (normal esophageal mucosa) ($n = 12-27$); B: GSH levels were significantly increased in biopsies from patients with GERD symptoms, esophagitis and duodenal mucosa compared to biopsy samples of normal esophageal mucosa

($n = 10-12$); C: catalase activity was significantly increased in biopsies from both esophagitis and Barrett's esophagus patients when compared to normal esophageal mucosa. Duodenal catalase activity was also high ($n = 10-12$, ^a $P < 0.05$, ^b $P < 0.01$ vs normal esophageal mucosa).

patients (Figure 5B). Because MnSOD is a sensitive target of tyrosine nitration, anti-MnSOD Western analysis of nitrotyrosine immunoprecipitates was performed. Immunoblotting of nitrated protein and subsequent incubation with anti-MnSOD is present in all groups evaluated; however, the amount of nitrated protein increases in patients with esophagitis and Barrett's esophagus compared to normal subjects (Figure 5C).

Table 3 Quantification of Cu,ZnSOD expression in Barrett's esophagus, esophagitis and control patients

Group	mean±SE	Median (range)	Intensity ¹ /	Percent (%) ²
Control	1.30±0.15	1.00 (1.00-2.00)	1	70
			2	30
Esophagitis	2.00±0.27	2.00 (1.00-3.00)	1	36.4
			2	27.3
			3	36.4
Barrett's esophagus	2.5 ±0.15 ^a	2.50 (2.00-3.00)	2	50
			3	50

^a $P < 0.05$ vs control. ($n = 10$ -12 cases per group). ¹The intensity of immunohistochemistry was graded blinded according to the following scale: 0: absent, 1: weak, 2: moderate and 3: intense. ²Percentage of samples according to degree of staining.

DISCUSSION

Oxidative stress is defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative damage^[19]. In the present study we have shown that esophageal mucosal damage in patients with GERD is associated with increased oxidative stress characterized by enhanced superoxide anion and peroxynitrite generation, which is accompanied by a clear pattern of antioxidant activity characterized by a profound decrease in SOD activity and an increase in GSH content and catalase activity.

In our work, we found that the main oxidant product in reflux esophagitis was superoxide anion. There was also a positive correlation between the degree of mucosal damage and levels of superoxide anion, reaching the highest values in patients with erosive esophagitis and Barrett's esophagus. Generation of the superoxide anion as the main free radical involved in mucosal damage had been established in different experimental models of acute and chronic esophagitis^[2,3,5]. The involvement of free radicals in human esophagitis has been less explored. Olyaei^[7] and Westcher^[8], who measured

mucosal ROS concentrations by LCEL (luminol enhanced concentrations), found increased mucosal production of chemiluminescence levels in patients with esophagitis and Barrett's esophagus.

Superoxide anion, although directly promoting inflammation, also has other interactions that might perpetuate the inflammatory process. The superoxide anion can be scavenged by nitric oxide (NO) and to form the powerful oxidant peroxynitrite anion (ONOO⁻), which would amplify the inflammatory process. Then, under pathological conditions, NO competes with endogenous SOD for superoxide anion^[20]. Therefore, the next logical step in this study was to determine the presence of peroxynitrite in esophageal mucosa. This study has shown that peroxynitrite anion is formed in esophageal mucosa and that this radical seems to be particularly involved in the pathogenesis of Barrett's esophagus. Although this aspect had not been examined in humans, these findings agree with those reported in an experimental model of esophagitis where peroxynitrite formation is a common event in the presence of excess of superoxide anion radicals^[3]. Furthermore, in a rat model for esophageal adenocarcinoma associated with Barrett's esophagus, positive nitrotyrosine staining was found, suggesting that peroxynitrite generation may contribute to the progression of esophageal cancer^[21]. In conclusion, both ROS and reactive nitrogen species play a significant role in reflux esophagitis and Barrett's esophagus.

When oxidative stress occurs, a defense system promotes the regulation and expression of several antioxidant enzymes such as SOD, catalase and glutathione peroxidase. In humans, there are three forms of SOD: cytosolic Cu,ZnSOD, mitochondrial MnSOD, and extracellular SOD (ECSOD). It is well known that oxidative stress increases SOD expression^[22]. In our study, Cu,ZnSOD and MnSOD were present in both control patients and patients with esophagitis or Barrett's esophagus and its expression increased according to the severity of lesion, reaching the highest level in Barrett's esophagus.

However, although an increase in SOD expression was observed, the activity of the total SOD was found to be dramatically decreased in esophagitis and Barrett's esophagus compared to normal esophageal epithelium. In experimental esophagitis, superoxide anion was involved in the induction of esophagitis and SOD activity levels were negligible where mucosal damage was severe^[3]. On the other hand, in the low-

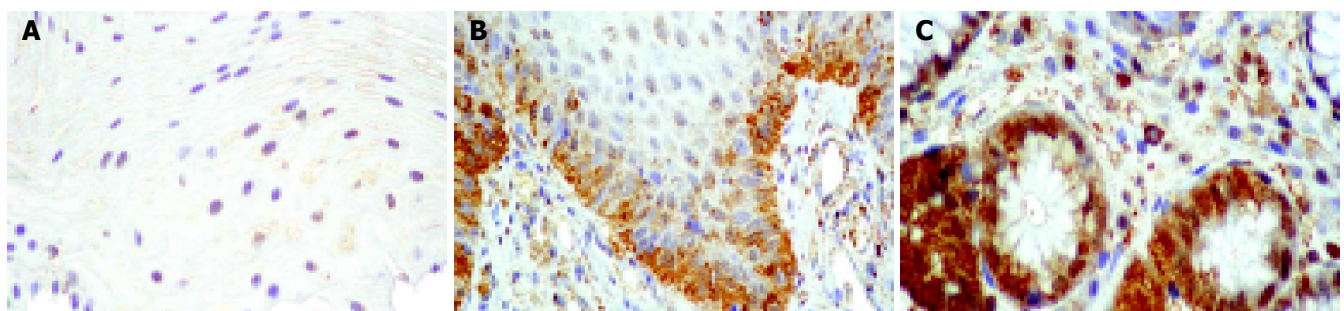


Figure 4 Microphotograph of immunohistochemical Cu,ZnSOD staining. Cu,ZnSOD was present both in control subjects (normal esophageal mucosa) (A) and in

patients with esophagitis (B) or Barrett's esophagus (C), but its expression increased according to the severity of lesion, reaching the highest level in Barrett esophagus.

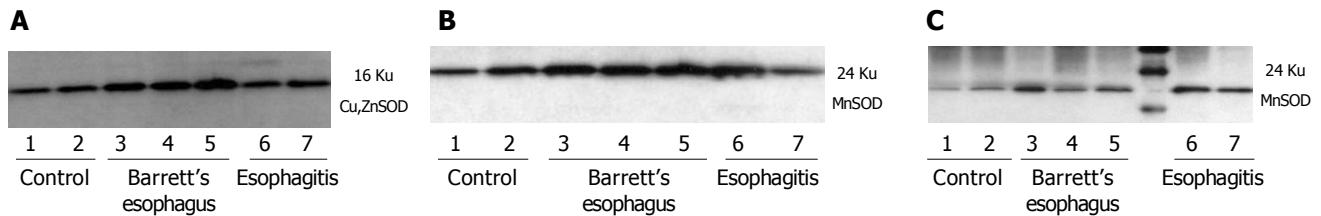


Figure 5 Representative Western analysis of tissue lysates (22 µg/lane) from control subjects (normal esophageal mucosa) and patients with esophagitis and Barrett's esophagus. **A:** Western blot analysis of Cu,ZnSOD expression in esophageal biopsies demonstrated an increase of Cu,ZnSOD expression in Barrett's esophagus biopsies (lanes 3-5) compared to control subjects (lanes 1, 2) and patients with esophagitis (lanes 6,7); **B:** A band at 24 ku corresponding

to monomeric MnSOD was observed in all groups evaluated. Increased staining for MnSOD was observed in Barrett's esophagus (lanes 3-5) and esophagitis (lanes 6,7) compared to control patients (lanes 1,2); **C:** Immunoprecipitated tyrosine-nitrated proteins and subsequent incubation with anti-MnSOD demonstrated that MnSOD nitrated is increased in patients with esophagitis (lanes 6,7) and Barrett's esophagus (lanes 3-5) compared to normal subjects (lanes 1,2).

grade model, where superoxide anion was not a key factor, no differences in endogenous SOD activity were observed^[4]. In human esophagitis, Wetscher *et al*^[8], showed that SOD decreased with the severity of esophagitis, although in patients with Barrett's esophagus and mild associated esophagitis high levels of esophageal mucosal SOD were found. In clear contrast with those findings, Sihvo *et al*^[9], found no differences in SOD activity in patients with esophagitis and Barrett's esophagus when compared to control patients (normal esophagus).

In our study, we found discrepancy between the increased expression for Cu,ZnSOD or MnSOD and the reduced activity levels (total SOD), which could be attributable to endogenous inhibitors of the SOD enzymes. It is known that peroxynitrites are potent oxidants and nitrating agents that cause depletion of cellular antioxidant defenses and inactivation of enzymes (by nitration of tyrosine residues) that may adversely affect their function^[23]. Human MnSOD contains nine tyrosine residues^[24]. Tyr34, which is present in the active site, appeared to be the most susceptible amino acid to peroxynitrite-mediated nitration^[24,25]. In this study, incubation of nitrotyrosine immunoprecipitates with a polyclonal antibody against MnSOD revealed that nitrated MnSOD was present and that its expression increased in patients with esophagitis compared to control patients. The nitration of MnSOD can partially contribute to a loss of enzymatic activity, since it has been described that both nitration and oxidation of critical tyrosine residues of MnSOD are required for complete inactivation of the enzyme^[26]. Although human Cu,ZnSOD has no tyrosine residues in its amino acid sequence, it is probable that other amino acids (such as cysteine, tryptophan) in these enzymes are subject to modification by peroxynitrites^[24,27]. The reaction of human Cu,ZnSOD with peroxynitrite may cause partial inactivation of the enzyme activity by nitration and/or oxidation of the single tryptophan 32^[28]. Other sources of enzymatic inactivation may also not be excluded since SOD inactivation by hydrogen peroxide^[29] and by peroxyl radicals^[30] has been described.

Therefore, the net effect of reduced activity of SOD is the increase of superoxide anion concentration in the cytosol and the toxic potential of superoxide-derived oxygen radicals such as peroxynitrite, aggravating esophageal damage. In addition, we have found that this process is accompanied by additional changes in the antioxidant system which may be regarded as an adaptive response to mucosal damage in

an effort to neutralize the oxidative load and it is characterized by an increase of GSH levels and catalase activity. In this study, mucosal GSH levels increased in patients with esophagitis, but they were not in the mucosa of Barrett's esophagus. Studies regarding the role of endogenous GSH in reflux esophagitis are contradictory. Wetscher^[6] and Katada^[31] found an increase of esophageal tissue GSH levels in a model of acute damage in rats, whereas inhibition of esophageal damage decreased the production of GSH. These authors suggest that the increase of GSH production is a marker of prolonged oxidative stress. However, Lee *et al*^[32], observed depletions of reduced glutathione in experimentally induced reflux esophagitis. In contrast to our findings in humans, it has been reported that GSH content was lower both in Barrett's esophagus and esophagitis when compared to patients without symptoms or endoscopic evidence of esophageal damage^[9] and when compared to normal adjacent squamous epithelium^[33,34].

The GSH-dependent system is a scavenger of great importance, since it acts at different levels of the antioxidant defense system: it is a scavenger for hydrogen peroxide, peroxides and superoxide anion and it also helps to maintain the sulfhydryl groups of proteins in the reduced form for their normal function^[35]. Furthermore, GSH is a substrate for the antioxidant enzyme glutathione peroxidase, which has activity as peroxynitrite reductase and therefore GSH can scavenge peroxynitrite with the formation of oxidized glutathione which is converted back to GSH. The increase of GSH content in esophageal inflammation, which was found in our study, may represent an adaptive response both to the oxidant stress in order to neutralize the oxidative load (superoxide anion and peroxynitrites), and to protect against peroxynitrite-mediated protein oxidation. The absence of increase of GSH levels in the mucosa of Barrett's esophagus may be a consequence of the presence of the metaplastic epithelium which shows higher SOD activity and/or loss of that particular adaptive response over time.

Although other free radicals such as hydrogen peroxide have not been investigated in the present study, we cannot exclude a potential role in the pathogenesis of esophageal oxidative damage^[2]. Experimental studies carried out in a model of acute esophagitis in rabbits showed that hydrogen peroxide generation was not detected in mucosal cells isolated after mucosal damage. In addition, the exogenous administration of catalase, a hydrogen peroxide scavenger, showed a trend

to improvement, but it was much less effective than SOD in preventing or reducing the mucosal damage. In this work, the highest values of catalase activity were found in duodenal mucosa. However, as compared to normal esophagus, catalase activity is increased in esophagitis and Barrett's esophagus and might play a role in the acquisition of tolerance to oxidative stress in an adaptive response of cells^[10].

In conclusion, a decrease in SOD antioxidant activity may contribute to the development of esophageal damage and Barrett's esophagus in GERD patients, leading to an increase of mucosal generation of superoxide anion and peroxynitrite radicals and the subsequent cell damage. Glutathione levels and catalase activity increase in response to this oxidative overload. We propose that administration of SOD may be a therapeutic target in the treatment of reflux esophagitis and complications as Barrett's esophagus.

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Expression of lysosome-associated protein transmembrane 4B-35 in cancer and its correlation with the differentiation status of hepatocellular carcinoma

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Abstract

AIM: To produce high-quality polyclonal antibody to lysosome-associated protein transmembrane 4B-35 and to identify LAPTMB-35 expression in cancer tissues and its correlation with differentiation status of hepatocellular carcinoma (HCC).

METHODS: The 297 bp 5' end of *LAPTMB* cDNA was obtained by PCR and inserted into prokaryotic expression vector pGEX-KG. Then the recombinant pGEX-KG-N₁₋₉₉ was transformed into *E. coli* JM109 to express GST-fusion protein. The fusion protein was purified by glutathione sepharose™ 4B agarose. The purified GST-LAPTMB-N₁₋₉₉ was characterized by SDS-PAGE, and used to immunize rabbits. The titer and specificity of antisera were detected by ELISA and Western blot, respectively. The correlation between the expression levels of LAPTMB-35 and the differentiation status of HCC was analyzed via Western blot. The expression of LAPTMB-35 in HCC and other six cancer tissues was investigated via tissue chip and immunohistochemical analysis.

RESULTS: About 6.2 mg of pure GST-LAPTMB-N₁₋₉₉ was isolated from 1 L of bacteria. The GST-LAPTMB-N₁₋₉₉ produced high titer antisera in rabbits and showed good immunity. Western blot showed specific reactions for the antibody to the LAPTMB-35 in the total proteins from HCC tissues and BEL-7402 cells, also to the fusion protein purified or in the transformed bacteria. LAPTMB-35 was remarkably expressed in several cancers, such as HCC, breast cancer, gastric carcinoma, lung cancer, and colon carcinoma, but not commonly expressed in esophageal cancer and rectum carcinoma. Notably, the expression levels of LAPTMB-35 were significantly and inversely correlated

to the differentiation of HCCs in a 20 case analysis.

CONCLUSION: Specific polyclonal antibody (LAPTMB-N₁₋₉₉-pAb) to LAPTMB-35 was produced. It identified the expression of LAPTMB-35 in some cancer tissues originated from single layer cuboidal and columnar epithelial cells and firmly demonstrated that the expression of LAPTMB-35 in HCC was inversely correlated with the differentiation of HCC.

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Key words: LAPTMB; LAPTMB-N₁₋₉₉-pAb; HCC; Tissue microarray

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INTRODUCTION

Hepatocellular carcinoma (HCC) remains one of the commonest internal malignancies of mankind. It ranks fifth in frequency in the world with an estimated number of 0.5-1 million cases per year, most of which occur in sub-Saharan Africa and south-east Asia including China^[1-3]. The vast majority can be attributed to chronic hepatitis B virus (HBV) infection and there has been almost no change in incidence. It has also become evident that the number of HCC is increasing in low to middle-incidence countries such as USA^[4-6], France^[7,8], UK^[9,10] and Japan^[11,12]. The rising number of cases in these countries is most likely to be due to chronic hepatitis C virus (HCV) infection^[4,6,12].

LAPTMB, which was first cloned by our research group as a novel oncogene candidate, is highly expressed in vast majority of HCCs^[13,14]. BLAST program analysis showed that the LAPTMB was mapped to chromosome 8q22.1, the gain region in HCC shown by CGH analysis. The ORF of LAPTMB contains two ATGs, indicating that this gene may encode two putative proteins with 35 kDa (317 aa) and 24 kDa (226 aa)^[14,15], respectively. The former study confirmed that the LAPTMB-35 was translated from the whole ORF and initiated from the first ATG, whereas the LAPTMB-24 was translated from the second ATG^[14]. The integral membrane protein characteristics were indicated by computer

analysis and demonstrated by experiments. The *LAPTM4B* gene was widely expressed in normal human tissues shown by Northern blot^[14]. Its expression was high in heart, skeletal muscle and testis; moderate in ovary, kidney; and pancreas; low in liver, spleen, and thymus; but lowest in lung and peripheral leukocytes. It was remarkably overexpressed (48 over 55 cases) in HCC when compared with PNL. Furthermore, the expression levels of *LAPTM4B* mRNA were significantly related to the differentiation status of HCCs: the highest in poorly-differentiated HCCs, higher in moderately-differentiated HCCs, and low in well-differentiated HCCs^[14].

The biological effects of LAPTM4B were studied by transient and stable transfection. The result showed that cell proliferation was promoted via LAPTM4B stable transfection of both mouse NIH3T3 cells^[16] and human HLE cells (manuscripts in preparation). Also, the LAPTM4B transfected NIH3T3 cells were tumorigenic when the transfectants were inoculated into NIH mice. Coimmunoprecipitation assay indicated that LAPTM4B interacted with integrin- $\alpha 6\beta 1$ in BEL-7402 cells, which were enhanced by LN-1^[15], and might play an important role in the integrin- $\alpha 6$ mediating signal transduction pathways. It was also found that the sequences of 91 amino acids at the N-terminus of LAPTM4B-35 were essential for its functions on cell survival and growth, which was revealed via transient transfection of plasmids containing full length and truncated sequences (273 bp) at the 5' end of LAPTM4B ORF into HLE cells^[14]. After 2-3 wk of G418 selection, colonies in pCDNA3-BE (containing truncated ORF) transfected cells were almost completely disappeared, whereas the pCDNA3-AE (containing full ORF) transfected cells formed lots of colonies^[14]. These results indicate that LAPTM4B-35 plays an important role in the regulation of cell survival, proliferation, and may involve in carcinogenesis.

It was evidenced that the overexpression of LAPTM4B-35 promoted malignant transformation of some cell lines, including accelerated proliferation, migration and invasion of cells, and activated some protooncogenes, including immediate early genes, such as c-myc, c-fos and c-jun (manuscripts preparation).

To investigate the function and expression of LAPTM4B-35 in HCC and HCC cell lines, specific antibody to LAPTM4B-35, but not LAPTM4B-24, the 297 bp at 5' end of LAPTM4B cDNA encoding LAPTM4B-N₁₋₉₉ was cloned into donor vector pGEX-KG^[17-19] and the recombinant plasmid was transformed into competent *E. coli* cells JM109. The GST-LAPTM4B-N₁₋₉₉ fusion protein was produced in JM109 cells after induced with IPTG, and purified using glutathione sepharoseTM 4B agarose^[20,21]. After rabbits were immunized, specific polyclonal antibodies, LAPTM4B-N₁₋₉₉-pAb, against the N-terminus of LAPTM4B-35 were obtained. With this antibody, the expressions of LAPTM4B-35 in HCC and several cancer tissues were performed via Western blot and TMA^[21-24], respectively. The correlation between expression levels of LAPTM4B-35 and HCC differentiation status were analyzed.

MATERIALS AND METHODS

Subjects

A total of 20 pairs of specimens were obtained from patients with HCC (aged from 35 to 70 years) who underwent

hepatectomy at the Peking Union Medical College Hospital (the West Hospital) in Beijing. Histopathological analyses were independently performed by pathologists. Tumor differentiation was graded as I-III, according to the Edmondson Grading System^[25,26]. Specimens were frozen immediately after surgical resection and stored in liquid nitrogen.

Chemicals

Molecular biological enzymes were purchased from Sangon Biotechnology Ltd. Peroxidase-conjugated goat anti-rabbit IgG (H+L) was purchased from Zhongshan Biotechnology Ltd. Glutathione-sepharose 4B agarose was purchased from Pharmacia Biotech Ltd.

Tissue microarray (TMA)

A TMA slide was purchased from Chengdu Phargentech Ltd. It contained 60 species of tissues, including two normal esophageal epithelial and eight esophageal cancer tissues, two normal mammary gland and eight breast cancer tissues, two normal lung and eight lung cancer tissues, two normal gastric epithelial and eight gastric carcinoma tissues, two normal colonic epithelial and eight colon carcinoma tissues, two normal rectal epithelial and eight rectum carcinoma tissues. All specimens were embedded in paraffin.

Cloning of LAPTM4B cDNA encoding LAPTM4B-N₁₋₉₈

To clone the 5' end of LAPTM4B ORF, PCR method was used. Based on the published *LAPTM4B* gene sequence^[14], two oligonucleotide primers (P1: 5' GGGATCCGCCACCA-TGACGTCACGGACTCGG 3'; P2: 5' GCGAAGCTTC-GTCCAGGGCGCGACCATC 3') were synthesized (Sangon Biotechnology Ltd). P1 was extended to the 5' end containing recognition sequences for endonuclease *Bam*HI (underlined). P2 was extended to the 5' end containing recognition sequences for endonuclease *Hind*III (underlined). PCR amplification was performed for 30 cycles at 94 °C for 30 s, at 55 °C for 30 s and at 72 °C for 50 s.

Expression vector construction

The pGEX-KG vector was digested with *Bam*HI and *Hind*III. cDNA fragment at the 5' end of *LAPTM4B* ORF was subcloned into the corresponding site of pGEX-KG to obtain a plasmid pGEX-KG-N₁₋₉₉. Then the recombinant plasmid was transformed into the *E. coli* strain JM109. The transformed colonies containing the cloned cDNA were screened by restriction enzyme analysis and DNA sequencing.

Expression and purification of GST-LAPTM4B-N₁₋₉₉ in *E. coli*

An overnight LB culture of *E. coli* containing pGEX-KG-N₁₋₉₉ was inoculated at a dilution of 1:100, and incubated at 37 °C with shaking. When the absorbance at 600 nm reached 0.6, GST-LAPTM4B-N₁₋₉₉ expression was induced by the addition of 0.5 mmol/L IPTG. Further 4 h for growing, the *E. coli* strains were harvested by spinning at 4 000 r/min for 15 min at 4 °C. The pellet was resuspended in PBS containing 0.05% Tween-20 of the original volume and lysed by sonication on ice, and then centrifuged at 10 000 g for 20 min at 4 °C. The supernatant was passed through glutathione-sepharose 4 B beads according to the manufacturer's instructions. SDS-PAGE was performed to analyze the purified protein from the infected cells.

Immunogenicity assay on recombinant protein

Two rabbits were used to generate polyclonal antibodies against the fusion protein. Each rabbit was injected with 1 mg purified fusion protein in complete adjuvant in the initial injection and with uncompleted adjuvant for the following injection. Before injection, the rabbit blood was collected for producing pre-immune serum. The titer of antisera was measured by ELISA.

Western blot

Proteins in cell lysates and tissues were fractionated by 10% SDS-PAGE and followed by electrotransferring onto nitrocellulose filters (Bio-Rad). The filters were blocked at 4 °C overnight with a blocking buffer (pH 7.6) containing 5% non-fat dry milk. Then the filters were incubated with indicated antibody (1:300) for 2 h at room temperature. After being washed with TBST (pH 6.0), the filters were incubated at room temperature for 1 h with horseradish peroxidase conjugated goat anti-rabbit IgG at 1:2 000 dilution in TBS-0.5% non-fat dry milk. Immunoreactive bands were visualized using ECL detection reagents (Santa Cruz).

Immunohistochemistry

HCC tissue and TMA slide were fixed in 4% PFA in PBS for 10 min, washed twice with PBS, and incubated in 3% H₂O₂ to eliminate the endogenous peroxidase activities. The tissues were then incubated either at room temperature for 1 h or at 4 °C overnight with 1% goat serum in PBS to block the nonspecific binding of antibodies. The slides were further incubated sequentially with polyclonal antibody and goat anti-mouse IgG conjugated to horseradish peroxidase. The pre-immune serum was used as a control. Color developments were performed by incubation with 3,3'-diaminobenzidine tetrahydrochloride in 0.03% H₂O₂ and 50 mmol/L Tris-HCl, pH 7.4. Hematoxylin was used for counterstaining.

RESULTS

Expression of fusion protein and protein purification

Confirmed by restriction endonuclease digestion and DNA sequencing, pGEX-KG-N₁₋₉₉ plasmid was successfully constructed and GST-LAPTM4B-N₁₋₉₉ was expressed in *E. coli* JM109 by IPTG induction. A 36 kDa fusion protein was stained in high intensity by 10% SDS-PAGE (Figure 1). This fusion protein was majored as a soluble pattern.

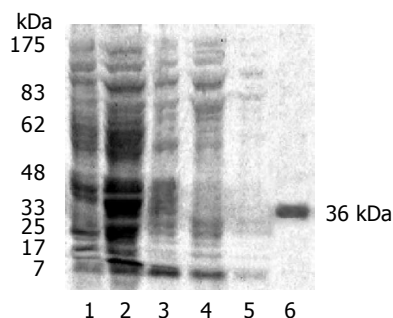


Figure 1 Purification of GST-LAPTM4B-N₁₋₉₉. 1: Uninduced pGEX-KG-N₁₋₉₉/JM109; 2: induced pGEX-KG-N₁₋₉₉/JM109; 3: supernatant of induced pGEX-KG-N₁₋₉₉/JM109; 4: unbinding fragment of glutathione sepharose™ 4B column; 5: eluted by PBS; 6: purified fusion protein GST-LAPTM4B-N₁₋₉₉.

Titer and specification of LAPTM4B-N₁₋₉₉-pAb

After being immunized thrice with purified GST-LAPTM4B-N₁₋₉₉, two rabbits both generated antisera at a titer of 5×10⁴, measured by ELISA. Western blot analysis showed that there was a specific Ag-Ab binding band at 36 kDa in the unpurified and purified GST-LAPTM4B-N₁₋₉₉ (Figure 2A, lanes 1 and 2). The BEL-7402 cell lysate was also identified by LAPTM4B-N₁₋₉₉-pAb (Figure 2B, lane 4).

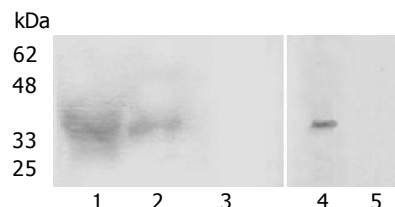


Figure 2 Western blot analysis of the specificity of LAPTM4B-N₁₋₉₉-pAb. 1: pGEX-KG-N₁₋₉₉/JM109 induced with IPTG (36 ku); 2: Purified infused protein (36 ku); 3: pGEX-KG-N₁₋₉₉/JM109 induced without IPTG; 4: BEL-7402 cell lysis, Western blot by LAPTM4B-N₁₋₉₉-pAb; 5: BEL-7402 cell lysis, Western blot by preimmunized antisera.

Expression analysis of LAPTM4B-35 in HCC

The expression levels of LAPTM4B-35 were significantly related to the differentiation status of HCCs: highest in poorly-differentiated HCCs (grade III, 12/20), high in moderately-differentiated HCCs (grade II, 4/20), and low in well-differentiated HCCs (grade I, 4/20) (Figure 3).

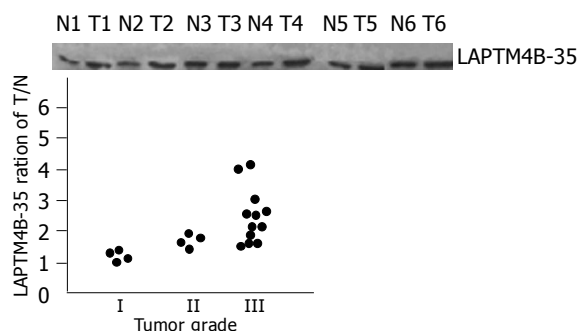


Figure 3 Analysis of the expression of LAPTM4B-35 with the differentiation status of HCCs. (a) Western blot analysis of LAPTM4B-35 in HCC tumor tissues (T), paired noncancerous liver tissues (N). (Tumor grade I: T3, T5; grade II: T1, T6; grade III: T2, T4) (b) Correlation between LAPTM4B-35 protein level and tumor grade. Paired tumor (T) vs adjacent noncancerous liver tissues (N) from 20 HCC patients were compared for their LAPTM4B expression by Western blot. Each spot in the figure represents the ratio (T/N) of the LAPTM4B-35 expression (tumor vs adjacent noncancerous liver tissue) from one patient ($P < 0.05$).

Immunohistochemical analysis of LAPTM4B-35 on HCC tissue and TMA

Immunohistochemical analysis was undertaken to characterize the expression of LAPTM4B-35 in HCC and TMA. In HCC (Figure 4A), positive brown signals were seen only in HCC cells and negative signals in the interstitial tissue of liver. The result was well coincident with the mRNA detection via in situ hybridization (Figure 4C)^[14]. Moreover, the expression of LAPTM4B-35 was also analyzed via TMA in six types of cancer tissues: esophageal carcinoma, breast cancer, pulmonary carcinoma, gastric carcinoma, colon carcinoma,

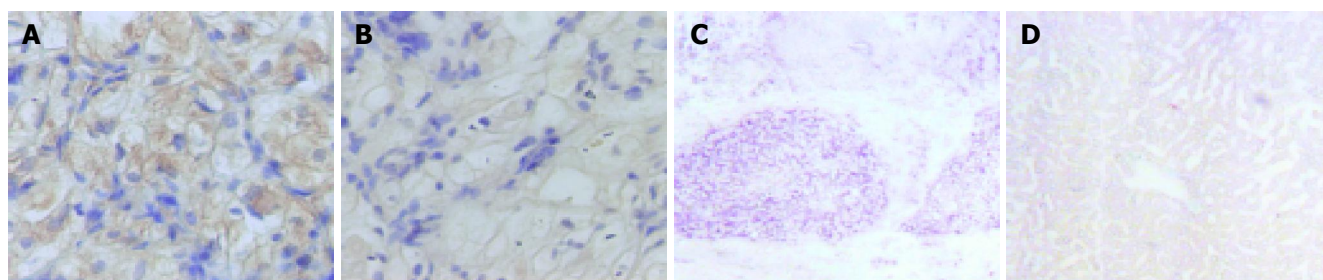


Figure 4 Analysis of LAPTM4B-35 and LAPTM4B mRNA expression by ISH in HCCs. **A:** immunized antisera (20 $\times$); **B:** preimmunized antisera (20 $\times$);

C: LAPTM4B mRNA expression in HCCs (20 $\times$); **D:** paired noncancerous tissue (20 $\times$).

and rectum carcinoma. There were no significant LAPTM4B-35 expressions in six counterparts of normal tissues but obvious high and weak expression accounted for the majority of breast cancers and gastric carcinomas. Positive

staining could only be observed in minority of pulmonary carcinomas and colon carcinomas and almost negative staining in all of esophageal carcinomas and rectum carcinomas tested (Figure 5).

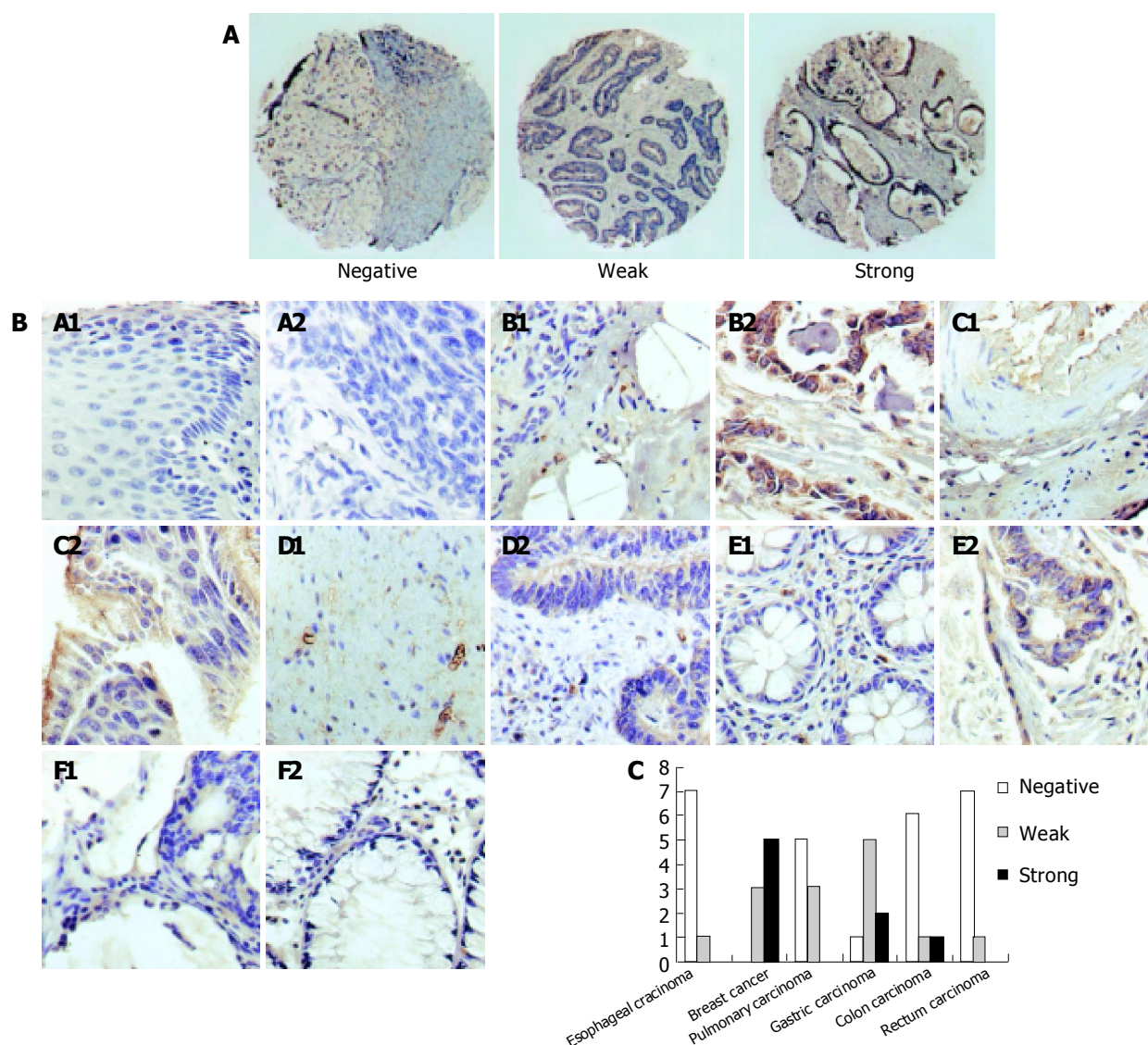


Figure 5 Analysis of LAPTM4B-35 via TMA. **A:** Scores assigned to each tissue spot according to three different staining coverages. Negative represents the complete negative staining. Weak represents scores assigned to tissue disks with borderline and partial positive staining. The complete positive staining was designated as strong; **B:** Representative elements of a TMA stained with LAPTM4B-N₁₋₉₉-pAb. Magnification $\times 200$. Normal tissue stained with LAPTM4B-

N₁₋₉₉-pAb (A1: esophageal mucous; B1: mammary gland tissue; C1: normal lung tissue; D1: gastric mucous; E1: colonic mucous; F1: rectal mucous). Tumor tissue stained with LAPTM4B-N₁₋₉₉-pAb (A2: esophageal carcinoma; B2: breast cancer; C2: pulmonary carcinoma; D2: gastric carcinoma; E2: colon carcinoma; F2: rectal carcinoma.); **C:** Histogram of LAPTM4B expression assessed using 6 TMAs.

DISCUSSION

In our previous work, we have produced a polyclonal antibody: LAPTM4B-EC2-pAb, which was prepared by immunization with KLH-conjugated 10-peptides whose sequences are localized at the second extracellular loop between the third and fourth transmembrane regions. By Western blot, expressions of LAPTM4B-35 and LAPTM4B-24 were identified in HCC, PNL and NL tissues with LAPTM4B-EC2-pAb, indicating that LAPTM4B-35 may initiate from the first ATG, whereas LAPTM4B-24 is translated from the second ATG. Notably, the expression levels of LAPTM4B-35 were primarily shown by Western blot with LAPTM4B-EC2-pAb to correlate with the differentiation status of 12 cases of HCC tissues, but the expression levels of LAPTM4B-24 did not relate to the differentiation status of HCC tissues.

To further clarify the correlation between upregulation of LAPTM4B-35 and differentiation of HCC and the function of LAPTM4B-35, we generated a specific antibody: LAPTM4B-N₁₋₉₉-pAb, against LAPTM4B-35 but not against LAPTM4B-24. Applying this antibody, it was firmly demonstrated that the expression of LAPTM4B-35 protein was inversely related with the status of differentiation status of HCC, suggesting its importance in carcinogenesis and progress of HCC as well as a possibility of being a criterion of HCC differentiation and pathological grading. The expression levels of LAPTM4B-35 were not found to correlate with the AFP levels of sera.

Using LAPTM4B-N₁₋₉₉-pAb, the expression of LAPTM4B-35 in some cancers originated from single layer cuboidal and columnar epithelia, such as HCC, breast cancer, gastric cancer and pulmonary cancer. We have previously reported that the high metastatic cell lines, such as the giant cell pulmonary carcinoma and prostate carcinoma cell lines highly expressed LAPTM4B-35. However, cancers originated from stratified epithelia, such as esophageal carcinoma and rectum carcinoma, did not practically express LAPTM4B-35. Malignant tumors from mesenchymal cells did not express LAPTM4B-35 either. The result of TMA gave us a clue that LAPTM4B-35 might also play an important role in breast cancer, gastric cancer or pulmonary carcinoma.

In conclusion, specific polyclonal antibody to the cytoplasmic N-terminal tail of LAPTM4B-35 was obtained and used for Western blot and immunohistochemistry. The expression of LAPTM4B-35 is associated with the differentiation status of HCC. In addition, the expression of LAPTM4B-35 in other cancer tissues may pave the way for further study on the function of N-terminal of LAPTM4B-35 for the development of tumors and their clinical diagnosis.

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• LIVER CANCER •

Expression and clinical significance of TAp73 α , p53, PCNA and apoptosis in hepatocellular carcinoma

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Abstract

AIM: To study the prognostic role of TAp73 α , p53, proliferating cell nuclear antigen (PCNA) and apoptosis in patients with hepatocellular carcinoma (HCC) after surgical tumor ablation.

METHODS: Forty-seven human resected HCC tissues and 42 adjacent non-cancerous tissues were studied with 10 normal liver tissues as control group. TAp73 α , p53, and PCNA were detected with Elivision immunohistochemistry. Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick-end labeling (TUNEL) method was used to detect the apoptosis cells. All clinical and pathological materials were analyzed by SPSS10.0 statistical package.

RESULTS: TAp73 α overexpressed in HCC tissues (36.2%) when compared with adjacent non-cancerous tissues (2.38%, $P < 0.005$) and normal liver tissues (0, $P < 0.01$). Mutant type p53 (mt-p53) overexpressed in HCC tissues (38.3%) when contracted with adjacent non-cancerous tissues (16.7%, $P < 0.05$) and normal liver tissues (0, $P < 0.01$). Proliferation index (PI) level in HCC tissues was significantly higher than that in adjacent non-cancerous tissues ($30.34\% \pm 4.46\%$ vs $27.88\% \pm 5.89\%$, t , $P = 0.028$). Apoptosis index (AI) level in HCC tissues was higher than that in adjacent non-cancerous tissues ($8.62\% \pm 2.28\%$ vs $7.38\% \pm 2.61\%$, t , $P = 0.019$). Expression of TAp73 α was associated with lymph node metastasis and mt-p53, with $r = 0.407$ and 0.265 , respectively. Expression of mt-p53 was associated with Edmondson's stage and AFP, with $r = 0.295$ and -0.357 , respectively. In Kaplan-Meier univariate analysis, TAp73 α , AFP, TNM stage, portal vein invasion, liver membrane invasion and HBsAg correlated with prognosis (log rank, $P = 0.039, 0.012, 0.002, 0.000,$

$0.014, 0.007$, respectively). Multivariate Cox regression analysis showed that TAp73 α , AFP, TNM stage, portal vein invasion, liver membrane invasion and age were independent factors of prognosis.

CONCLUSION: These results suggest that TAp73 α can be used as a prognostic indicator of patients with HCC undergoing surgical tumor ablation. AFP, TNM, portal vein invasion, liver membrane invasion and age also have a potency of predicting the prognosis of HCC.

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Key words: HCC; TAp73 α ; PCNA; Apoptosis; p53; Prognosis; TUNEL

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INTRODUCTION

Primary liver cancer (PLC) is one of the most malignant tumors in China. It is often in the late stage when diagnosed, with a poor prognosis even through positive treatments^[1,2]. Hepatocellular carcinoma (HCC) is the main pathological type of PLC, accounting for about 90% of all PLCs. Recently, the incidence of HCC has been found to be increasing particularly in males in countries such as Japan, Italy, France, Switzerland, UK and the USA^[3,4]. It is urgent to find some biomarkers to predict the biological character of HCC. Although many biomarkers have been tried, such as AFP mRNA, circulating VEGF and PD-ECGF, human macrophage metalloelastase gene^[5], p27^[6], p53 mutation^[7], expression of p73^[8], telomerase activity^[9], etc., a routine biomarker for prediction of metastasis and recurrence is not yet available.

As it is a multi-step progress of the occurrence and development of HCC involving with multi-genes, it is impossible to predict the prognosis of HCC patients accurately by detecting only one biomarker. TAp73 α and p53 play their roles in the development and invasion of HCC^[10-13]. Cell proliferation and apoptosis are two different but associated processes in HCC^[14]. Moreover, proliferation index (PI) and apoptosis index (AI) can reflect the biological characters of tumor cells^[15]. So we try to predict the prognosis of HCC

patients more accurately by detecting TAp73 α , p53, PI and AI together.

MATERIALS AND METHODS

Materials

Forty-seven human resected HCC tissues and 42 adjacent non-cancerous tissues were collected and studied in the First Hospital of Xi'an Jiaotong University from January 2001 to 2002 (clinicopathologic characteristics of HCC tissues are listed in Table 1). Ten normal liver tissues were used as control group acquired by autopsy from healthy adult male, who died from accidental death simultaneously.

Table 1 Kaplan-Meier for survival

Factor	n	%	P	Factor	n	%	P
Sex			0.910	Node metastasis			0.197
Female	9	19.15		Positive	8	17.02	
Male	38	80.85		Negative	39	82.98	
Age (yr)			0.258	PV invasion			0.000 ^b
>45	29	61.70		Positive	7	14.89	
≤45	18	38.30		Negative	40	85.11	
HBsAg			0.007 ^b	AI			0.938
Positive	44	93.62		<Mean	25	53.19	
Negative	3	6.38		>Mean	22	46.81	
AFP			0.012 ^a	PI			0.052
<400 μg/L	23	48.94		<Mean	25	53.19	
>400 μg/L	24	51.06		>Mean	22	46.81	
Size			0.281	p53			0.402
≤5 cm	19	40.43		Negative	29	61.70	
>5 cm	28	59.57		Positive	18	38.30	
TNM stage			0.002 ^b	TAp73 α			0.039 ^a
II	13	27.66		Negative	30	63.83	
III+IV	34	72.34		Positive	17	36.17	
Differentiation			0.566	Liver membrane invasion			0.014 ^a
Well	39	82.98		Negative	28	59.57	
Poor	8	17.02		Positive	19	40.43	

^aP<0.05; ^bP<0.01 vs control group.

Elivision IHC study of p53, PCNA and TAp73 α

Resected specimens were routinely fixed in 40 g/L formaldehyde solution immediately after the resection. Paraffin blocks were prepared as follows. The tumor tissues were sliced vertically at a length of 4 μm through the central site of the tissues. After paraffin was removed from the slice with alcohol and xylol, endogenous peroxidase activity was blocked with 30 mL/L hydrogen peroxide at room temperature for 5-10 min. The preparation was subsequently treated in a 600-W microwave oven for 5 min in 0.01 mol/L citric acid buffer (pH 6.0), repeated thrice. After blocking with goat serum, every section was added according Ab₁ [p53 (ZM-0407) and proliferating cell nuclear antigen (PCNA) (ZM-0213), Zymed Biotechnology, Zymed, CA] 50 μL (dilution rate 1:50), done at 37 °C for 1-2 h. Then staining was performed by ElivisionTM plus two-step System (kit-9902, Dako, Carpinteria, CA). After treated with 3,3'-diaminobenzidine (DAB) at room temperature for 7 min, every section was submitted to chromatin staining, dehydrated and mounted.

In this study, we used a-specific polyclonal p73 raised against a C-terminal peptide [poly-TAp73 α (BA-1327), Wuhan Boster Biological Technology Co., Ltd, PRC] to detect TAp73 α protein. After the hot plerosis of antigen,

the preparation was added, antigen-plerosis, done at room temperature for 20 min and rinsed with PBS thrice for 3 min. Fifty microliters of X-Triton solution was subsequently applied, done at room temperature for 20 min. Following this, the preparation was rinsed with PBS thrice for 3 min; other steps were the same to detect p53 and PCNA.

The positive specimen of TAp73 α and p53 protein was defined as nuclear was stained over 5%, while the plasma was not. Further, 5-25% was defined as (+), 25-50% as (++) and over 50% as (+++). As to PCNA, positive cell was defined as the nuclear was stained while the plasma not. We counted up to 500 cells to calculate PI.

TUNEL staining

Apoptosis cell was detected as followed. TUNEL kit was purchased from Roche Applied Science. After being dewaxed with alcohol and xylol, the preparation was reacted with 30 mL/L hydrogen peroxide to block endogenous peroxidase activity for 30 min, and rinsed with PBS. The preparation was subsequently reacted with proteolytic enzyme (0.5 μg/mL potassium cacodylate), and rinsed with deionized distilled water (DDW). Then the preparation was reacted with 50 μL TdT mixture at 37 °C for 1 h, which was combined with reagents 1 and 2, containing: 200 mmol/L potassium cacodylate; 25 mmol/L Tris-HCl (pH 6.5); 0.25 g/L bovine serum albumin; 1 mmol/L COCl₂; 5 μmol/L biotin-dUTP; and 100 U/mL TdT. After being rinsed with PBS, to the preparation 350 μL of reagent was added at 37 °C for 40 min and stained with BCIP/NBT. Every section was subsequently treated with DAB coloration substrate solution, rinsed with running water and added to a nuclear staining solution, after which it was dehydrated, cleared, and mounted. Apoptosis cells were stained as blue, while others were stained pink. We counted up to 500 cells to calculate AI.

Negative control of p53, TAp73 α and PCNA were prepared by replacing Ab₁ with PBS, while using the known positive specimens as the positive control. As to the detecting of apoptosis cell, negative control was prepared by omitting the TdT-labeled reagent solution and the positive control was prepared by pre-treating the specimens with DNAase I.

Follow-up method

All patients were followed up every 3 mo after the surgical tumor ablation. Survival length was defined as the time from the surgical ablation to death or the last day of our follow-up, 1st March 2004.

Statistical analysis

All the clinical and pathological data were put on computer and analyzed by SPSS 10.0 statistical packet. Results were examined using χ^2 test, exact probabilities in 2×2 table, Spearman rank correlation, log-rank and *t*-test. Statistical significance was defined by a *P*-value of less than 0.05 in two-sides.

RESULTS

Expression of protein TAp73 α

In the cancer tissues, 17 specimens were positive (17/47, 36.2%), including 12+ and 5++ (Figure 1). In the adjacent non-cancerous tissues, only one of 42 was positive (2.38%);

while in the normal liver tissues none was positive. TAp73 α in the cancer tissues was higher than that in the adjacent non-cancerous tissues ($P < 0.005$) and normal liver tissues ($P < 0.01$). Furthermore, TAp73 α was associated with node metastasis ($r = 0.265$; $P = 0.032$) and p53 ($r = 0.407$; $P = 0.005$), but it showed no correlation with other clinicopathological characteristics.

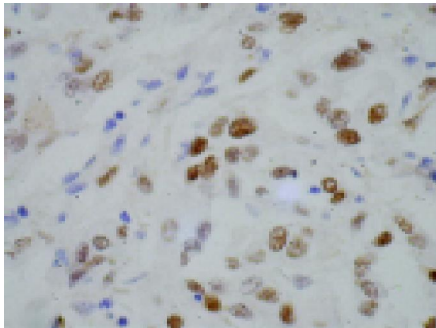


Figure 1 TAp73 α protein in HCC, nuclei of tumor cells were stained with yellow or brown ($\times 400$).

Expression of protein p53

In the cancer tissues, 18 specimens were positive (18/47, 38.3%), including 8+, 5++ and 5+++ (Figure 2), while only 7 of 42 were positive (16.7%) in the adjacent non-cancerous tissues and none in the normal liver tissues. Expression of protein p53 in the cancer tissues was significantly higher than that in the adjacent non-cancerous tissues ($P = 0.023$) and normal liver tissues ($P < 0.01$). p53 was associated with AFP ($r = -0.357$; $P = 0.014$) and the differentiation of tumor cells ($r = 0.295$; $P = 0.044$), but it showed no correlation with PI and AI.

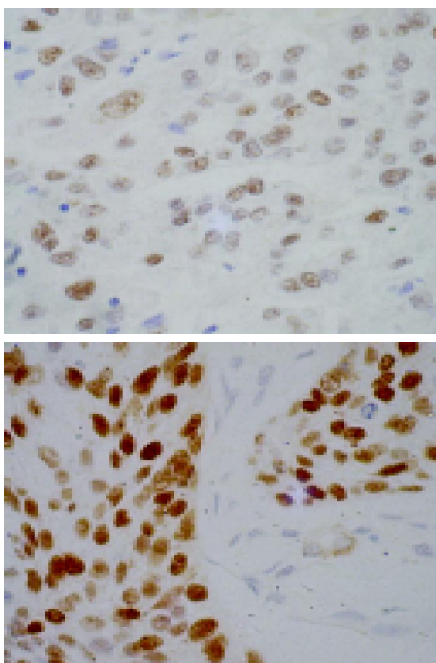


Figure 2 p53 protein in HCC, nuclei of tumor cells were stained with yellow or brown ($\times 400$).

Expression of PCNA and apoptosis

Positive cells of PCNA and apoptosis are seen in Figures 3 and 4, respectively. The mean value of PI in the cancer tissues was higher than that in the adjacent non-cancerous tissues ($30.34\% \pm 4.46\%$ vs $27.88\% \pm 5.89\%$; t , $P = 0.028$). PI in portal vein invasion subgroup was higher than that without portal vein invasion ($33.43\% \pm 3.21\%$ vs $29.82\% \pm 4.48\%$; t , $P = 0.048$). But PI showed no correlation with other clinicopathological characteristics.

AI in the HCC tissues was higher than that in the adjacent non-cancerous tissues ($8.62\% \pm 2.28\%$ vs $7.38\% \pm 2.61\%$; t , $P = 0.019$). AI in the late stage was higher than that in the early stage ($7.46\% \pm 2.30\%$ vs $9.06\% \pm 2.15\%$, t , $P = 0.030$), but it showed no correlation with other clinicopathological characters.

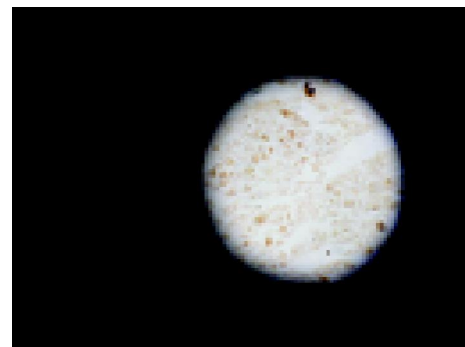


Figure 3 PCNA protein in HCC, nuclei of tumor cells were stained with brown ($\times 400$).



Figure 4 Apoptosis cells were stained in blue, while non-apoptosis cells were stained in pink ($\times 400$).

Survival analysis

We further classified the staining results of PI and AI into two subgroups, over or under the mean values in survival analysis. Until 1st March 2004, 35 patients died of HCC, 1 died of brain accident and 11 lived well. Survival time ranged from less than 1 mo to more than 34 mo (15.00 ± 1.21 mo). In Kaplan-Meier univariate analysis, survival between different TAp73 α , AFP, TNM stage, portal vein invasion, liver membrane invasion and HBsAg groups had statistically significant difference by log-rank test (Table 1).

As most patients in this study had positive HBsAg and

the degree of variation was almost 0, HBsAg was excluded in Cox regression analysis. It showed that TAp73 α , AFP, TNM stage, portal vein invasion and liver membrane invasion were independent dangerous factors influencing the survival of HCC patients ($P < 0.05$, Table 2).

Table 2 Variables in the equation method = backward stepwise (conditional LR)

	SE	Wald
AFP (>400 $\mu\text{g/L}$, <400 $\mu\text{g/L}$)	0.417	8.475
PV invasion (positive, negative)	0.519	5.684
AI (high, low)	0.380	3.495
TAp73 α (positive, negative)	0.367	4.323
Liver membrane invasion (y, n)	0.367	8.434
Age (>45, ≤ 45)	0.392	4.360
TNM stage (II, III+IV)	0.477	6.180

DISCUSSION

p53, a transcription factor, connects to a specific base sequence, inhibits the expression of various genes, and plays a major role in regulating the cell cycle and preventing the malignant transformation of cells. In HCC, there is always p53 gene mutant or inactive^[16], which leads to the abnormal growth of cells and ultimately the occurrence of cell conversion and carcinoma changes^[17]. In spite of these important roles, the relationship between the pathological significance of p53 mutation and prognosis in HCC has not been fully established.

Because of the short $T_{1/2}$ of wide-type p53 (wt-p53) protein, p53 protein detected by immunohistochemistry (IHC) is almost mutant-type p53 (mt-p53) protein. In this study, 38.3% HCC tissues expressed mt-p53 protein, higher than that in the adjacent non-cancerous tissues and normal liver tissues, indicating that p53 mutation is frequent in HCC. Furthermore, the different level of mt-p53 in HCC correlated with the differentiation of tumor cells^[18-20], that is, with the increasing of p53 level, the differentiation of tumor cells becomes poorer. It can be regarded that p53 level can reflect the differentiation of tumor cells. In this study mt-p53 did not show its correlation with the prognosis. So, we think, though mt-p53 is frequent in HCC, correlating with the occurrence and development of HCC, and can be used as an index of tumor cell differentiation, it is not an ideal index of prognosis.

p73 gene is a new family member of p53, it shares 63% identity with the DNA-binding region of p53 including the conservation of all DNA contact residues, 38% identity with the tetramerization domain, and 29% identity with the transactivation domain^[21]. It plays an important role in several cancers, such as lung cancer, colorectal cancer and bladder cancer^[22-25].

In this study, TAp73 α overexpressed in HCC tissues, indicating that TAp73 α may play its role in regulating occurrence and development of HCC. Similarly, Zemel and Pan found that p73 protein highly expressed and accumulated in HCC tissues, and overexpression of p73 may in some way be associated with the pathogenesis of HCC^[26,27].

In 1999, Tannapfel found that p73 expression status

was statistically significantly related to prognosis ($P < 0.0001$). Patients with p73-positive tumors had a poorer prognosis than those with p73-negative carcinomas. Multivariate Cox survival analysis identified the age of the patient, p73 expression status, co-existing cirrhosis, and Edmondson grade as independent prognostic factors. The protein p73 is overexpressed by a subset of HCCs and could serve as a useful indicator of prognosis in patients with this disease^[8]. In this study, the expression of p73 also influences the prognosis of HCC patients. Patients who overexpressed p73 had a poorer prognosis than that did not. In the Cox regression, p73 protein expression was still an independent factor of prognosis of HCC patients. Besides, the expression of p73 correlated with node metastasis, which indicated a late stage and poor prognosis^[8,28]. In some reports, p73 can induce apoptosis by p53-dependent and p53-independent way^[29,30], but in this study p73 does not show any relationship with AI.

There is not only overproliferation of tumor cells, but also over apoptosis of tumor cells^[14]. But as PI overcomes AI, cancer develops in some degree. PCNA can be used as the index of proliferation and be detected by IHC^[15]. Nakano *et al*^[18], find that the poorer the differentiation of the tumor cells, the higher the PI is ($P < 0.001$). In this study, though there was no correlation between PI and differentiation, PI in the cancer tissues was higher than that in the adjacent non-cancerous tissues, indicating a tendency of higher proliferating potency in tumor cells ($P = 0.028$). Moreover, PI correlated with portal vein invasion, indicating that patients with portal vein invasion had a worse biological character than that did not. Soini^[31] finds that patients with a higher AI have a better survival than patients with a lower one after HCC tissue resection. In this study, AI in the HCC tissues was higher than that in the adjacent non-cancerous tissues ($P = 0.019$). And AI in late stage was higher than that in early stage. But as there are more proliferating cells than apoptosis ones, cancer has a tendency of being advanced. So we suggest that AI may be an index of late stage.

In this study, Kaplan-Meier univariant analysis showed that patients in stage II had a better prognosis than patients in III and IV, indicating that TNM stage is one of the useful independent indices of prognosis^[32]. The status of liver membrane and portal vein invasion also influences the survival of HCC patients^[10,33].

Besides pathological characters, AFP is a most useful index of prognosis of HCC patients. In Kaplan-Meier univariant analysis, AFP correlated with prognosis significantly ($P < 0.05$), and in the Cox regression analysis, AFP was also an independent index of prognosis.

Together, these data suggest that TAp73 α can be used as a prognostic indicator for patients with HCC undergoing surgical tumor ablation; AFP, TNM stage, portal vein invasion and liver membrane invasion also have a potency of predicting the prognosis of HCC.

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YMDD variants of HBV DNA polymerase gene: Rapid detection and clinicopathological analysis with long-term lamivudine therapy after liver transplantation

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Abstract

AIM: To look for a rapid low-cost technique for the detection of HBV variants.

METHODS: Two patients who underwent orthotopic liver transplantation (OLT) for HBV infection were treated with lamivudine (100 mg daily) and HBV infection recurred in the grafted livers. The patients were monitored intensively for liver enzymes, hepatitis B surface antigen (HBsAg) and HBV DNA in serum. Liver biopsy was performed regularly. HBV DNA in a conserved polymerase domain (the YMDD locus) was amplified from serum of each patient by PCR and sequenced. HBV genotypes were analyzed by restriction fragment length polymorphism (RFLP) of the PCR products generated from a fragment of the polymerase gene.

RESULTS: YMDD wild-type HBV was detected in one patient by PCR-RFLP and DNA sequencing 19 mo after OLT, and YIDD mutant-type HBV in the other patient, 16 mo after OLT.

CONCLUSION: PCR-RFLP assay is an accurate and simple method for genotyping lamivudine-resistant HBV variants.

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Key words: Liver transplantation; HBV DNA; Polymerase gene; YMDD variants; PCR-RFLP

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INTRODUCTION

Chronic hepatitis B is a common disease with about 350 million HBV carriers according to the figures of World Health Organization^[1]. The persistence of viremia in patients often leads to progressive liver diseases including cirrhosis and hepatocellular carcinoma. For HBV-infected patients with end-stage liver disease, antiviral treatment cannot significantly alter their clinical course; orthotopic liver transplantation (OLT) is the only option. Because of the very high HBV reinfection rate of hepatic allografts and the rapid progression of HBV-induced disease in allografts^[2], it is essential to prevent HBV reinfection of hepatic allografts after OLT. Interferon has been shown to be beneficial to some patients with chronic HBV infection; however, the overall response rate to interferon alpha is less than 40%^[3]. Lamivudine, an enantiomer of β -L-2', 3'-dideoxy-3'-thiacytidine (3TC), is a potent inhibitor of RNA-dependent DNA polymerase of HBV and HIV reverse transcriptase^[4,5]. Treatment of HBV with oral lamivudine can suppress virus replication and reduce hepatic necro-inflammatory activity^[6].

Although lamivudine can prevent HBV reinfection of hepatic allografts, long-term therapy can lead to lamivudine-resistance of HBV^[7-9]. Furthermore, lamivudine-resistant HBV strains, with amino acid substitution in the YMDD (tyrosine, methionine, aspartate) motif of DNA polymerase, have been found in patients after OLT and immunosuppressive therapy^[5]. The amino acid substitution from methionine to valine or isoleucine at amino acid 552 of the YMDD motif of the HBV polymerase gene is the main mutation responsible for resistance to lamivudine treatment. The reported rate of lamivudine resistance is 14% in patients with chronic HBV infection and 27% in patients with recurrent HBV infection after OLT^[10-13]. The low incidence and late occurrence of YMDD mutant viruses suggest that such mutant strains originate from wild-type by point mutation during the course of therapy. Once lamivudine resistance occurs, a combination antiviral therapy (such as interferon and hepatitis B immunoglobulin) may be necessary to prevent exacerbation of hepatitis^[14-17].

Occurrence of lamivudine-resistant HBV variants is commonly detected by direct sequencing of HBV DNA.

However, this assay is excessively time-consuming for a large number of clinical samples. In this study, the frequency and characteristics of variants at codon position 552 of the HBV polymerase gene were examined by restriction fragment length polymorphism (RFLP) of the products generated from the HBV genome after amplification by PCR. The results of this analysis were compared with those obtained by direct HBV DNA sequencing, alanine aminotransferase levels and liver biopsy.

MATERIALS AND METHODS

Patients

Between 2000 and 2004, 72 patients underwent OLT for the end-stage liver disease secondary to HBV infection in our institute. Seven of these patients developed recurrent HBV infection in the grafted liver confirmed by serological examination and liver biopsy. All patients received standard immunosuppression treatment with cyclosporine, azathioprine, corticosteroids, anti-HBsAg immunoglobulin injection and oral lamivudine (100 mg daily). The patients were closely monitored for serum liver enzymes, hepatitis B surface antigen (HBsAg), HBeAg, and HBV DNA. Liver biopsies were performed before and after OLT. YMDD variants of HBV DNA polymerase gene were detected by direct sequencing in the YMDD motif of HBV and PCR-RFLP. YIDD mutant was found in one and wild-type YMDD in six of the seven patients. The following two patients served as their example.

Patient 1 was a 34-year-old man, his liver function was abnormal 15 years ago without any treatment. Two years ago, he developed oliguria, abdominal distension, anorexia, profound fatigue and edema and was diagnosed as cirrhosis secondary to chronic HBV infection, liver dysfunction and portal vein hypertension. He received liver protective and urinitive therapy. He developed headache, furor and unconscious behavior after oral lasec and was diagnosed as stage II hepatic encephalopathy 6 mo ago. His condition improved and was discharged after 20 d. Fifteen days ago, he was found to have a 1.8 cm×1.7 cm solid mass in the anterior lobe of right liver by B ultrasound, and MRI of portal vein showed occlusion and tumor thrombosis of the right liver branch of portal vein, and he was admitted on September 11, 2000. Blood test showed HBsAg (+), anti-HBs (-), HBeAg (+), anti-HBe (-), anti-HBc (+), HBV DNA (+). He underwent OLT on November 3, 2000. The resected liver showed poorly differentiated hepatocellular

carcinoma with cirrhosis. Liver biopsy showed mild to moderate acute hepatic allograft rejection 1 wk and 9 mo after OLT, and acute viral hepatitis 19 mo after OLT, the immunohistological staining of HBsAg was positive. Metastatic hepatocellular carcinoma of the right adrenal gland was found 20 mo after OLT, which was confirmed by pathology. The patient stopped lamivudine therapy himself because of bad financial condition.

Patient 2 was a 56-year-old man. He suffered from viral hepatitis 22 years ago and improved after treatment. He developed cirrhosis 6 years ago. CT and B ultrasound examination showed a 10.3 cm×10.3 cm solid mass in liver and tumor thrombosis in portal vein. He was admitted on December 26, 2000. Blood test showed HBsAg (+), anti-HBs (-) HBeAg (-), anti-HBe (+), anti-HBc (+), HBV DNA (+). He underwent OLT on January 16, 2001. The resected liver showed cirrhosis secondary to viral hepatitis with massive coagulative necrosis. Liver biopsy showed no acute cellular rejection and other lesions 3 wk after OLT, and acute viral hepatitis 16 mo after OLT, the immunohistological staining of HBsAg was positive. He died of upper gastrointestinal hemorrhage and hepatic encephalopathy 25 mo after OLT.

Amplification and sequencing of HBV DNA polymerase gene fragment including YMDD motif

HBV DNA was extracted from 200 µL of serum samples after digestion with proteinase K (0.8 g/L) at 56 °C for 4 h in 20 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 1 g/L sodium dodecyl sulfate. After a final extraction by chloroform, DNA was precipitated by absolute ethanol and dissolved in 50 µL of H₂O. For amplification of the DNA polymerase gene including YMDD motif, PCR was performed using primers BF105 and BR112 (Table 1 and Figure 1)^[18]. The PCR reaction mixture contained 1 µL DNA template, 0.5 µL primers, 1.5 mmol/L MgCl₂, 200 µmol/L dNTP, 0.5 u pfu polymerase, 12.5 mmol/L KCl in a total volume of 25 µL. The amplification conditions included an initial denaturation for 4 min at 94 °C, 35 cycles of amplification with denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 3 min, followed by a final extension at 72 °C for 7 min. PCR-negative samples were further amplified by nested PCR using 1 µL of the above PCR products by primers BF111 and BR109 under the same PCR conditions. PCR-amplified DNA was purified after agarose gel electrophoresis and sequenced directly.

Table 1 Primers used in the present study

Primer	Nucleotide Sequence	Nucleotide	Direction
BF105	5'-TCCTGCTGCTATGCCTCATC-3'	411-430	Sense
BF107	5'-CCAGGAACATCAACCACCAG-3'	485-504	Sense
BR112	5'-TTCCGTCGACATATCCCATGAAGTTAAGGA-3'	895-865	Antisense
BF111	5'-TCGGACGGAACCTGCACTTG-3'	581-600	Sense
BF108	5'-ATGCCGTTTCTCCTGGCTCA-3'	655-674	Sense
BR109	5'-AAGGGAGTAGCCCCAACGTT-3'	870-851	Antisense
YNSspI	5'-TTTCCCCCACTGTTTGGCTTTCAGTAATAT-3'	711-740	Sense
TMPpu10I	5'-CAGACTTGGCCCCCAATACCACATCATGCA-3'	769-740	Antisense
TMApaLI	5'-CAGACTTGGCCCCCAATACCACATCGTGCA-3'	769-740	Antisense

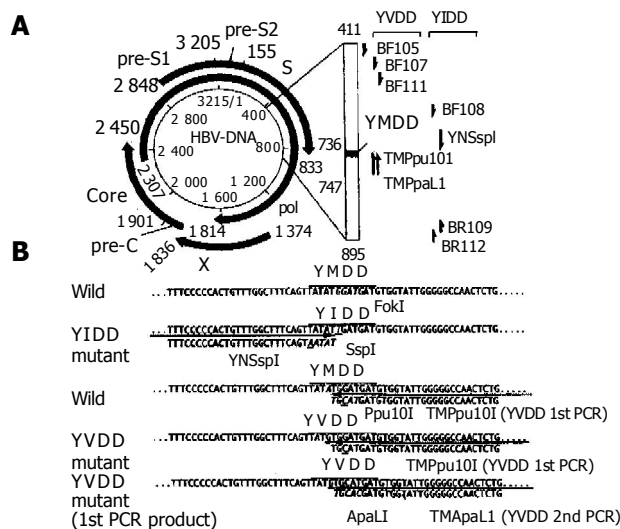


Figure 1 Schematic representation of analyzed partial DNA polymerase gene and primers used to detect two YMDD mutants (A) and nucleotide sequences of DNA polymerase gene including YMDD motif and primers for specific detection of both YIDD and YVDD mutants (B).

Sensitive and specific detection of YMDD mutant by PCR-RFLP-based assay

To detect two YMDD mutants, YIDD and YVDD, we designed PCR primers that could introduce a restriction enzyme recognition site into only PCR products amplified from mutant viruses. To detect YIDD mutant, 1 μ L of the extracted HBV DNA solution was initially amplified using primers BF108 and BR112. Twenty-five cycles of PCR (at 94 $^{\circ}$ C for 1 min, at 58 $^{\circ}$ C for 1 min, at 72 $^{\circ}$ C for 1.5 min) were performed after initial denaturation at 94 $^{\circ}$ C for 4 min, followed by a final extension at 72 $^{\circ}$ C for 7 min. The second PCR was performed with primer YNSspI, which introduced SspI site into only mutant PCR products (Figure 1), and BR109 using 1 μ L of the first PCR product and similar time-temperature conditions. Five microliters of the second PCR product was digested with 5 units of restriction enzyme SspI (Takara, Shuzo Co., Kusatsu, Japan) and was subjected to electrophoresis on 40 g/L agarose gel.

To detect YVDD mutant, similar amplification was

performed using primers BF107 and TMPpu 10I in the first PCR, and TMApaL I and BF111 in the second PCR. Digestion by the restriction enzyme *Apa*LI, which digested only DNA fragment from YVDD mutant, was performed before agarose gel electrophoresis.

To enhance the detection of both YMD mutants, we used the restriction enzyme *Fok*I that could digest amplified DNA from wild type but not YIDD mutant, and *Ppu*10 I that could also digest DNA fragment amplified from the wild type using primer TMPpu 10I but not YVDD mutant. The first PCR products (1 μ L) were digested by either of the two enzymes, and used in the second amplification as described above.

RESULTS

Clinicopathological data

The clinicopathological data of patient 1 before and after OLT are summarized in Figure 2A. The patient developed HBV reinfection 19 mo after OLT. Liver biopsy showed that the portal tracts were slightly enlarged because of mild to moderate chronic inflammatory cell infiltration with focal piecemeal necrosis. Disarray of single necrotic hepatocytes and chronic inflammation were found in the parenchyma (Figure 3A). Cholestasis was evident in both hepatocytes and canaliculi. Knodell score was 3 (1 score for periportal/bridging necrosis, portal inflammation, and lobular necrosis respectively; 0 score for fibrosis; the total score was 3). Immunohistochemical staining was positive for HBsAg in most hepatocytes (Figure 3B).

The clinicopathological data of patient 2 before and after OLT are summarized in Figure 2B. The patient developed HBV reinfection 16 mo after OLT. Liver biopsy showed that the portal tracts were enlarged because of moderate chronic inflammatory cell infiltration and mild fibrosis with obvious piecemeal necrosis. Disarray of many necrotic hepatocytes and chronic inflammation were found in the parenchyma (Figure 3C). Knodell score was 7 (2 scores for periportal/bridging necrosis, portal inflammation, and lobular necrosis respectively; 1 score for fibrosis; the total score was 7). Immunohistochemical staining was positive for HBsAg in hepatocytes (Figure 3D).

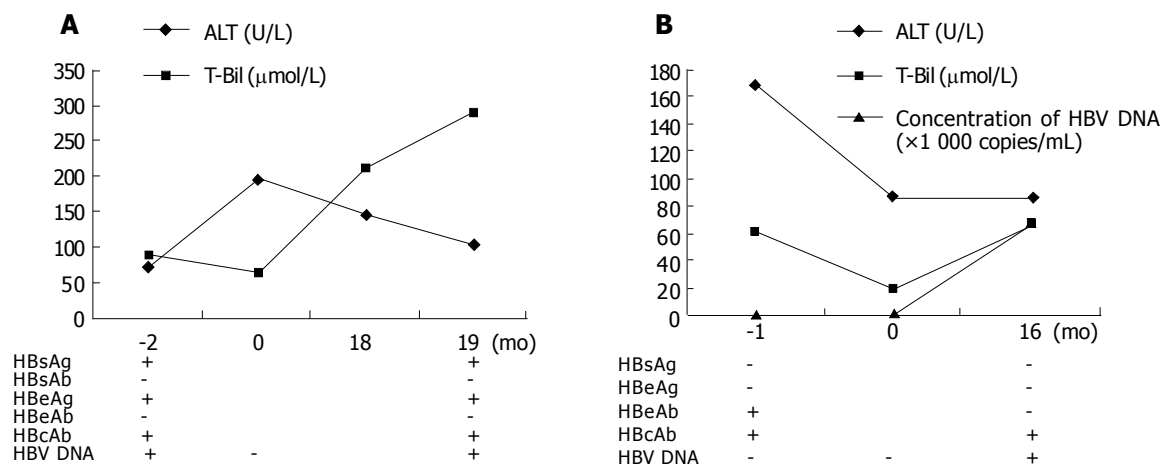


Figure 2 Clinicopathological data of patient 1 (A) and patient 2 (B) before and after OLT.

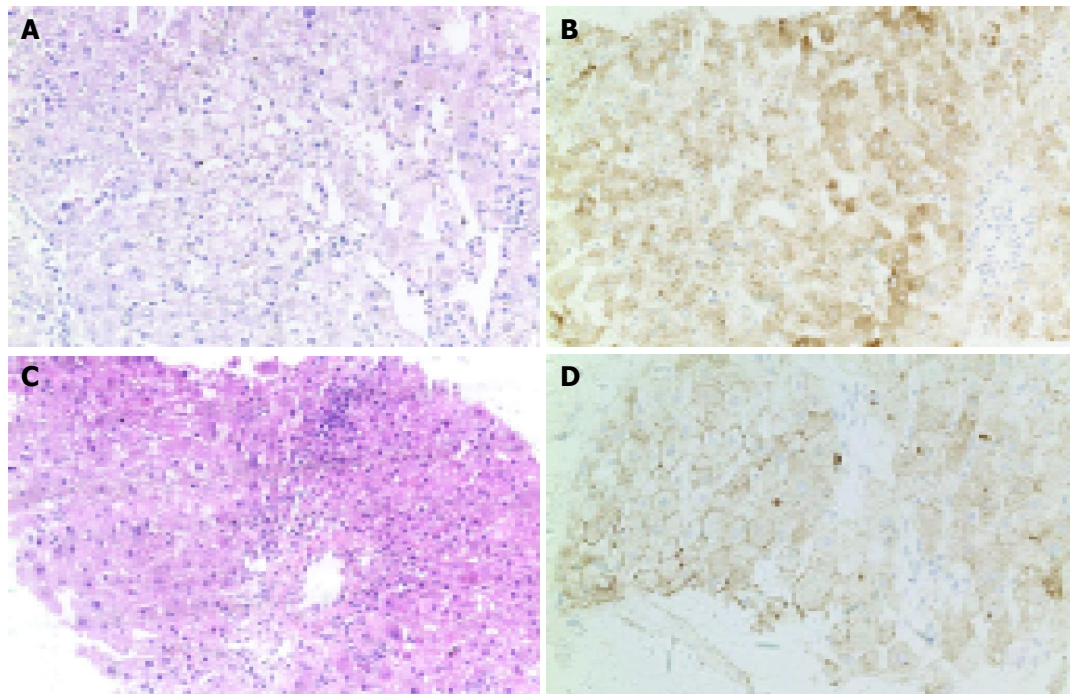


Figure 3 Liver biopsy of patients 1 (A and B) and 2 (C and D) 19 and 16 mo after OLT. A and C: HE, ×20; B and D: positive immunohistochemical staining

for HBsAg in hepatocytes (IHC, ×20).

In short, patient 2 had more obvious hepatic lesions than patient 1, showing more evident degeneration and necrosis of hepatocytes, piecemeal necrosis and fibrosis of portal tracts. However, the immunohistochemical staining for HBsAg in patient 2 was weaker than that in patient 1.

Result of PCR-amplified HBV DNA sequencing and PCR-RFLP

The PCR-amplified products of a 289-bp fragment of the DNA polymerase gene of HBV including YMDD motif were seen in patients 1 and 2, but not in control (Figure 4). They were purified and analyzed by direct sequencing.

Detection of YIDD mutant A weak 159-bp and a strong 159-bp products were amplified by nested PCR with and without *FokI* restriction enzyme digestion after first PCR. The nested PCR product was digested with *SspI* restriction enzyme, which did not cut the sequence, suggesting that HBV of patient 1 was YMDD wild without YIDD mutant (Figure 5A).

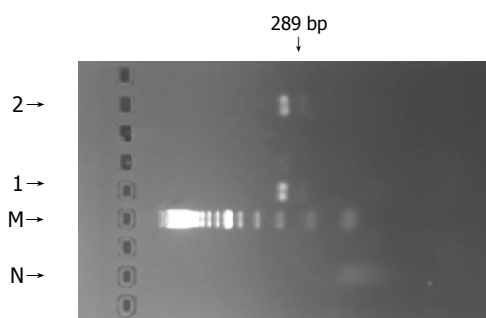


Figure 4 PCR-amplified products of a 289-bp fragment of DNA polymerase gene of HBV including YMDD motif in patients 1 and 2. N: normal; M: DNA marker; 1: patient 1; 2: patient 2.

Detection of YVDD mutant A strong 188-bp product was amplified by nested PCR with and without *FokI* restriction enzyme digestion after first PCR. The nested PCR product was digested with *ApaLI* restriction enzyme, which did not cut the sequence compared with IIC, suggesting that HBV of patient 1 was YMDD wild, without YVDD mutant (Figure 5A). The DNA sequence of patient 1 was TAT ATG GAT GAT from positions 132 to 142 of nested PCR product, its corresponding amino acid sequence was YMDD (Figure 6A).

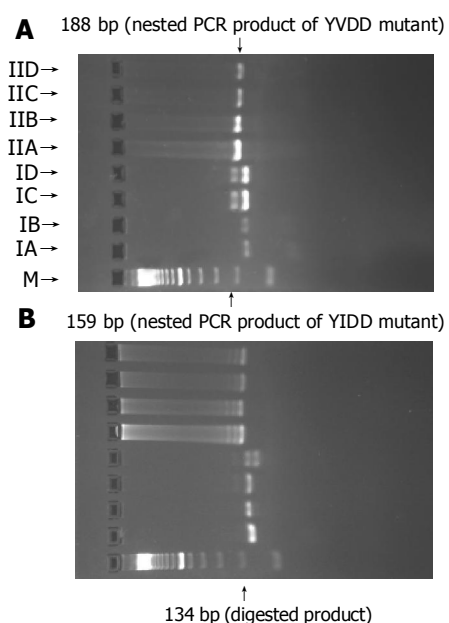


Figure 5 PCR-RFLP analysis pattern of patient 1 (A) and patient 2 (B).

Both patients developed HBV reinfection 19 and 16 mo after OLT, respectively. HBV was wild-type YMDD in patient 1, and YIDD mutant in patient 2. The clinicopathological data of patient 2 showed a sudden rise in HBV DNA titer during administration of lamivudine, suggesting the occurrence of YMDD mutant of HBV DNA polymerase gene and lamivudine resistance. Synthesis of HBV DNA genome occurs within the viral nucleocapsid in a mechanically ordered fashion. The nucleocapsid contains small pores that permit influx of nucleotide triphosphates and metabolites of nucleoside analogs such as lamivudine for DNA synthesis^[19-22]. Lamivudine is a cytidine analog that has been shown to be effective in inhibiting HIV and HBV replication^[23-26]. Lamivudine inhibits HIV and HBV replication by suppressing the activity of viral reverse transcriptase and acts as a chain terminator. HBV DNA polymerase activity in the wild-

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• *Helicobacter pylori* •

Increased activity of Pgp multidrug transporter in patients with *Helicobacter pylori* infection

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Abstract

AIM: To determine whether local antibiotic resistance involves P-glycoprotein (Pgp)-mediated active drug out-pumping during *Helicobacter pylori* (*H. pylori*) infection treatment with classic antibiotic therapy.

METHODS: Pgp activity was determined in gastric mucosa biopsy specimens obtained from 53 patients with pathohistologically verified gastritis and microbiologically confirmed *H. pylori* infection, and compared with the Pgp activity in 12 control subjects with normal endoscopic findings. The *H. pylori* positive patients were treated with short-term 7-d therapy consisting of two antibiotics (amoxicillin and azithromycin/metronidazole and clarithromycin) and a proton pump inhibitor. Pgp activity was determined by flow cytometry in the test of rhodamine dye efflux and quantified as mean fluorescence ratio (RMF).

RESULTS: Upon the first cycle, *H. pylori* was successfully eradicated in 20 patients, whereas therapy was continued in 33 patients. In the course of antibiotic therapy, RMF increased ($P < 0.05$) and gastric cells showed higher rhodamine dye efflux. The mean pre-treatment RMF values were also higher ($P < 0.0001$) in patients with multiple therapeutic failure than in those with successful *H. pylori* eradication and control subjects.

CONCLUSION: Pgp might be one of the causes of therapy failure in patients with *H. pylori* and antibiotic therapy could be chosen and followed up on the basis of the Pgp transporter local activity.

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Key words: *H. pylori*; RMF; Pgp

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INTRODUCTION

Protocols that include a small number of antibiotics (metronidazole, tetracycline, amoxicillin, clarithromycin and azithromycin) and other drugs with antimicrobial effect (bismuth and proton pump inhibitors, PPI) are used in the management of *Helicobacter pylori* (*H. pylori*) infection. According to the Maastricht Consensus, successful treatment of *H. pylori* infection implies a combination of two antibiotics and one antisecretory drug^[1,2]. At present, *H. pylori* infection is successfully treated in about 90% of cases; however, 10% of patients remain *H. pylori* positive. Successful *H. pylori* eradication depends on numerous factors. Better success is achieved if there is coexistence of antral gastritis or gastritis of the gastric body^[3], and in patients with peptic ulcer infected with *H. pylori* *cagA* and *vacA*s1 positive strains than in those with functional dyspepsia^[4-6]. The efficacy of *H. pylori* eradication has been shown to be influenced by gastric pH^[7], geographic location, and patient compliance during treatment^[8]. It should be noted that the development of primary and secondary bacterial resistance depends on the length of treatment, combination of antibiotics, and drug concentration^[9,10]. *H. pylori* has developed resistance to many drugs, e.g., metronidazole, clarithromycin, etc.^[5,8,11]. Treatment failure has been ascribed to metabolic changes in *H. pylori* itself^[5,12]. Repeat drug administration at a higher concentration has been demonstrated to produce better result^[7,10,12], suggesting that local conditions are responsible for therapeutic success^[8,12].

It is known that a high concentration of P-glycoprotein (Pgp), a multidrug transporter that actively pumps out all potentially cytotoxic substances, is found on the gastrointestinal tract epithelium. Multidrug transporters are transmembranously located, their mechanism of action being based on binding a broad spectrum of drugs to the membrane polypeptide chain and extruding them to the surroundings by use of ATP energy. All ATP dependent efflux proteins belong to the large ABC superfamily^[13-15] and are homologous in many animal species^[16]. Although the efflux as a mechanism of antibiotic resistance was demonstrated for gram-negative bacteria in the early 1980s^[17], the antimicrobial efflux transporter for *H. pylori*, LmrA, homologous to human multidrug transporter has only recently been described^[18-20].

MATERIALS AND METHODS

Materials

Gastric biopsy specimens (GBS) were collected from 65 subjects upon their informed consent in writing, according to the Helsinki Declaration and approved by the Hospital Ethics Committee. Fifty-three patients (24 male and 29 female), mean age 47.4 years (range 18–75 years), with endoscopically and pathohistologically verified antral gastritis had *H pylori* infection and were treated as outpatients at the University Department of Medicine, Sveti Duh General Hospital in Zagreb.

Methods

H pylori was verified by two parallel tests, i.e. microbiology and pathohistology. Microbiologic diagnosis included isolation and typing, whereas antibiotic sensitivity was determined by E test (B-biodisk) and Chaves' method^[21–23]. Histologic diagnosis included material staining according to Giemsa^[24]. Control group had 12 patients with dyspepsia as an indication for upper gastrointestinal endoscopy, in whom *H pylori* infection was neither microbiologically nor histologically demonstrated^[21–24].

Treatment of *H pylori* infection As the study was not aimed at comparison of different therapeutic protocols, it included a higher proportion of patients with multiple therapeutic failure than would otherwise be expected according to the efficacy of *H pylori* eradication. All patients underwent standard short-term triple therapy consisting of pantoprazole (P), a PPI, at a dose of 40 mg b.i.d., and two antibiotics, according to Maastricht recommendations^[1,29]. The following antibiotics were used: metronidazole (M), 500 mg; amoxicillin (A), 1 000 mg; and clarithromycin (C), 500 mg b.i.d. for 7 d, in randomized combinations. The patients were randomly allocated to the PAM ($n = 39$) or PAC ($n = 14$) protocol. Following initial therapeutic failure in eradication of *H pylori* infection, the patients were administered a new combination of the listed antibiotics for 10 d, or azithromycin (AZ) at a dose of 500 mg s.i.d. for 5 d in addition to one of the listed antibiotics for 10 d^[1,2,25]. So, in the second course after initial failure with the PAM protocol, there were 15 patients in the PAC protocol and PAAZ protocol each. Also, in the second course after initial failure with the PAC protocol, there were three patients on the PAAZ protocol^[1,2,9,25]. Following second failure of *H pylori* eradication, a third combination was introduced, consisting of P 40 mg b.i.d., in combination with bismuth citrate (B) t.i.d., M 500 mg s.i.d., and tetracycline (T) 500 mg q.i.d. for 14 d^[1,2,25]. This final protocol included 22 patients. The treatment with pantoprazole, 40 g/d, was continued for another 6 wk after each of these protocols^[1,2,9,25]. *H pylori* eradication was simultaneously assessed by microbiology and pathohistology 7 wk of the completion of each protocol^[1,2,9]. Patients who had been receiving anti-inflammatory or MDR-dependent drugs (that may have influenced the efflux) within one year preceding the study were not included^[13–20].

Collection of biopsy specimens GBS for microbiology and histology as well as for the assessment of Pgp activity were obtained from the oxyntic area during gastrointestinal endoscopy using an Olympus Q20 and Pentax endoscopy video system, in line with recommendations of the Sydney

classification^[24]. Two specimens from the antrum and body of the stomach each, and one from the angulus were obtained for microbiologic and histologic analysis, whereas two specimens from the anterior and posterior wall of the antrum each were obtained for the study of Pgp activity.

Microbiologic and histologic analysis Density of intraepithelial neutrophils (DIEn) and *H pylori* density (DHP) were determined in biopsy specimens as sensitive parameters of mucosal lesion and *H pylori* presence^[24]. *H pylori* and neutrophil density was scored 1–4 according to Sydney classification.

Determination of Pgp activity. Reagents Rhodamine-123 (Rh123, 2-6-amino 3 imino 3H xanthene 9 y benzoic acid methyl ester, Sigma, St. Louis, MO, USA) is a cationic lipophilic fluorescent dye frequently used to study Pgp activity. The cells were labeled with 1:100 of stock solution in PBS, a final concentration 20 µg/mL. Non-toxic substrates for Pgp, a fungal metabolite Cyclosporine A (CsA, from *Tolypocladium inflatum*, Sandoz Pharmaceuticals, Switzerland) and homoharringtonine (HHT, an alkaloid from *Cephalotaxus hainanensis*, Sigma) were used in a final concentration of 2.5 and 150 ng/mL, respectively.

Cell lines For determination of Pgp activity, exponentially growing human erythroleukemic K562 cell line with an optimal density of 5×10^5 /mL were prepared as control cells. The cell lines were grown in an RPMI 1640 nutrient mixture supplemented with 2 µmol/L L-glutamine, 1 µmol/L sodium pyruvate, 1 000 units/mL penicillin, 100 mg/mL streptomycin, and 10% fetal calf serum at 37 °C in a humidified atmosphere of 50 mL/L CO₂ in air^[26]. Negative control: drug-sensitive K562 line was purchased from the ATCC (American Type Culture Collection, Rockville, MD, USA). Positive control: the resistant HHT/K562 cell line obtained by continuous exposure to HHT was a kind gift from Professor J.P. Marie (Hotel Dieu, France) and was cultured in the presence of 150 ng/mL of HHT.

Preparation of GBS Upon endoscopy, the GBS were immediately placed in transport media and then into transport containers with ice at a constant temperature of 4 °C. GBS was dissociated mechanically by mincing the sample with little scissors, then washed with cold buffer and centrifuged at 3 000 r/min for 5 min at 4 °C. The viability and yield were assessed by trypan blue exclusion.

Rhodamine efflux measurement A direct functional assay for Pgp activity was performed by the Rh123 uptake/retention method^[27] adapted for stomach tissue analysis. Briefly, aliquoted GBS ($5\text{--}8 \times 10^5$ cells/tube) were stained for 60 min with 5 µL of Rh123 in the presence or absence of MDR-reversing agent CsA (5 µL). After Rh123 uptake, cells were washed and fed with Rh123-free culture medium, cultured for 60 min at 37 °C, again in the presence or absence of CsA to evaluate its effect on Rh123 efflux (retention). Afterwards, GBS was centrifuged in culture medium, resuspended and kept in ice and darkness for 10 min until flow cytometry analysis. As a reference, K562 and HHT 150/K562 cells were processed in parallel with the patient samples. The analysis was performed on an FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission

at 530/30 and 570/30 nm band-pass filters). Analysis of 10^4 cells *per* sample was carried out in log histogram and is illustrated in Figure 1. When Rh123 was being diffused into the cell, Pgp actively pumped out the fluorochrome, and cellular fluorescence was determined by the rates of influx and efflux. If another compound, a substrate and/or inhibitor, was presented in the same manner together with Rh123, the efflux was blocked and the fluorescent dye accumulated in the cell, producing higher mean fluorescence intensity. The result can be quantitatively analyzed and expressed as the ratio of the two mean fluorescences. RMF represents the ratio of MF of Rh123 with CsA modulator divided by MF of Rh123 without modulator after subtraction of the fluorescence of the blanks (autofluorescence of cells <1%). As the amount of intracellular Rh123 dye content upon the addition of modulator correlated with Pgp activity, $\text{RMF} \geq 1$ was considered positive.

Variation due to test conditions Upon endoscopy, GBS was transported at a constant temperature of 4 °C and analyzed within 1 h. Gastric cells were mechanically dissociated by use of small scissors or by enzymatic digestion (trypsin), whereby the viability and gain were controlled by trypan blue exclusion. The cells obtained by mechanical dissociation (mincing with scissors) were more numerous and showed higher viability than those obtained by trypsin enzymatic dispersion. In order to assess the impact of storage on test reproducibility, 12 randomly chosen GBS were divided into two groups. One group was analyzed on the day of sampling, and the other was stored overnight at room temperature in 10 mL of cold buffer. The minimal concentration of GBS was 0.5×10^5 cells/mL.

Statistical analysis

We used STATISTICA software for the analysis of variance, multiple regression analysis and ROC analysis in the interpretation of results. The level of significance was set at $P < 0.001$. The mean value of the scores obtained on antrum biopsy (anterior and posterior wall/2) was used for statistical analysis of DIEn and DHP.

RESULTS

Variation due to test conditions

The activity of Pgp was measured in GBS of 53 patients with *H. pylori* infection and 12 dyspeptic patients without *H. pylori* infection (control group). The two patient groups were matched according to mean age (47.39 *vs* 48.33 years) and sex (M/F = 5/7 *vs* 24/29).

In order to assess the impact of storage on test reproducibility, as shown in Table 1, the mean RMF was 1.427 ± 1.034 and 2.521 ± 2.067 on d 1 and 2, respectively. RMF was elevated in most of the samples (10/12; *t* test for dependent samples: $t = 1.932$; $\gamma = 11$; $P < 0.0795$; SIGN test: $\chi = 1.443$; $P < 0.148$). In the samples with the addition of modulator, fluorescence varied on d 2 and it seemed that there was no overexpression of Pgp function, although the cells were viable. As storage results in changed drug transport functions, for reasons of cellular metabolic and membrane integrity it is better to use fresh cells, within a few hours of sampling.

Measurement of Pgp activity in patients with *H. pylori* infection Pgp activity was determined in 53 patients with *H. pylori* infection and 12 dyspeptic patients without *H. pylori* infection (control group). The intensity of rhodamine dye extrusion by GBS with *H. pylori* infection was higher as compared with *H. pylori* negative control GBS (0.8 ± 0.30 *vs* 1.38 ± 0.66 ; $\gamma = 1$; $F = 8.6$, $P < 0.0046$). *H. pylori* infection stimulated Pgp activity. In patients with *H. pylori* infection, RMF values were statistically increased significantly ($P < 0.005$) as compared with control patients (Figure 2). Based on ROC analysis, $\text{RMF} \geq 1$ was considered positive.

Therapy stimulating Pgp activity Pre- and post-therapeutic Pgp activity was assessed in 10 randomly chosen *H. pylori* positive patients irrespective of therapeutic success. After 7-wk treatment, stronger GBS extrusion of rhodamine dye was recorded in 6/10 patients, with a statistically significant increase in RMF values (Figure 3). Wilcoxon pair test yielded a significant difference between the pre- and post-therapeutic values ($t = 9.0$; $\chi = 1.885$; $P < 0.05$).

Neither is repeat therapy efficacious in enhanced Pgp activity As shown in Table 2, the course of therapy was

Table 1 Effect of sample storage

<i>H. pylori</i> positive patients	D1			D2		
	Rh123 MF	Rh123+CsA MF	RMF	Rh123 MF	Rh123+CsA MF	RMF
1	73.14	124.58	1.703	25.00	44.76	1.790
2	75.25	17.86	0.237	27.83	4.28	0.157
3	5.47	20.85	3.404	17.25	41.72	2.419
4	83.40	113.79	1.364	43.03	61.20	1.423
5	83.40	113.79	1.364	90.44	172.18	1.920
6	141.21	68.47	0.485	72.91	80.63	1.106
7	72.31	223.19	3.087	46.72	223.44	4.783
8	262.30	220.78	0.842	308.4	326.90	1.060
9	135.53	93.90	0.693	36.71	255.95	6.972
10	170.21	225.35	1.324	220.95	223.71	1.012
11	115.20	165.20	1.434	37.88	94.26	2.288
12	11.30	25.10	2.221	25.76	134.78	5.332
RMF (mean $\pm$ SD)	1.427 $\pm$ 1.034			2.522 $\pm$ 2.067		

D 1 = fresh cells, several hours of sampling; d 2 = cells stored overnight at room temperature; Rh123 = rhodamine; CyA = cyclosporine A; MF = mean fluorescence; RMF = ratio of mean fluorescence.

Table 2 Role of pretherapeutic determination of Pgp activity

Variables	Therapy cycle			B/A and controls	
	<i>H. pylori</i> positive		<i>H. pylori</i> negative		
	1 = A (<i>n</i> = 20)	2 or more = B (<i>n</i> = 33)	NO (<i>n</i> = 12)	<i>P</i> ^a	Beta <i>P</i> ^b
Age (yr)	47.5±8.8	47.3±13.7	48.3±16.5	0.973	0.152
Sex (M/F)	9/11	15/18	5/7	0.975	0.153
DHP	2.75±0.91	2.90±0.97	1.0±0.0	0.00001	0.00065
DIEn	2.65±0.81	3.06±0.86	1.0±0.0	0.00001	0.450
RMF (mean±SD)	0.889±0.28	1.647±0.65	0.787±0.29	0.00001	0.000005

H. pylori positive patients: A = one therapy cycle, B = two or more therapy cycles; NO = no therapy, M = male; F = female; DHP = *H. pylori* density; DIEn = density of intraepithelial neutrophil infiltration; RMF = ratio of mean fluorescences, *P*^a for analysis of variance, *P*^b for multiple regression analysis.

not affected by either age or sex. The patients requiring multiple courses at *H. pylori* eradication (group B, *n* = 33) initially had a more severe infection (higher *H. pylori* concentration and intraepithelial neutrophil infiltration), however, neither these variables were found crucial for course of treatment. Therapeutic success was influenced by Pgp activity: the patients with failure of *H. pylori* eradication had a statistically higher (*P* < 0.00001) pretherapeutic Pgp activity (1.647±0.65) than either patients with efficient *H. pylori* eradication (0.889±0.28) or control group (0.787±0.29).

Multiple regression analysis yielded a significantly highest beta coefficient for RMF and DIEn (*P* < 0.001). ROC analysis demonstrated higher sensitivity, specificity and accuracy for RMF (90.90%, 71.87% and 81.54%, respectively, for borderline RMF value of 1.0) than for DHP (48%, 71% and 60%, respectively, for borderline DHP of score 2), and DIEn (66%, 71% and 69.23% respectively, for borderline DIEn of score 2).

Combined therapy has no effect in case of enhanced Pgp activity

Although the number of patients included in the study was too small for statistical analysis, the antibiotics used appeared to differ in stimulating Pgp activity, i.e. the first successful course with amoxicillin in combination with metronidazole or clarithromycin (RMF±SD for PAM 0.83±0.3 and for PAC 0.95±0.25) was less dependent on Pgp activity than the repeat combination with azithromycin (RMF±SD 1.706±0.62; *n* = 18). The patients with multiple attempts at *H. pylori* eradication had higher pretherapeutic RMF values,

however, there was no significant difference in the choice of antibiotics (between-group analysis of variance: *f* = 5.031; *P* < 0.000898) (Figure 4).

Factors influencing therapeutic efficacy Upon therapy completion, the effects of all parameters on the efficacy of *H. pylori* eradication were assessed. Comparison of pretherapeutic RMF values between the patients with single *H. pylori* eradication and those requiring two or more attempts at *H. pylori* eradication showed no difference (*P* < 0.13418). There was no significant difference in pretherapeutic Pgp activity between the patients with ultimately successful and unsuccessful *H. pylori* eradication (1.26±0.715 *vs* 1.54±0.66). As there was no such difference in other clinical parameters either (age, sex, DIEn and DHP, *P* < 0.189), it seems that, although influencing the duration and course of treatment, Pgp cannot be used to predict the ultimate treatment outcome (Table 3).

Table 3 Impact of initial values on *H. pylori* eradication efficacy

Variable	Final eradication of <i>H. pylori</i>		<i>P</i>
	Successful (<i>n</i> = 31)	Unsuccessful (<i>n</i> = 22)	
Age (yr)	46.54±12.51	48.54±11.44	0.556
Sex (M/F)	15/16	9/13	0.598
DHP	2.90±0.97	2.77±0.92	0.626
DIEn	2.77±0.846	3.09±0.86	0.189
RMF (mean±SD)	1.26±0.715	1.54±0.66	0.13418

M = male; F = female; DHP = *H. pylori* density according to Sydney criteria; DIEn = density of intraepithelial neutrophil infiltration according to Sydney criteria; RMF = ratio of means.

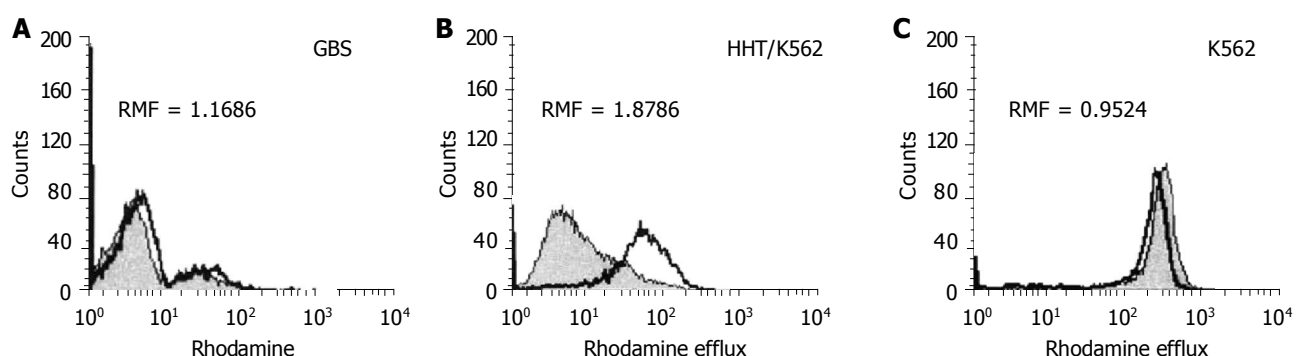


Figure 1 FACS analysis of Rh123 accumulation as a function of Pgp activity (A–C): (A) GBS were stained with a fluorescent substrate Rh123 in the presence (overlaid histogram) or absence (filled histogram) of the MDR reversing agent

CyA. Pgp activity is expressed as RMF, a ratio of mean fluorescences obtained from the gated viable cell population. RMF ≥ 1 considered positive; (B) resistant (Pgp-positive/active) and (C) sensitive (Pgp-negative/inactive) control cell lines.

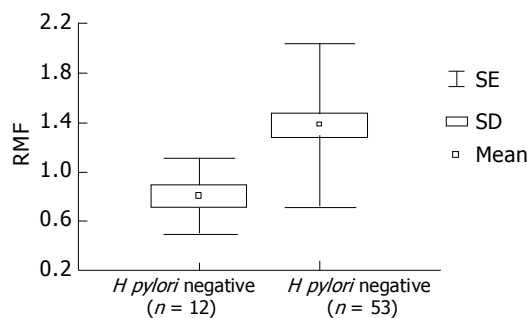


Figure 2 Enhanced Pgp activity in patients with *H pylori* infection. RMF values of 53 *H pylori* positive patients and 12 *H pylori* negative (control) patients are presented ($\bar{x}$ = mean; SD = standard deviation; SE = standard error).

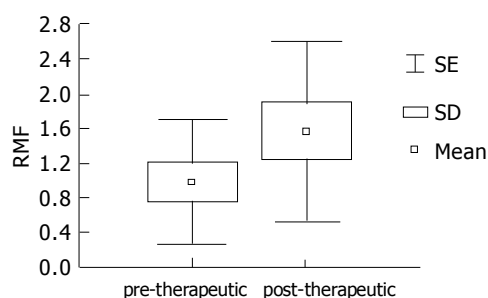


Figure 3 Therapeutic effect on Pgp activity in 10 randomly selected *H pylori* positive patients: significantly increased post-therapeutic RMF values.

DISCUSSION

Although *H pylori* infection is a well-characterized disease, many questions about the mechanisms of drug failures to cure the disease remain to be answered. The increased prevalence of antibiotic-resistant *H pylori* strains has serious implications, apart from patient compliance, as antimicrobial resistance is the most important factor determining the outcome of antibiotic therapy. The present article speculates about the potential mechanism limiting the gastrointestinal availability of antibiotics by Pgp mediated decrease in drug accumulation. Pgp is expressed on a wide variety of normal cells, especially in secretory tissues including the liver, intestinal tract epithelia from jejunum and colon, adrenal cortex, kidney, certain capillary endothelia, peripheral blood lymphocytes and hemopoietic precursor cells^[28]. In gastrointestinal tract, Pgp acts as an ATP-consuming efflux pump extruding the natural toxins across the epithelial surface to the intestine, hepatocytes in biliary canaliculi and small ductules of the pancreas^[29]. Direct evidence that Pgp inhibits the gastrointestinal absorption of orally administered drugs comes from several sources^[30-33]. To the best of our knowledge, this is the first report analyzing Pgp activity in *H pylori* infection. We evaluated Pgp function in gastric cells from patients with *H pylori* infection by means of intracellular accumulation and efflux of a fluorescent dye. In the GBS loaded with Rh123 dye an increased efflux was observed in 33 *H pylori* positive patients who failed to respond to treatment. The patients with enhanced Pgp activity (RMF > 1.5) failed to respond to either first or second therapy with the same or another antibiotic. As Pgp activity is low (RMF

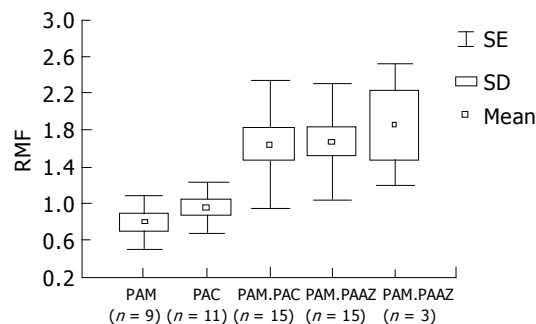


Figure 4 RMF values with five therapeutic protocols: combined therapy had no effect with enhanced Pgp activity. First course: PAM (pantoprazole+amoxicillin+metronidazole), PAC (pantoprazole+amoxicillin+clarithromycin); second course: PAM+PAC, PAM+PAAZ (pantoprazole+amoxicillin+azithromycin), PAC+PAAZ.

<1) in patients who respond favorably to the first course of treatment, it is of most importance to assess Pgp activity before therapy introduction and to monitor it before repeat antibiotic cycles. Pgp activity is generally low at the time of diagnosis, i.e. before the patient receives any treatment. These findings indicate that an increased Pgp activity may contribute to the drug-induced effect on gastric cells long after therapy completion. Although *H pylori* infection per se stimulates Pgp pump activity, this activity seems to additionally rise during the course of disease, because RMF index is lower at the disease onset. And the last but not the least, the lower RMF values recorded in patients with remission support the hypothesis that inappropriate therapeutic combinations lead to eradication failure. Therefore, Pgp activity testing might be used as a sign of therapeutic failure and need of multiple therapeutic protocols in a particular patient. Although Rh123 efflux could not predict final outcome of the attempts at *H pylori* eradication, it did show that elevated RMF values had greater impact on *H pylori* eradication failure with the use of AZ than with other antibiotics. Other parameters of *H pylori* infection (DHP, DIEn) did not prove useful in predicting either the need of an increased number of therapeutic protocols or the final treatment outcome.

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• *Helicobacter pylori* •

Do patients with non-ulcer dyspepsia respond differently to *Helicobacter pylori* eradication treatments from those with peptic ulcer disease? A systematic review

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Abstract

AIM: It is controversial whether patients with non-ulcer dyspepsia (NUD) respond differently to *Helicobacter pylori* (*H. pylori*) eradication treatment than those with peptic ulcer disease (PUD). To review the evidence for any difference in *H. pylori* eradication rates between PUD and NUD patients.

METHODS: A literature search for full articles and meeting abstracts to July 2004 was conducted. We included studies evaluating the efficacy of a proton pump inhibitor (P) or ranitidine bismuth citrate (RBC) plus two antibiotics of clarithromycin (C), amoxicillin (A), metronidazole (M), or P-based quadruple therapies for eradicating the infection.

RESULTS: Twenty-two studies met the criteria. No significant difference in eradication rates was found between PUD and NUD patients when treated with 7-d RBCCA, 10-d PCA or P-based quadruple therapies. When the 7-d PCA was used, the pooled *H. pylori* eradication rate was 82.1% (431/525) and 72.6% (448/617) for PUD and NUD patients, respectively, yielding a RR of 1.15 (95%CI 1.01-1.29). However, the statistically significant difference was seen only in meeting abstracts, but not in full publications.

CONCLUSION: There is no convincing evidence to suggest that NUD patients respond to *H. pylori* eradication treatments differently from those with PUD, although a trend exists with the 7-d PCA therapy.

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Key words: *H. pylori* eradication; Non-ulcer dyspepsia; Peptic ulcer disease; Meta-analysis

INTRODUCTION

It has been over 17 years since the first randomized, placebo-controlled clinical trial of *Helicobacter pylori* (*H. pylori*) eradication was published^[1]. Treatment to cure the infection has evolved from single agents to combination treatment regimens with up to four drugs. Currently, the most commonly recommended combination treatments consist of a proton pump inhibitor (P), clarithromycin (C) and amoxicillin (A) or metronidazole (M)^[2-4]. However, the success of treatment with these combination regimens varies considerably between reports^[5]. Several factors have been identified to contribute to the variability of the treatment outcomes. These include patient- and treatment-related factors^[5]. However, conflicting results have been reported with regard to whether *H. pylori* eradication treatment is more effective in patients with peptic ulcer disease (PUD) than in those with non-ulcer dyspepsia (NUD) or other non-ulcer gastric diseases^[6-11], especially when patients are treated with the PCA combination regimen. We undertook this meta-analysis to review systematically the literature on *H. pylori* eradication in patients with PUD or with NUD and to evaluate the magnitude of any difference in *H. pylori* eradication rates between the two groups of patients when treated with the most commonly recommended treatment regimens.

MATERIALS AND METHODS

Search strategy

A computerized literature search for relevant studies was performed in Cochrane Controlled Trials Register, MEDLINE, PubMed, and EMBASE databases to June 2004 in all languages using the following MeSH terms or textwords in various combinations: dyspepsia, NUD, functional dyspepsia, peptic ulcer, PUD, *H. pylori*, duodenal ulcer, gastric ulcer, eradication. Several other online trials' registries were also searched including United Kingdom National Research Register (<http://www.doh.gov.uk/research/nrr.htm>), Current Science register of controlled trials (<http://www.controlled-trials.com>), ClinicalTrials.gov

developed by the National Library of Medicine (<http://www.clinicaltrials.gov/ct>). A recursive hand search of reference lists, review articles, editorials and abstracts from major relevant international meetings was also conducted to supplement electronic searches: American Digestive Disease Week 1997-2004, the United European Gastroenterological Week 1997-2003, American College of Gastroenterology Annual Meetings 1997-2003, the European *H. pylori* Study Group Meetings 1997-2003 and the World Congress of Gastroenterology 1998-2002.

Inclusion and exclusion criteria

Because the research question of this study sought any difference in *H. pylori* eradication rates between patients with PUD or NUD, the relative efficacy of different treatment regimens was neither compared nor analyzed. Therefore, we included all clinical trial designs if studies met the following criteria: (1) any clinical trial in adults involving one of the following combination regimens given in the recommended doses: a PPI or ranitidine bismuth citrate (RBC) plus two antibiotics (clarithromycin, amoxicillin, metronidazole or tinidazole), and proton pump inhibitor-based quadruple therapies; (2) studies which provided comparative data on *H. pylori* eradication rates between patients with PUD and those with NUD; (3) studies which provided raw data for intention-to-treat (ITT) analysis or ITT data could be derived from the paper; (4) *H. pylori* infection was diagnosed by at least one of the following methods: rapid urease test, histology, culture or urea breath test; eradication was confirmed at least 4 wk after stopping all medications. We excluded studies that reported mixed eradication data or were identified as duplicate publications. Studies of patients with chronic gastritis alone were also excluded.

The eligibility criteria were assessed by two independent reviewers (JQH and GFZ). Any disagreement about the decision was solved through discussion to reach consensus between the reviewers.

Data extraction

Two independent reviewers (JQH and GFZ) extracted the data from the qualified studies and any disagreement was resolved by discussions to reach consensus between the reviewers. The following major items were extracted from each qualified study: name of the first author, source and date of publication, study design, demographics of patients, diagnosis of *H. pylori* infection and methods used for confirmation of eradication, details of treatment regimen (number of drugs, dose, dose frequency and treatment duration), number of patients randomized, treated and dropped-out, and number of patients with *H. pylori* infection eradicated by ITT analysis and by disease category (e.g., PUD, or duodenal ulcer or gastric ulcer or NUD).

Outcome measures

The only outcome concerned in this study was the *H. pylori* eradication rate by ITT analysis in patients with PUD compared to those with NUD. Although we also extracted eradication rates by per-protocol analysis, the data were not presented due to concerns over the methodological

problems, such as information on drop-outs which was poorly reported.

Assessment of study quality

The quality assessment was done independently by JQH and GFZ. Because of the nature of our research question, no existing scoring instruments could be used for conducting the quality assessment. Therefore, we focused primarily on the quality of presentation of study results such as whether ITT *H. pylori* eradication rates were clearly presented, *etc.*

Subgroup analysis

Studies were grouped by combination regimens including different doses, treatment durations and types of publication (meeting abstract, full article).

Statistical analysis

The minimum number of studies required for statistical pooling was arbitrarily set at three and above. If only two studies were available for any subgroup analysis, results from the two studies were described and no pooling was undertaken. The following statistical techniques were used to analyze the data, wherever appropriate. Summary relative risk, risk difference and 95% CI were calculated from the raw data of the studies using a random-effects model. The number-needed-to-treat (NNT) was also calculated. The Cochrane Q method was used to test for homogeneity. $P < 0.1$ was considered to be statistically significant for test of homogeneity. In the presence of statistical heterogeneity, we searched for sources of methodological heterogeneity. We also examined publication bias by the method of Egger's regression^[12]. All analyses were performed with EasyMa software 2001 (M. Cucherat, Lyon, France).

RESULTS

The literature search generated 502 citations. Browsing the titles and abstracts reduced the number of citations to 246. Of these potentially useful citations, 224 were subsequently excluded for various reasons including review articles ($n = 8$), no raw data ($n = 104$), unclear patient population ($n = 68$), unlisted treatment regimens ($n = 17$), re-treatment ($n = 15$) and duplicate publications ($n = 13$). A detailed list of the excluded studies is available upon request.

Twenty-one studies met the predefined inclusion and exclusion criteria and were subject to analysis^[6-11,13-27].

RBC-containing regimens

RBC-containing triple or quadruple regimens were studied in 6 trials involving 10 treatment comparisons^[6,9,13-16]. Five studies were published in full^[6,13-16] and one in abstract form^[9]. The treatment-related information from these studies is tabulated in Table 1.

A total of 7 different combination regimens and 10 treatment arms were reported. Among the 10 treatment arms, 7 were given for 7 d and three for 5 d. Because of the large variability in drug combinations, meta-analysis was not possible for most combination treatments (Table 1). Triple therapies consisting of RBC (R), clarithromycin (C)

Table 1 Characteristics of RBC-containing triple and quadruple therapies

Refs	Combination regimens	Dose	Treatment duration	Eradication rate (ITT)		P
				PUD	NUD	
				N/T.N (%)	N/T.N (%)	
	Triple therapies					
	RBCCA					
6	RBC/Cla/Amo	400 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7 d	20/23 (86.96)	18/22 (81.8)	
15	RBC/Cla/Amo	400 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7 d	13/18 (72.2)	48/57 (84.2)	
16	RBC/Cla/Amo	400 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7 d	19/23 (82.6)	66/76 (86.8)	
	Others			52/64 (81.3)	132/155 (85.2) [†]	
13	RBC/Cla/Tin	400 mg b.i.d./500 mg b.i.d./500 mg b.i.d.	5 d	20/27 (74.1)	23/39 (58.97)	0.32
9	RBC/Cla/Tin	400 mg b.i.d./500 mg b.i.d./500 mg b.i.d.	5 d	6/9 (66.7)	9/12 (75)	0.95
16	RBC/Cla/Met	400 mg b.i.d./500 mg b.i.d./400 mg b.i.d.	7 d	20/21 (95.2)	69/78 (88.5)	0.61
16	RBC/Met/Tet	400 mg b.i.d./400 mg b.i.d./1 g b.i.d.	7 d	38/46 (82.6)	43/54 (79.6)	0.90
14	RBC/Fur/Tet	350 mg b.i.d./100 mg b.i.d./500 mg b.i.d.	7 d	20/24 (83.3)	31/36 (86.1)	0.94
14	RBC/Fur/Amo	350 mg b.i.d./100 mg b.i.d./1 g b.i.d.	7 d	15/19 (78.95)	34/41 (82.9)	0.99
	Quadruple therapy					
6	RBC/Cla/Amo/Met	400 mg b.i.d./400 mg b.i.d./1 g b.i.d./500 mg b.i.d.	5 d	174/174 (100)	62/71 (87.3)	<0.05

RBC, ranitidine bismuth citrate; Cla, clarithromycin; Tin, tinidazole; Fur, furazolidone; Tet, tetracycline; Amo, amoxicillin; Met, metronidazole; b.i.d., twice daily; PUD, peptic ulcer disease; NUD, non-ulcer dyspepsia; N/T.N, number/total number. ¹Relative risk: 0.96 (95%CI 0.84–1.11).

and tinidazole (T) were reported in two studies from the same lead author. However, personal communication with the corresponding author has excluded any duplication of patients. No statistically significant difference in *H. pylori* eradication rate was observed in patients with PUD or NUD treated with the 5-d RBT combination regimen in both reports^[9,13].

Three reports, involving RBC 400 mg, clarithromycin 500 mg and amoxicillin 1 g, all given twice daily for 7 d, were considered appropriate for meta-analysis^[6,15,16]. *H. pylori* eradication was achieved in 81.3% (52/64) of patients with PUD and 85.2% (132/155) in those with NUD, yielding a summary relative risk of 0.96 (95%CI 0.84–1.11); indicating that triple therapy consisting of RBC, clarithromycin and amoxicillin was similarly effective for eradicating *H. pylori* infection in patients with PUD compared to those with NUD. The test of homogeneity was not statistically significant (Q value = 1.1429, P = 0.56).

PPI-based quadruple combination treatments

We found five studies consisting of six treatment arms that provided comparative *H. pylori* eradication data in PUD and NUD patients^[8,13,17–19]. Of these, four were published in

full^[13,17–19] and one in abstract form^[8]. Table 2 tabulates the treatment-related characteristics of these studies. Because of the large variability in the drug combinations, dose and treatment duration among these studies, meta-analysis was not possible. However, within study comparison did not reveal any statistically significant difference in *H. pylori* eradication rates between patients with PUD and those with NUD in any of the combination regimens (Table 2).

PPI-based triple therapies

Nine studies and 10 treatment arms reported comparative data involving triple therapies consisting of a PPI, clarithromycin and amoxicillin^[7–11,20–22,27]. Of these, seven studies were only in abstract form^[7–10,20–22] and two were published in full^[11,27].

Table 3 summarizes treatment-related information from these studies.

Seven-day treatment involving a standard dose of PPI, clarithromycin 500 mg and amoxicillin 1 g, all given twice daily for 7 d, was used in six studies including 525 PUD patients and 617 NUD patients^[7,9,11,20,22,27]. *H. pylori* eradication was achieved in 82.1% of the 525 patients with PUD and 72.6% of the 617 patients with NUD, yielding a RR of

Table 2 Characteristics of bismuth- or PPI-based quadruple therapies

Refs	Combination regimens	Dose	Treatment duration	Eradication rate (ITT)		P
				PUD	NUD	
				N/T.N (%)	N/T.N (%)	
17	CBS/Met/Tet/Lan	120 mg ×8/250 mg ×8/250 mg ×8/30 mg b.i.d.	1d	22/33 (66.7)	5/13 (38.5)	0.16
17	CBS/Met/Tet/Lan	120 mg ×8/250 mg ×8/250 mg ×8/30 mg b.i.d.	2 d	26/35 (74.3)	20/40 (50)	0.06
13	Amo/Cla/Tin/Ome	1 g b.i.d./500 mg b.i.d./500 mg b.i.d./20 mg b.i.d.	5 d ^{1,2}	30/31 (96.8)	31/34 (91.2)	0.68
18	Bis/Met/Tet/Lan	120 mg q.i.d./400 mg t.i.d./500 mg q.i.d./30 mg q.d.	7 d	91/101 (90.1)	107/118 (90.7)	0.93
19	Bis/Met/Tet/Ome	120 mg q.i.d./400 mg t.i.d./500 mg q.i.d./30 mg q.d.	10 d	53/57 (93)	46/49 (93.9)	0.84
8	Bis/Met/Tet/Ome	120 mg q.i.d./500 mg t.i.d./500 mg q.i.d./20 mg b.i.d.	14 d	9/11 (81.8)	8/13 (61.5)	0.53

CBS, colloidal bismuth subcitrate; Met, metronidazole; Tet, tetracycline; Lan, lansoprazole; Bis, bismuth; Cla, clarithromycin; Rab, rabeprazole; Tin, tinidazole; Amo, amoxicillin; ×8, 8 times daily; q.i.d., four times daily; q.d., once daily; b.i.d., twice daily; Ome, omeprazole. ¹Rabeprazole and amoxicillin were used on d 1–5, clarithromycin and tinidazole on d 6–10; ²amoxicillin was used on d 1–5, clarithromycin and tinidazole on d 6–10, and omeprazole on d 1–10. PUD, peptic ulcer disease; NUD, non-ulcer dyspepsia; N/T.N, number/total number.

Table 3 Characteristics of triple therapies consisting of a PPI, clarithromycin and amoxicillin

Refs	Combination regimens	Dose	Treatment duration (d)	Eradication rate (ITT)		P
				PUD	NUD	
				N/T.N (%)	N/T.N (%)	
22	Ome/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7	134/161 (83.2)	34/42 (81)	
7	Ome/Cla/Amo	20 mg b.i.d. ¹ /500 mg b.i.d./1 g b.i.d.	7	60/65 (92.3)	30/50 (60)	
9	PPI/Cla/Amo	PPI b.i.d./500 mg b.i.d./1 g b.i.d.	7	27/34 (79.4)	15/21 (71.4)	
11	Rab/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7	101/135 (74.8)	288/392 (73.5)	
20	Pan/Cla/Amo	40 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7	27/29 (93.1)	13/19 (68.4)	
27	Rab/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7	82/101 (81.2)	68/93 (73)	
				431/525 (82.1)	448/617 (72.6) ²	
10	Ome/Cla/Amo	40 mg b.i.d./800 mg b.i.d./2 g b.i.d.	7	87/98 (88.8)	6/6 (100)	0.388
10	Ome/Cla/Amo	40 mg b.i.d./1.6 g b.i.d./2 g b.i.d.	7	51/51 (100)	49/50 (98)	0.313
8	Lan/Cla/Amo	30 mg b.i.d./500 mg b.i.d./1 g b.i.d.	10	13/13 (100)	65/71 (91.6)	
21	Ome/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	10	63/96 (65.6)	20/27 (74.1)	
27	Rab/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	10	75/97 (77.3)	78/99 (78.8)	
27	Ome/Cla/Amo	20 mg b.i.d./500 mg b.i.d./1 g b.i.d.	10	77/103 (74.8)	68/103 (73.1)	
				231/309 (74.8)	231/300 (77) ³	

Ome, omeprazole; Lan, lansoprazole; Pan, pantoprazole; Rab, rabeprazole; Cla, clarithromycin; Amo, amoxicillin; daily; q.d., daily; b.i.d., twice daily. ¹Omeprazole was used for 2 wk. PUD, peptic ulcer disease; NUD, non-ulcer dyspepsia; PPI b.i.d., PPI in standard dose. ²RR (95%CI): 1.15 (1.01-1.29). ³RR (95%CI): 1.03 (0.95-1.12). N/T.N, number/total number.

1.15 (95%CI 1.01-1.29). However, the test of homogeneity showed the presence of statistically significant between-study heterogeneity (Q Cochrane = 11.81, df = 5, $P = 0.037$). Scrutiny of these studies found that the heterogeneity was most likely caused by a large differential difference in *H. pylori* eradication rates between studies. Therefore, a random-effects model was used, which yielded a RR of 1.15 (95%CI 1.01-1.29). The result suggests that the 7-d PCA combination regimen given in the recommended doses cured significantly more *H. pylori* infections in patients with PUD than in those with NUD. The summary risk difference was estimated at 0.11 (95%CI -0.01-0.22), giving an estimated NNT of 9. The Egger's regression test for the detection of publication bias was not statistically significant ($r = -0.64$, $P = 0.08$) for the 7-d PCA triple therapy.

Figure 1 depicts the individual and summary relative risks and 95%CI.

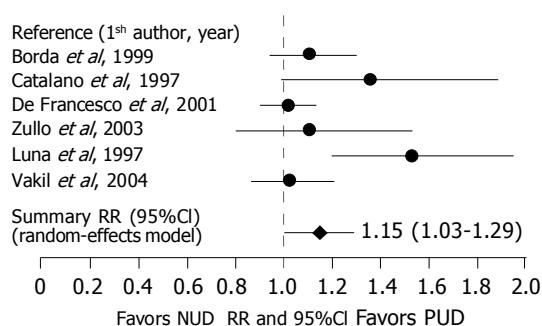


Figure 1 Individual and summary relative risks and 95% CI comparing the efficacy of 7 d PCA combination regimen in patients with PUD or NUD.

A subgroup analysis was conducted according to the type of publication. Of the six studies, four were published in abstract form^[7,9,20,22] and two as full articles^[11,27]. The

pooled eradication rates from the four meeting abstracts were 85.8% for patients with PUD and 69.7% for those with NUD, yielding a RR of 1.19 (95%CI 1.06-1.33). However, the test of homogeneity was statistically significant (Q Cochrane: 8.47, $P = 0.037$). A reanalysis of the data using a random-effects model generated a RR of 1.23 (95%CI 1.00-1.52), demonstrating a borderline statistically significant difference, indicating a trend that patients with PUD respond to the 7-d PCA combination treatment better than those with NUD.

Similar eradication rates were observed in the two reports published as full articles^[11,27]. An Italian study reported by Zullo and colleagues showed similar *H. pylori* eradication rates between patients with PUD (74.8%) and those with NUD (73.5%)^[11] (Table 3). A US study reported by Vakil *et al.*^[27], demonstrated that PCA eradicated 8.2% more *H. pylori* infections in patients with PUD than in those with NUD (Table 3). However, this difference was not statistically significant.

Sato and colleagues reported one study, involving two treatment arms, in which higher doses of antibiotics were used in 7-d PCA combination^[10]. However, no significant difference in *H. pylori* eradication rates between the two groups of patients was reported (Table 3).

Three studies reported efficacy data obtained with a 10-d standard dose PCA combination regimen involving 309 PUD patients and 300 NUD patients^[8,21,27]. The pooled eradication rate was 74.8% (231/309) for PUD patients and 77% (231/300) for NUD patients, respectively, yielding a RR of 1.03 (95%CI 0.95-1.12). The test of homogeneity was not statistically significant (Q Cochrane: 2.81, $P = 0.42$). Thus, there was no statistically significant difference in *H. pylori* eradication rate between the two groups of patients.

Four studies (five arms) reported comparative eradication data involving triple therapies consisting of a PPI, metronidazole and clarithromycin or amoxicillin^[23-26]. The results of these studies are tabulated in Table 4. However, no statistically significant difference in *H. pylori* eradication

Table 4 Characteristics of triple therapies consisting of a PPI, metronidazole and clarithromycin or amoxicillin

Refs	Combination regimens	Dose	Treatment duration	Eradication rate (ITT)		P
				PUD	NUD	
				N/T.N (%)	N/T.N (%)	
23	Pan/Met/Cla	40 mg q.d./400 mg b.i.d./250 mg b.i.d.	7 d	54/65 (83.1)	21/24 (87.5)	0.613
23	Pan/Met/Cla	40 mg b.i.d./400 mg b.i.d./250 mg b.i.d.	7 d	54/64 (84.4)	18/22 (81.8)	0.781
24	Ome/Met/Cla	40 mg q.d./500 mg b.i.d./250 mg b.i.d.	7 d	15/19 (78.95)	5/6 (83.3)	0.819
25	Pan/Met/Amo	40 mg b.i.d./500 mg b.i.d./1 g b.i.d.	7 d	14/19 (73.7)	7/11 (63.6)	0.569
26	Ome/Met/Amo	40 mg q.d./400 mg t.i.d. ¹ /750 mg b.i.d.	14 d	22/27 (81.5)	22/37 (59.5)	0.063

Ome, omeprazole; Pan, pantoprazole; Cla, clarithromycin; Amo, amoxicillin; Met, metronidazole; daily; q.d., daily; b.i.d., twice daily. ¹Metronidazole was used for 5 d only. PUD, peptic ulcer disease; NUD, non-ulcer dyspepsia.

rates between patients with PUD and those with NUD was reported in any of the studies^[23-26].

DISCUSSION

Individual studies suggest that patients with PUD may respond better to *H. pylori* eradication treatment than patients with NUD. However, the results have been conflicting between studies^[6-11]. Several factors may have contributed to the conflicting reports, which include a small sample size^[10,17,24,25], use of sub-optimal doses or inadequate treatment duration^[13,17]. In order to systematically examine this question, we comprehensively reviewed all relevant clinical trials that provided data on *H. pylori* eradication between patients with PUD and those with NUD.

Although there are numerous combination treatment regimens for eradicating *H. pylori* infection, only a few choices of treatments are currently recommended^[2-4,28,29]. Therefore, we focused on the combination treatment regimens that are currently recommended by the majority of authorities or consensus groups. These include triple therapies consisting of a PPI or RBC plus two antibiotics (clarithromycin, amoxicillin or metronidazole) or PPI-based quadruple therapies^[2-4,28,29].

No significant difference in the *H. pylori* eradication rate was observed between patients with PUD and those with NUD when RBC-based triple therapies were used, irrespective of the combination of antibiotics, dose regimens and treatment duration (Table 1). Similar results were also found in patients treated with PPI-based quadruple therapies (Table 2), suggesting that both RBC-based triple therapies and PPI-based quadruple combination therapies are similarly effective for eradicating *H. pylori* infection in patients with PUD or with NUD.

PPI-based triple therapies are the most commonly recommended treatment choice for eradicating *H. pylori* infection because of their highly consistent efficacy and excellent safety record^[30,31]. However, the number of studies that included both PUD and NUD patients was limited and this has prohibited us from conducting a reliable meta-analysis of all the recommended PPI-based triple therapies. We found that, with the exception of the 7-d PCA combination, no significant differences in *H. pylori* eradication rates have been observed in patients with PUD or NUD when other PPI-based triple therapies were used (Table 4). Among the six studies using the recommended PCA treatment, 9.5%

more *H. pylori* infections were cured in patients with PUD compared to those with NUD, which is statistically significant. However, the results from the subgroup analysis by publication type showed that the difference was caused by a large differential difference (16.1%) observed in the meeting abstracts because there was no statistically significant difference in the *H. pylori* eradication rates between the two groups of patients in the two full articles^[11,27]. Therefore, according to the current literature, there is no convincing evidence that patients with NUD respond to the 7-d PCA combination treatment significantly different from those with PUD. This is especially the case when the PCA combination treatment is used for 10 d and similar *H. pylori* eradication rates are achieved in both groups of patients^[8,21,27].

Nevertheless, Houben and colleagues observed that PPI-based triple therapies eradicated 8.8% more *H. pylori* infections in patients with PUD than in those with NUD ($P = 0.036$)^[32]. However, this study was published in abstract form and no detailed information on treatment regimens was provided^[32]. A similar, but statistically non-significant difference was also reported by Kamberoglou *et al*^[33], in a study involving 106 NUD and 142 PUD patients treated with either PPI- or RBC-based triple therapies in which 8.1% more *H. pylori* infections were cured in patients with PUD than in those with NUD. Similarly, the study was only available in abstract form. Furthermore, the authors did not separate patients treated with PPI-based therapies from those receiving RBC-based regimens, making it difficult to ascertain which combination treatment contributed most to the observed difference.

Why patients with NUD and PUD respond differently to the 7-d PCA combination regimen remains unclear. Several hypotheses suggested by Gisbert and colleagues indicate that infection with different strains of *H. pylori*, different severities of inflammation or patient compliance, age and gender may contribute to differences in *H. pylori* eradication rates between patients with PUD and NUD^[6]. Nevertheless, it is not clear why the difference was only seen with the 7-d PCA combination treatment, but not with other treatment regimens.

We combined patients with duodenal ulcer or gastric ulcer in this study because the evidence from individual studies suggests no significant difference in *H. pylori* eradication rates between patients with duodenal or gastric ulcer when the PCA combination^[10,21,22] or other PPI-based

triple therapy^[19] was used. The results show that *H. pylori* infection-related gastric or duodenal ulcers respond similarly to eradication treatment.

There are several limitations to our study. First, the nature of the study question which we assessed indicates that there are no randomized controlled clinical trials. Therefore, we included all study designs in the analysis and these may suffer from certain methodological weaknesses. Second, the number of trials available for analysis was limited especially for non-PCA combination regimens. Therefore, a type II error cannot be excluded. The small number of studies also prohibited us from conducting several clinically important subgroup analyses to assess the potential impact of bacterial resistance, different strains of *H. pylori* and the country of study on the difference in *H. pylori* eradication rates.

In conclusion, although individual studies may have suggested that patients with PUD respond better to *H. pylori* eradication treatment than those with NUD, we have not found any convincing evidence that this is the case across different combination treatment regimens although a trend exists with the recommended 7-d triple therapy consisting of a standard dose proton pump inhibitor, clarithromycin and amoxicillin.

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• BASIC RESEARCH •

Effects of mycophenolate mofetil vs cyclosporine administration on graft survival and function after islet allotransplantation in diabetic rats

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Abstract

AIM: To develop an experimental model of islet allotransplantation in diabetic rats and to determine the positive or adverse effects of MMF as a single agent.

METHODS: Thirty-six male Wistar rats and 18 male Lewis rats were used as recipients and donors respectively. Diabetes was induced by the use of streptozotocin (60 mg/kg) intraperitoneally. Unpurified islets were isolated using the collagenase digestion technique and transplanted into the splenic parenchyma. The recipients were randomly assigned to one of the following three groups: group A (control group) had no immunosuppression; group B received cyclosporine (CsA) (5 mg/kg); group C received mycophenolate mofetil (MMF) (20 mg/kg). The animals were killed on the 12th d. Blood and grafted tissues were obtained for laboratory and histological assessment.

RESULTS: Median allograft survival was significantly higher in the two therapy groups than that in the controls (10 and 12 d for CsA and MMF respectively vs 0 d for the control group, $P < 0.01$). No difference in allograft survival between the CsA and MMF groups was found. However, MMF had less renal and hepatic toxicity and allowed weight gain.

CONCLUSION: Monotherapy with MMF for immunosuppression was safe in an experimental model of islet allotransplantation and was equally effective with cyclosporine, with less toxicity.

INTRODUCTION

Islet cell transplantation can provide a minimally invasive method of restoring euglycemia and insulin independence early in the course of diabetes mellitus^[1]. This alternative treatment comprises a very promising therapeutic approach since the early 1970s^[2-4] and recent reports in the literature support this hypothesis^[5-9]. However, the results “fall short” compared to the expectations mainly due to the loss of thousands of islets during the three-stage islet isolation process, the need for immunosuppressive agents having significant diabetogenic side effects and the difficulty to detect graft rejection early^[10]. Thus, the reported 1-year insulin-independent survival after islet transplantation in 1999 was only 14%^[11].

A major issue in transplantation is graft rejection. The administration of immunosuppressive drugs such as cyclosporine (CsA), tacrolimus and corticosteroids is essential in order to prevent this complication^[12]. Nevertheless immunosuppressants are not devoid of serious side effects such as the induction of diabetes, nephrotoxicity and carcinogenesis^[13-17].

In 1993 mycophenolate mofetil (MMF), the 2-4-morpholino ethyl ester of mycophenolic acid, the biologically active component, was introduced as a novel immunosuppressive agent^[18]. MMF reversibly inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), an important enzyme in de novo synthesis of purine building blocks of DNA, namely guanine and adenine^[19]. Lymphocytes, which play a significant role in graft rejection, have no way of producing adequate amounts of purines if IMPDH is not available^[20]. Thus, MMF prevents proliferation of both T cells and B cells and thereby inhibits antibody production. Moreover, through depletion of intracellular GTP levels in lymphocytes, MMF suppresses glycosylation and the expression of some adhesion molecules, thereby reducing

lymphocyte migration to the transplant^[21]. However, its effect on T cell proliferation received more attention because of the importance of T cells in the allogeneic response^[22].

MMF is considered as a safe drug and the most frequently reported side effects are mild and involve mainly the gastrointestinal system (diarrhea, abdominal pain, nausea and vomiting)^[23-25]. Its major advantages are the lack of nephrotoxicity and diabetogenic effects, which makes MMF an important agent in renal and islet transplantation. Although its use in renal transplantation has been established^[26-28], its use in clinical islet transplantation is still limited^[29,30]. There are also a few reports regarding the use of MMF in combination with other regimens in experimental models of islet xeno- and allotransplantation^[31-37] but in very few cases as monotherapy.

The aim of this study was to evaluate the efficacy of MMF as monotherapy on islet graft function and development in a period as early as 12 d after allogeneic islet transplantation in chemically induced diabetic rats, with a dose lower than the usually administered and to compare it with the efficacy of a widely used immunosuppressant such as cyclosporine A.

MATERIALS AND METHODS

Animals and experimental groups

Thirty-six male Wistar rats and 18 male Lewis rats weighing 220-300 g were used as recipients and donors respectively (animals were obtained from Pasteur Institute, Athens, Greece and Democretos Research Center, Athens, Greece). All the principles for laboratory animal care were followed according to the European Union Regulations and the Greek law for the use of laboratory animals (Act 160, Volume 64, No. A, May 1991, License Ref. 1267/2570). The recipients were randomly allotted to three groups of 12 animals: group A (control group) had no immunosuppression, group B received CsA (Neoral - Novartis) at a dose of 5 mg/kg, and group C received a low dose MMF (CellCept - Roche) (20 mg/kg), which is half the usual dose.

Induction of diabetes

Diabetes was induced by single intraperitoneal injection of 60 mg/kg streptozotocin (STZ - Sigma - S-0130) freshly resolved in a solution of PBS (phosphate buffer solution Sigma 1000-3) at a concentration of 10 mg/mL (pH 4.5 using citric acid) 7 d prior to transplantation. It has been documented that doses bigger than 50 mg/kg cause irreversible and complete destruction of beta cells in adult rats^[38].

Isolation of unpurified islets

Islets were acquired with a modification to the technique previously described by Papalois *et al*^[39]. Briefly, after the animals were anesthetized a midline abdominal incision was performed and the common bile duct was recognized and ligated in its middle. Then the duct was catheterized with a fine catheter (polyethylene tubing, PE 10 - ID 0.28 mm and A 0.61 mm - Becton Dickinson) and 6 mL of collagenase (Sigma - Type XI - C 7657) solution (0.9 ng/mL) were infused slowly until the pancreas was distended. Subsequently pancreatic resection was performed and the donor was killed.

The pancreatic specimen was incubated in water bath (at 37 °C) for 20 min. After incubation the distended pancreas was washed twice in cold Hanks solution in order to terminate collagenase activity and to wash out collagenase and fat tissue. The product was then filtered through a 400- μ L filter in order to retain duct remnants, sutures, lymph nodes and pancreatic capsule remnants. The cell suspension was then considered ready for transplantation. We omitted the separation of the endocrine from the exocrine tissue in order to avoid the loss of islet yield during the purification process. A 100- μ L sample was taken and islets were counted by dithizone staining. The mean $\pm$ SE islet yield for transplantation was 1 812 $\pm$ 145.

Transplantation technique

Spleen was used as the site of transplantation^[40-42]. The rats were anesthetized as previously described and 0.7 cc of the isolated cell suspension was injected slowly using an insulin syringe into the splenic parenchyma. Leakage was avoided with a 2-0 silk suture tied at the spleen's pole.

Determination of biochemical indices

Blood glucose levels were measured in blood obtained from rat-tails using a Glucometer Elite blood glucose-measuring instrument (Bayer AB, Gothenburg, Sweden). Measurements were performed one week prior to transplantation (d -7), right after transplantation (d 0) and at the 3rd, 5th, 7th, 10th and 12th d post-transplantation. Diabetes was diagnosed when two consecutive glucose readings were over 180 mg/dL. Grafts were considered as functional when blood glucose levels were below 200 mg/dL after the 2nd post-operative d for two consecutive measurements. The 2 d interval was allowed for islets to become functional. Liver enzymes (SGOT, SGPT, γ GT) as well as creatinine values were determined from blood samples obtained on d 12 from 11 animals of the two therapy groups and from 5 animals of the control group that were still alive. The normal range of each of the biochemical indices for the Wistar rats used in our laboratory is as follows: SGOT: 116-278 IU/L, SGPT: 29-80 IU/L, γ GT: 0.6-2.1 IU/L, creatinine: 0.4-0.8 mg/dL.

Animals sacrifice

The overall time of observation was 12 d. On the last day the animals were killed and blood as well as grafted tissues were obtained for laboratory assessments and histological examination.

Histological examination of the grafted tissues

Spleens carrying the transplanted islet grafts were removed and fixed in formalin saline. Paraffin tissue sections (3- μ m thick) were stained with hematoxylin-eosin. Intracellular content of insulin was demonstrated immunohistochemically using the peroxidase technique by means of polyclonal anti-insulin antibodies (rabbit anti-insulin, Monosan, Netherlands) and with an indirect biotin streptavidin detection kit (iView™ DAB detection kit, Ventana Medical Systems, SA, France), which detects mouse IgG, mouse IgM, and rabbit polyclonal primary antibodies. Pancreas tissue was used as positive control. We estimated the intensity of staining as weak and intense. Evaluation was performed using light microscopy

(HE $\times 200$). The presence of insulin positive cells and intact islets was assessed using a semi-quantitative scoring system: none (0-1), occasional (1-8), many (9-18) and plentiful (>18) (numbers representing count of islets per 10 big optic fields). The presence of infiltrating inflammatory cells was also assessed using a semi-quantitative method (0 = occasional infiltrating cells, + = few, ++ = moderate, +++ = many, ++++ = massive infiltration)^[35]. Eleven out of the twelve grafted sites were available for histological examination from the CsA and MMF groups (one rat from each group died during the transplantation procedure). All grafted tissues from the control group were obtained for histological assessment regardless of the animal's day of death.

Statistical analysis

All values were presented as mean $\pm$ SD. Statistical analysis was performed using the Student's *t* test comparing group means. To evaluate the differences in graft survival, the Kaplan-Meier survival analysis was performed and a log rank test was used for the evaluation of differences. *P* values less than 0.05 were considered significant. Comparisons among time measurements of each variable during treatment period for each study group were analyzed using one factor repeated measures ANOVA (pair wise multiple comparisons were performed using Tukey critical difference). Mann-Whitney *U* test (exact significance) was used for the comparisons between groups.

RESULTS

The operation-related mortality in the three groups was as follows: no rat died in the control group, 1 out of 12 rats died in group B (8.3%) and 1 out of 12 died in group C (8.3%). All rats in CsA and MMF groups that survived the operation were alive at the 12th d (the day of killing). In the control group, however, mean survival was 8.1 d and only five rats reached the day of killing (41.7%). This difference was statistically significant ($P < 0.01$, Table 1).

Table 1 Islet allograft survival and animal survival in recipients treated with CsA and MMF

Variable	Group A Controls <i>n</i> = 12	Group B CsA <i>n</i> = 11	Group C MMF <i>n</i> = 11	<i>P</i> χ^2
3 rd -d graft survival (%)	3 (25)	9 (82)	9 (82)	<0.01
Graft survival in days after Tx (median SD)	0 (1.2)	10 (4.9)	12 (5.1)	Kruskal Wallis <0.01
Animal survival in days after Tx (median SD)	8.1 (3.7)	12 (0.0)	12 (0.0)	Kruskal Wallis <0.01

Tx: transplantation, CsA: cyclosporine A, MMF: mycophenolate mofetil.

The functional outcome of islet allografts was evaluated after d 3 (Table 1). In the control group, 3-d allograft survival was significantly less than both cyclosporine and MMF groups (25% *vs* 82%, $P < 0.01$, Table 1). Median allograft survival was also significantly higher in the two therapy groups than that in the controls (10 and 12 d for CsA and MMF respectively *vs* 0 d for the control group, $P < 0.01$, Table 1). Actuarial allograft survival was calculated for all

three groups and the Kaplan-Meier curves were constructed (Figure 1). In both therapy cases the differences in allograft survival in comparison to the control group were statistically significant (log rank test, $P < 0.01$ for MMF *vs* control and $P < 0.01$ for CsA *vs* control). In contrast, the difference in actuarial allograft survival between the CsA and MMF groups was not statistically significant (log rank test, $P = 0.505$).

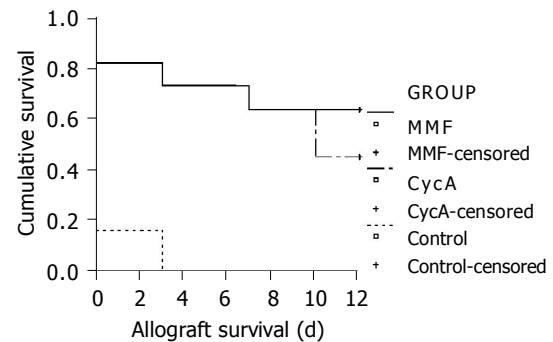


Figure 1 Actuarial allograft survival curves in the three study groups based on serum glucose levels, showing significantly prolonged survival in the two therapy arms in comparison to the controls.

Glucose changes from baseline were recorded (Figure 2). Significant overall differences between the three groups were observed at the 3rd, 5th, 7th, 10th and 12th post-transplantation day (ANOVA, Figure 2). These differences, at all times but one, were attributed to higher glucose levels in the control groups (Tukey). The difference between the CsA and the control group was not significant only on the 3rd post-transplantation day and consequently the overall difference was attributed to lower glucose levels in the MMF group. The glucose values over time tended to be at lower levels in the MMF group compared to the CsA group although this observation was not statistically significant ($P = 0.747$).

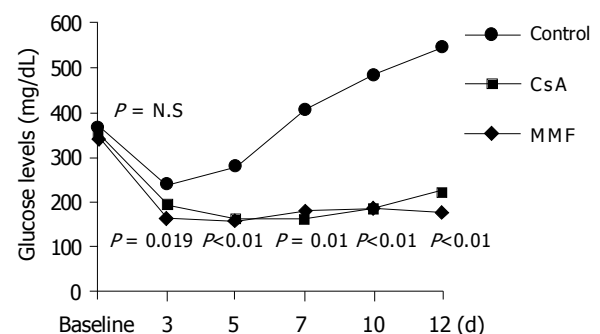


Figure 2 Effect of MMF and CsA on the blood glucose levels of the recipients over time after transplantation.

Weight changes were also recorded for all recipients (Figure 3). Statistically significant differences in the proportion of weight change from baseline were found between the three groups after the 7th post-operative day. These changes were significant also in the 10th and the 12th d. The MMF

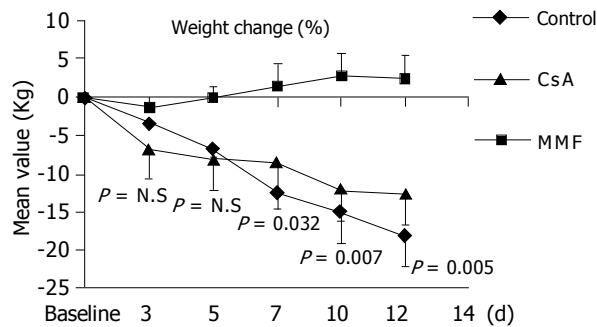


Figure 3 Effect of MMF and CsA on the percentage of weight change of the recipients.

group (Figure 3) was the only one in which weight gain was recorded, although this observation was not statistically significant.

Post-transplantation biochemical data (SGOT, SGPT, γ GT and creatinine) in the two therapy cases and in the control group were also recorded. All the post-transplantation biochemical parameters in the control group and in the MMF group were within normal range. The same observation was made for the CsA group, except for γ GT values (Table 2). All biochemical data recorded, except for SGOT values, were significantly lower in the MMF group in comparison to corresponding values in the CsA group (Table 2). Compared with the control group creatinine levels were significantly lower in the MMF group ($P < 0.027$) and γ GT levels were significantly elevated in the CsA group ($P < 0.01$, Table 2).

Table 2 SGOT, SGPT, γ GT and creatinine measurements in the controls as well as in the two therapy groups

Variable	Group A Controls	Group B CsA	Group C MMF
SGOT (mean, SD)	144 (27.6)	119 (47)	131 (46)
SGPT (mean, SD)	48.4 (12.9)	66 (27)	33 (15) ^f
γ GT (mean, SD)	1.14 (0.42)	2.8 (0.5) ^b	0.7 (0.5) ^f
Creatinine (mean, SD)	0.6 (0.16)	0.6 (0.1)	0.4 (0.1) ^{df}

^b $P < 0.01$ vs controls, ^d $P < 0.027$ vs controls, ^f $P < 0.01$ vs CsA.

Light microscopy histological assessment of the grafts from the three different groups revealed the appearance of exocrine tissue, islets clumped together or free-lying in the surrounding exocrine tissue, peri-islet tissue inflammatory infiltration and areas of necrosis (Table 3). In untreated rats a massive infiltrate covered most of the allografts and completely destroying most of them (Table 3). Twelve days after transplantation various numbers of islets and various degrees of infiltration were observed in allografts removed from animals treated with CsA or MMF alone. Immunohistochemical staining for insulin in the control group was relatively weak (Figure 4A), whereas in both treatment groups a marked number of cells had more intense insulin staining (Figures 4B and C). Bigger and better developed islets were found in the MMF-treated group (Figure 4C).

Table 3 Histologic evaluation after transplantation

Group Animal	CsA Inf/End	MMF Inf/End	Controls Inf/End
1	0/Occasional	0/Occasional	0/Occasional
2	0/Occasional	+ /Occasional	+++ /None
3	0/Many	+ /Occasional	++++ /None
4	++++ /Occasional	+ /Plentiful	++++ /None
5	++++ /Occasional	+++ /Plentiful	++++ /None
6	++++ /Many	++++ /Occasional	++ /Occasional
7	++++ /Many	++++ /Occasional	++ /Occasional
8	++++ /Many	++++ /Occasional	+++ /Occasional
9	Necrosis	++++ /Occasional	++++ /Occasional
10	Necrosis	++++ /Occasional	Necrosis
11	Necrosis	Necrosis	Necrosis
12	-	-	Necrosis

Inf: infiltrating cells, End: endocrine cells. Sections with abundant necrosis were not evaluated.

DISCUSSION

In the present study we compared the efficacy of MMF and CsA in an experimental model of islet allotransplantation. The present study was based on a previous one^[45] in which the efficacy of two different doses of MMF (12 and 23 mg/kg) with CsA (5 mg/kg) was compared. In the previous study MMF in the dose of 23 mg/kg was equally effective with CsA in maintaining graft function. However, graft survival in the group of 12 mg/kg was not satisfactory. Consequently it was decided to omit the MMF group that was not effective, trying to have higher MMF levels and at the same time to test a dose that is half of that presented in many reports.

Immunosuppression with cyclosporine (5 mg/kg) maintained graft function for a median of 10 d, while administration of MMF at a dose of 20 mg/kg was equally effective prolonging graft survival for a median of 12 d. The present results are in accordance with those reported by other researchers demonstrating that treatment with CsA and MMF alone or in combination with other immunosuppressants reduces allograft and xenograft rejection. However, in these studies higher doses were employed (10-30 mg/kg for CsA and 40 mg/kg for MMF)^[32-37]. Previously reported data have shown that CsA increases insulin resistance and has nephrotoxic and hepatotoxic effects, especially when combined with other agents such as glucocorticoids and sirolimus^[41-43]. In clinical studies MMF has been shown to be a safe drug and furthermore there is evidence that it not only maintains the graft but improves renal function as well^[26-28].

The ability of MMF to preserve islet allo- and xenograft function is not only due to the selective antiproliferative effect on B and T cells, but also due to the protection of the microvasculature from the immune response, even from the 10th d after transplantation. As a consequence the grafts' nutritive microcirculation^[44] is preserved. In the present study, despite the limited observation period, the fact that larger and better-developed islets were found in the MMF group in comparison to the cyclosporine group suggests an immediate beneficial effect of MMF in the preservation of islet architecture. Interestingly, in our previous report

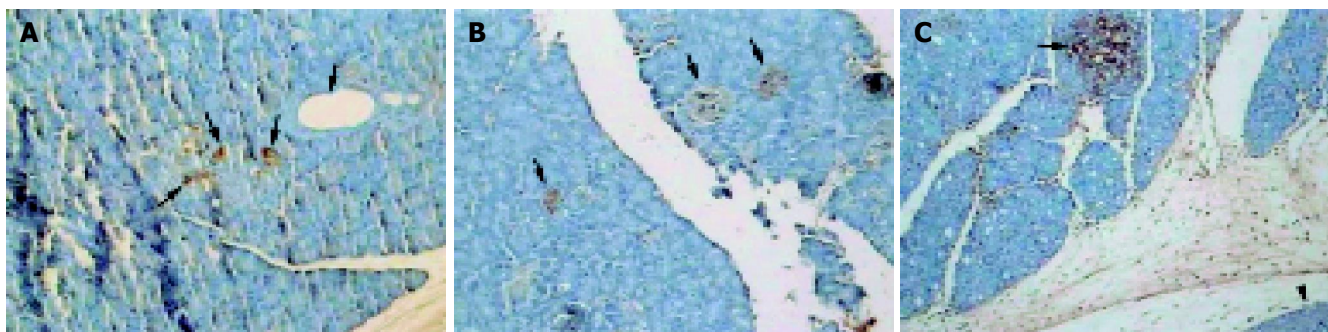


Figure 4 **A:** Three weak stained islets (long arrows) located into the exocrine tissue 12 d after transplantation without immunosuppression. A dilated pancreatic duct (short arrow) is also present (anti-insulin, $\times 200$); **B:** Intact islets into the exocrine tissue (long arrows) 12 d after engraftment and immunosuppression with CsA. The insulin staining is positive and intense (anti-insulin, $\times 200$); **C:** A

well-developed and large islet (long arrow) into the exocrine tissue 12 d after transplantation and immunosuppression with MMF. Anti-insulin stain shows beta cell granulation within islets. At the lower right corner of the figure splenic parenchyma is present (short arrow) (anti-insulin, $\times 200$).

such beneficial effect on islet architecture was observed even when approximately half of the present dose of MMF was used^[45].

It was observed that transplantation of allogeneic islet tissue without immunosuppression resulted in 100% rejection within few days. It has been well established that the islet allografts survive about 5 d in diabetic rats without immunosuppression^[37]. The fact that very few of the grafts in the control group became functional after the 3rd d of transplantation is probably due to the presence of exocrine tissue. Exocrine tissue contamination of freely transplanted pancreatic islets deteriorates the process of graft revascularization and induces additional injury by provoking a deleterious inflammatory response and consequently leading to graft destruction^[46,47]. Taking into account the massive infiltration detected in the grafts of untreated recipients in the present study, this might be the reason for the low graft survival in this group.

In this experimental model of islet transplantation creatinine, SGPT and γ GT values were found to be lower in the MMF group compared to those found in the CsA group and with the exception of γ GT values all other biochemical data in the CsA group did not exceed the normal range. The significantly increased γ GT values found in the CsA case are indicative of a cholestatic effect of the specific drug, a well-documented side effect^[48]. Interestingly, creatinine values were found to be lower in the MMF group compared to controls. This finding is in accordance with the published data that MMF may protect from and even reverse nephrotoxicity caused by other immunosuppressant agents, such as CsA^[26,49]. The fact that the MMF group was the only one in which weight gain was recorded indicates that this agent is well tolerated without serious side effects.

In conclusion, low dose MMF provided effective immunosuppression in an experimental allograft islet transplantation model and compared favorably to CsA in terms of islet morphology and side effects. Given the fact that complications of immunosuppressive therapy continues to be one of the major hurdles to successful islet transplantation, management of immunosuppression requires careful risk *vs* benefit assessment. Favorable benefit/side effects ratio for the biochemical and histological parameters with the low dose monotherapy of MMF was

observed in the present study, compared to data presented in other reports. This drug might represent a standard suitable immunosuppressive agent for improving the outcome of pancreatic islet allo-transplantation.

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• BASIC RESEARCH •

Protective role of metallothionein in stress-induced gastric ulcer in rats

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CONCLUSION: In WIR-stressed rats, the MT level was increased in serum and in stomach and liver tissues. Pre-administration of exogenous MT or pre-induction of endogenous MT can protect the gastric mucosa against stress-induced ulcers and inhibits the formation of stress-induced lipid peroxide. MT could have a gastroprotective effect and might be a new interventional and therapeutic target in stress-induced gastric ulcers.

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Key words: Metallothionein; Gastric ulcer

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Abstract

AIM: To illustrate the pathophysiological role of metallothionein (MT) in gastric ulcer induced by stress.

METHODS: Wistar rats underwent water-immersion-restraint (WIR) stress, ZnSO₄ (an MT inducer) treatment, WIR+ZnSO₄ or WIR+MT, and the ulcer index (UI) was estimated in excised stomach and liver tissues. The mRNA level of gastric MT was determined by semi-quantitative RT-PCR. The MT content in gastric and hepatic tissues was determined by Cd/hemoglobin affinity assay. The lipid peroxidation products malondialdehyde (MDA) and conjugated dienes (CD) were estimated by use of thiobarbituric acid reactive species and ultraviolet spectrophotometry.

RESULTS: WIR stress induced severe gastric mucosal lesions in rats. Compared with control rats, stressed rats had increased lipid peroxide content in serum and stomach and liver tissues. MDA content was increased by 34%, 21% and 29% and CD level by 270%, 83% and 28%, respectively. MT content in the stomach and liver was increased by 0.74- and 1.8-fold, and the MT-mRNA level in the stomach was increased by 26%. Pretreatment with ZnSO₄ prevented gastric lesion development (the UI was 87% lower than that without pretreatment), and the MDA and CD content in serum and tissues was lower. The MT content in the liver was double in rats that were not pretreated, and the MT mRNA level in the stomach was 35% higher. MT administration 1 h before the WIR stress prevented gastric lesion development (the UI decreased by 47% compared with that in rats not pretreated), and the MDA and CD content in serum and tissues was significantly lower.

INTRODUCTION

Physical or psychological stress is one of the common causes of upper gastrointestinal ulceration^[1]. Since Hans Selye's report established that gastric ulcer occurs under chronic stressful conditions, a plethora of investigations have been performed to unravel mechanisms underlying stress-induced gut lesions^[2]. Molecules and their receptors involved in the stress response are being characterized. Clinical observations indicate that various kinds of stress such as shock, burns, sepsis, and severe trauma as well as severe emotional distress are closely related to acute upper gastrointestinal erosions and ulcerations^[3]. Although the pathogenesis of gastric lesions is not completely understood, mucosal ischemia and enhanced back diffusion of hydrogen ions have been proposed as major pathophysiological phenomenon in these stress-induced injuries. The production of oxygen-free radicals via the xanthine-xanthine oxidase system and neutrophils and lipid peroxidation initiated by the produced reactive oxygen species (ROS) have been investigated to explain the mechanisms of acute gastric lesion formation associated with stress.

Lipid peroxidation induced by oxygen-free radicals may play a critical role in the pathogenesis of acute gastric mucosal injury in experimental animals and humans^[4,5]. As well, stress could agitate the injured and defensive capacity of the body. On one hand, stress can increase the injured substances; on the other, it can enhance the endogenous defense systems. So, it is important to reinforce the function of the defense system for the prevention and therapy of stress-induced diseases.

Metallothionein (MT) is a low-molecular-weight, heavy-metal-binding, cysteine-rich protein. The highest concentration of MT is found in the liver, kidney, intestine and pancreas. Called as an acute-phase protein or stress protein, MT responds to various stresses such as physiological and psychological stresses^[6], and MT induced by stressors has been shown to detoxify harmful heavy metals and ROS produced by the stressors^[7]. The ability of MT to capture hydroxyl radicals, which are primarily responsible for the toxicity of ROS, is 300 times greater than that of glutathione, the most abundant antioxidant in the cytosol^[8]. In addition, MT has been implicated in protecting cells from stress by regulating metabolism and the immunity reaction^[9].

We hypothesized that MT may play a central role as a protective substance in the development of gastric mucosal lesions in rats under stress. Therefore, we investigated the expression of MT in rats treated with a water immersion restraint (WIR) and examined the effects of exogenous MT and pre-induction of endogenous MT on the formation of stress-induced gastric ulcers. We attempted to confirm whether MT has a protective effect on the pathogenesis of gastric ulcer due to stress and to explore its mechanism.

MATERIALS AND METHODS

Materials

All animal care and experimental protocols were in compliance with the Animal Management Rule of the People's Republic of China and the Care and Use of the Laboratory Animals guide of the First Hospital, Peking University. Wistar rats weighing 180-220 g were maintained under standard conditions of temperature, humidity and light (12 h dark, 12 h light). They were provided with Purina chow and free access to water. MT (from rabbit liver), ZnSO₄ (an MT inducer) and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO); ¹⁰⁹Cd was purchased from Dupont NEN Inc. (Boston, MA); TRIzol reagent was from Gibco (Gaithersburg, MD); dNTP was from Clontech (Palo Alto, CA); and M-MuLV reverse transcriptase, Taq DNA polymerase, RNasin and oligo (dT) primer were from Promega (Madison, WI). Oligonucleotides used for amplification were synthesized by Sai Bai Sheng (Beijing, China). The MT-I sequences of oligonucleotide were MT-I sense (MT-S), 5'-GCG ATC TCT CGT TGA TCT CC 3'; and MT-I antisense (MT-A), 5'-CAG CTG CAC TTG TCC GAA G 3'. The β-actin sequences of oligonucleotide were sense, 5'-ATC TGG CAC CAC ACC TTC-3'; and antisense, 5'-AGC CAG GTC CAG ACG CA-3'. Other chemicals were of analytical grade.

Animal experiments

Rats were randomly divided into control, WIR, ZnSO₄, WIR+ZnSO₄ and WIR+MT groups (*n* = 6 for every group) and were starved for 24 h but were allowed free access to water. The ZnSO₄ and WIR+ZnSO₄ groups were injected subcutaneously with 0.8 nmol/kg ZnSO₄ 24 h before killing. Except for control rats, all rats were restrained in a wire cage and immersed up to the depth of the xiphoid process in a 23 °C water bath to create WIR stress^[10]. MT was injected intraperitoneally in the WIR+MT rats (2.5×10⁻⁷ mol/kg) 1 h

before the onset of WIR stress. Rats were killed under pentobarbital anesthesia after 6-h WIR stress. After infusion of 10 mL of saline and fixation with 40 g/L formaldehyde for 5 min, rats were cut open along the gastric greater curvature, and stomachs and livers were removed. The gastric mucosa was carefully examined for lesions linear breaks (erosions) at the mucosal surface of the glandular part under a stereoscopic microscope. The ulcer index (UI), extent of the lesion, is expressed as the sum of the length of these breaks per stomach^[11].

Determination of the MT concentration by ¹⁰⁹Cd/hemoglobin affinity assay

The MT concentration in tissues was determined by use of the ¹⁰⁹Cd/hemoglobin affinity assay^[12]. Tissues from the stomach and liver were homogenized in four volumes of 10 mmol/L Tris-HCl buffer (pH 7.4), and the homogenate was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was heated for 2 min in a boiling water bath, then centrifuged at 10 000 *g* for 2 min to remove heat-precipitated proteins. A 200-μL aliquot of each column fraction or heat-denatured tissue supernatant fraction was placed in a 1.5-mL microcentrifuge tube. The samples were 10-fold to keep the MT concentration in the range of the assay. An amount of 200 μL of radioactive cadmium (2.0 mg ¹⁰⁹CdCl₂/mL with radioactivity of 1.0 μCi/mL) was added to each centrifuge tube, mixed, and incubated for 10 min at 25 °C. The free cadmium was removed by the following procedure: 100 μL of 2% bovine hemoglobin solution was added to each tube, and the tubes were heated in boiling water for 2 min, cooled in ice, and centrifuged at 10 000 *g* for 5 min at 4 °C. This procedure was repeated twice to remove the excess radioactive cadmium. A clear supernatant fluid was transferred to a gamma counting tube. The amount of radioactivity in the last supernatant fraction was then measured by use of a gamma counter (Perkin Elmer 1470). The quantity of cadmium-binding proteins (MT equivalents) was calculated^[12] and expressed as micromoles of cadmium per gram of heat stable protein.

Measurement of malondialdehyde (MDA)

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species with use of 1,1,3,3-tetraethoxypropane as a standard. Briefly, an aliquot of 250 μL of serum or tissue homogenates was mixed thoroughly with 2.25 mL of fresh TBA (0.67%) and heated at 95 °C for 30 min in a water bath. The suspension was then cooled to room temperature, centrifuged at 3 000 r/min for 10 min, and the pink colored supernatant was taken for spectrophotometry measurement at 532 nm for MDA assay. MDA content was expressed in terms of nanomoles per liter for serum and nanomoles per gram wet weight of tissue^[13].

Measurement of conjugated dienes (CD)

Lipid peroxidation measurement by CD level was performed as described^[14]. An aliquot of 500 μL of serum and tissue homogenates was extracted in chloroform-methanol (2:1) and dried with N₂. The dried extract was re-dissolved in cyclohexane and absorbency measured at 234 nm by use of a spectrophotometer.

Semi-quantitative RT-PCR

Total RNA of gastric tissue was prepared by *in situ* lysis of the tissues with TRIzol reagent. One microgram total tissue RNA was reverse-transcribed into single-strand cDNA with use of M-MuLV reverse transcriptase and oligo (dT) 15 primers. PCR was performed in a 0.2-mL tube containing tissue cDNA (2 μ L), 5 μ mol/L per each MT-S and MT-A primer mixture (1 μ L), 2.5 mmol/L per each dNTP mixture (1 μ L), 1.5 mmol/L MgCl₂ (1.5 μ L), 10 $\times$ PCR buffer (2.5 μ L), and 1.25 unit Taq DNA polymerase, in a total volume of 25 μ L. After denaturing at 95 $^{\circ}$ C for 5 min, PCR was run at 94 $^{\circ}$ C for 30 s, 61 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s for 30 cycles (72 $^{\circ}$ C for 5 min). A total of 8 μ L of PCR product was separated in a 1.5% agarose gel, and stained with ethidium bromide. The optical density of the 256-bp band was measured with use of the Gel Documentation System (Bio-Rad, Hercules, CA). The amplification of MT cDNA was confirmed by digestion of the PCR products with restriction enzyme *Bgl*II. An amount of 2 μ L PCR product was amplified again with the two rat β -actin primers at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 40 s for 24 cycles (72 $^{\circ}$ C for 5 min), and the optical density of the β -actin band (291 bp) was measured. The ratio of MT mRNA to β -actin mRNA was considered as the relative amount of MT mRNA.

Statistical analysis

All data are expressed as mean $\pm$ SD. Statistical differences were evaluated by one-way ANOVA and then by the Student-Newman-Keuls test; comparisons between two groups involved the use of Student's *t*-test. Linear regression analysis was used to assess the correlation between variables. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Gastric mucosal lesions developed in rats subjected to WIR stress over 6 h, as shown in Figure 1. Macroscopic observation showed lesions, most often 1–2 mm in size, or petechial bleeding. The area of involvement was confined to the glandular part of the stomach. The mean UI was 42.8 ± 11.8 (Figure 2). In contrast, control rats had no lesions. As shown in Table 1, lipid peroxide content in the gastric mucosa of stressed rats was higher than that of the control rats. Compared with control rats, stressed rats showed increased MDA content in the serum and stomach and liver tissues, by 34%, 21% and 29%, respectively (all $P < 0.01$), and increased CD content, by 270%, 83% and 28%, respectively (all $P < 0.01$, Table 1).

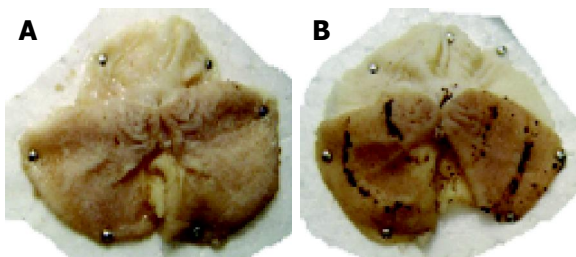


Figure 1 Gastric ulcers induced by WIR stress in the rat stomach. **A:** Stomach of normal rats; **B:** gastric ulcers in stomach of the WIR-stressed rats.

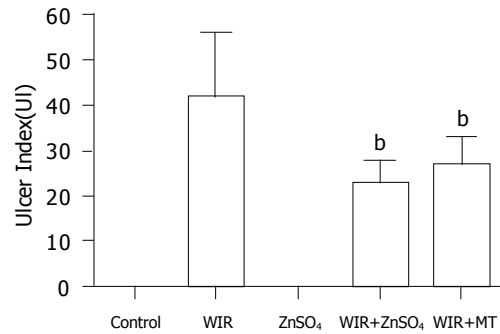


Figure 2 UI in WIR-stressed rats and ZnSO₄ and MT treatment. The UI was ^b $P < 0.01$ vs WIR group.

Compared with control rats, stressed rats showed increased MT content, by 0.74- and 1.8-fold (all $P < 0.01$), in the stomach and liver, respectively; the MT-I mRNA level in the stomach was increased by 26% ($P < 0.01$, Figure 3).

MT content in the stomach and liver of ZnSO₄-treated rats was increased by 74% and 180% compared with that in control rats (all $P < 0.01$). MT was generated effectively in the WIR+ZnSO₄ group and attenuated gastric lesion development (with an 87% lower UI than that of WIR-alone rats; $P < 0.01$). Compared with WIR-alone rats, the WIR+ZnSO₄-treated rats showed double the MT content in hepatic tissues ($P < 0.01$), and the MT-I mRNA level in the stomach was increased by 35% ($P < 0.01$). Pretreatment with ZnSO₄ also ameliorated the increased level of lipid peroxidation products induced by WIR stress in serum, and stomach and liver tissues, decreased the MDA content, by 14% ($P < 0.05$), 16% ($P < 0.05$) and 26% ($P < 0.01$), respectively, and decreased the CD content, by 49% ($P < 0.01$), 18% ($P < 0.05$) and 18% ($P < 0.05$, Table 1).

MT pretreatment not only prevented gastric lesion development (the UI decreased by 47% compared with that in WIR-alone rats; $P < 0.01$) but also inhibited the formation of MDA and CD. Compared with WIR-alone rats, MT-pretreated rats showed decreased MDA content in the stomach and liver, by 20% ($P < 0.05$) and 19% ($P < 0.05$), respectively, and decreased CD content in serum and stomach, by 15% ($P < 0.05$) and 49% ($P < 0.01$, Table 1), respectively.

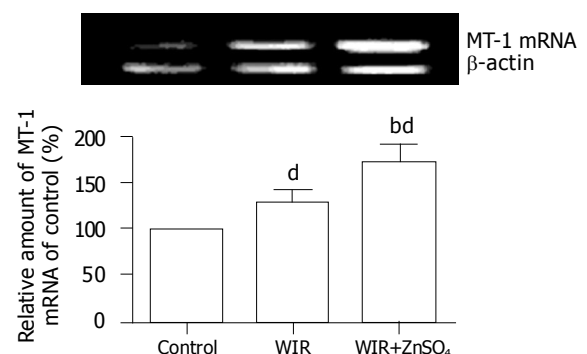


Figure 3 RT-PCR analysis of stomach MT-1 mRNA level. The data (expressed as ratio of MT-1 mRNA and β -actin mRNA) were shown as mean $\pm$ SD ($n = 3$ /group) ^b $P < 0.01$ vs control, ^d $P < 0.01$ vs WIR group.

Table 1 The content of MT, CD and MDA in rat plasma, stomach and liver (mean±SD)

	Control	WIR	ZnSO ₄	WIR+ ZnSO ₄	WIR+MT
CD content in plasma (A/mL)	10.19±1.81	37.37±2.04 ^b	10.33±2.07 ^a	19.08±1.52 ^{a,b}	18.64±1.32 ^{a,b}
CD content in stomach (A/g tissue)	39.96±6.74	73.09±3.71 ^b	51.39±4.41 ^{a,b}	60.48±3.93 ^{a,b}	62.43±4.99 ^{a,b}
CD content in liver (A/g tissue)	64.87±3.05	83.26±3.78 ^b	66.99±4.7 ^a	68.07±4.93 ^a	80.76±3.16 ^b
MDA content in plasma (nmol/mL)	3.65±0.41	4.88±0.30 ^b	3.98±0.35 ^a	4.20±0.21 ^a	4.77±0.18 ^b
MDA content in stomach (nmol/g tissue)	4.17±0.18	5.06±0.77	4.67±0.54	4.21±0.62	4.01±0.32 ^a
MDA content in liver (nmol/g tissue)	6.86±0.86	8.87±1.20 ^b	7.27±0.57 ^a	6.60±0.87 ^a	7.21±0.44 ^a
MT content in stomach (μg/g protein)	1.080±0.066	1.875±0.285 ^c	1.925±0.7 ^{c,e}	1.813±0.448 ^{c,e}	1.493±0.188 ^c
MT content in liver (μg/g protein)	9.955±1.358	28.325±7.117	54.165±19.224 ^{c,e}	56.63±15.218 ^{c,e}	12.575±6.106 ^c

n = 6 in each group. ^a*P*<0.05 vs stress group, ^b*P*<0.01 vs control group, ^c*P*<0.05 vs control, ^e*P*<0.05 vs stress group, respectively.

DISCUSSION

It is well known that oxygen-free radicals, primarily superoxide anion (O₂^{•-}) and hydroxyl radical (OH[•]), play an important role in the pathogenesis of acute gastric lesions induced by experimental stress^[15]. Such radicals cause tissue injury through lipid peroxidation. ROS can induce cellular membrane damage by peroxidation of phospholipid fatty acids. In rats subjected to WIR stress, the development of gastric mucosal lesions is closely related to the enhanced formation of lipid peroxide and enhanced oxidation of nonprotein sulfhydryl and nonprotein SH, which depends on an increased generation of oxygen-free radicals such as superoxide anion via the xanthine-xanthine oxidase system^[16-18].

MT was first isolated from horse kidney and characterized more than 40 years ago^[19]. Typically, MT has low molecular weight (<7 000 Da). There are four known isoforms. The MT-I and MT-II isoforms have a ubiquitous tissue distribution and are abundant in the liver, pancreas, gastrointestinal system and kidney, whereas MT-III and MT-IV are restricted to the brain and skin. The MT-I and MT-II isoforms are inducible by various stress conditions and compounds, including ROS, glucocorticoids, cytokines, and metal ions. In contrast, the MT-III and MT-IV isoforms are relatively unresponsive to these inducers.

Several lines of evidence suggest that MT can protect cells from damage caused by agents. For example, overexpression of MT in cells diminishes the sensitivity of the cells to compounds that generate oxygen-free radicals, DNA-damaging agents such as UV radiation, and nitric oxide, whereas targeted disruption of the MT gene renders the cells significantly more sensitive to stress damage^[20,21]. MT-I overexpression provides protection against ischemia-reperfusion injury of the heart, and pre-induction of hepatic MT by Zn can diminish hepatic injury by lipid peroxidation^[22]. These results suggest that MT could play a central role in protecting cells against oxidative stress. Growing experimental evidence suggests that lipid peroxidation is an important pathogenetic factor of gastric mucosal lesions induced by stress^[15]. However, the alteration of MT generation and its role during the pathogenesis of stress-induced gastric ulcer is unclear.

The present study has shown that rats treated with WIR stress showed greater development of gastric ulcer. Gastric mucosal injury, or stress ulceration, arose in conscious animals in response to psychological stress from immersion in water with physical restraint. The most common group of indices used to assess oxidative stress is peroxidation

products of lipids, usually polyunsaturated fatty acids, which are susceptible to attack by free radicals. The initial products of lipid peroxidation are CD hydroperoxides. These active substances decompose into various aldehydes or, if the original fatty acid is arachidonic acid, isoprostanes. All of these products of degradation and decomposition (CD and the widely used end product MDA) are used in assessing oxidative stress. In the present study, rats subjected to WIR stress for 6 h showed significantly increased levels of MDA and CD in serum and gastric and hepatic tissues.

As shown in our experiment, in WIR-stressed rats, the MT level in the stomach and liver was significantly increased and the MT mRNA expression up-regulated. ZnSO₄, a strong inducer of MT, markedly enhanced the MT generation in gastric and hepatic tissues and up-regulated MT mRNA expression. Moreover, pre-treatment with ZnSO₄ ameliorated the generation of stress-induced gastric ulcer and reduced the levels of lipid peroxide (MDA and CD) in the serum and gastric and hepatic tissues. Exogenous MT administered 1 h before WIR stress effectively suppressed the generation of gastric ulcer^[23] and significantly decreased the formation of lipid peroxidation products. These results indicate that MIR stress-induced endogenous MT generation, and endogenous MT pre-induced or supplemented inhibited the formation of lipid peroxide and gastric ulcer. MT could be a protective factor against stress-induced gastric mucosal injury. Other researchers^[24,25] treated the mice with ethanol or pylori, and they found that the gastric lesion indices were significantly higher in MT-null mice than in wild-type mice; these results suppose that MT may play a cytoprotective role under these conditions.

MT is an acute-phase protein induced by various stressors. The MT gene promoter has response elements to heavy metals (MRE), glucocorticoids (GRE), oxidative agents, electrophilic compounds and xenobiotics (ARE). The expression and synthesis of MT are induced for heavy metals (Zn, Cu, Cd and also Hg, Pb, As, Ni, Ag), glucocorticoids and other stress hormones, cytokines, and free radicals, as well as UV and ionizing radiation. Zn and Cu are physiological inducers of MT. Other inducers act more or less actively as stress agents. Receiving the earliest attention as regulators of MT expression, glucocorticoids are responsible in part for augmenting hepatocyte zinc pools during such stimulation. Two glucocorticoid response-element sequences are 17 kb upstream in the 5' flanking region of the mouse MT promoter. WIR stress promptly

enhances the release of glucocorticoid hormone^[26]. MT is known to be the strongest endogenous scavenger for hydroxyl radicals, and kinetic study *in vitro* demonstrated that MT was 38.5-fold more potent than glutathione in preventing hydroxyl radical-generated DNA degradation^[27].

Stress is a two-edged sword for the body: it can agitate the injury and defensive capacity of the body. So it is necessary to reinforce the function of the endogenous defensive system to prevent and treat disease. As a member of the endogenous defensive system, MT might play an important role in the pathogenesis of stress. It is worth investigating further whether MT could be a new target in preventing and treating gastric ulcer.

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• BASIC RESEARCH •

Clinicopathological significance of Bcl-2 and Bax protein expression in human pancreatic cancer

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in IDCs of the pancreas.

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Abstract

AIM: To assess the clinicopathological significance of the expression of the apoptosis-inhibitory Bcl-2 protein (pBcl-2) and the apoptosis-promoting Bax protein (pBax) in human invasive ductal carcinomas (IDCs) of the pancreas.

METHODS: Fifty-nine surgical specimens of IDCs of the pancreas were stained immunohistochemically to detect pBcl-2 and pBax expressions whose correlation to tumor classification, staging, and prognosis was analyzed by univariate and multivariate analyses.

RESULTS: The expression of pBcl-2 and pBax was detected in 21 of 59 (35.6%) and in 29 of 59 (49.2%) patients with IDCs of the pancreas, respectively. Neither pBcl-2 nor pBax alone was correlated to TNM staging and differentiation degree of IDCs of the pancreas according to univariate analysis. By Mantel-Cox test, the median survival time after surgery for pBcl-2(+) and pBcl-2(-) groups were 14.3 and 7.3 mo, respectively ($\chi^2 = 9.357$, $P = 0.002$) and that for pBax(+) and pBax(-) groups were 12.9 and 10.2 mo, respectively ($\chi^2 = 0.285$, $P > 0.05$). Contingency coefficient between pBcl-2 and pBax expression was 0.298, indicating that there was correlation between them ($\chi^2 = 5.74$, $P < 0.05$). The median survival time after surgery for pBcl-2(+)pBax(+) and pBcl-2(+)pBax(-) groups were 14.3 and 14.1 mo, respectively, and that for pBcl-2(-)pBax(+) and pBcl-2(-)pBax(-) groups were 5.9 and 9.9 mo, respectively. There was a significant difference between pBcl-2(+)pBax(+) and pBcl-2(-)pBax(+) ($\chi^2 = 5.06$, $P < 0.05$), such was the case for pBcl-2(+)pBax(+) and pBcl-2(-)pBax(-) ($\chi^2 = 7.18$, $P < 0.01$). Cox proportional hazards model for multivariate analysis was applied, indicating that pBcl-2, TNM staging, age and pBax were high risk factors of post-surgical survival time.

CONCLUSION: Both pBcl-2 and pBax have high expression in IDCs of the pancreas, indicating that co-expression of pBcl-2 and pBax is a good indicator of favorable prognosis

INTRODUCTION

In North America and Europe, pancreatic cancer is the fourth and sixth leading cause of adult deaths from cancer and was responsible for close to 5% of all cancer-related deaths. Pancreatic cancer is characterized by insidious onset and rapid progress with only 3 mo of the average survival time after diagnosis. Surgery is indicated in less than 15% of the patients and the 5-year-survival rate is less than 4%. The recent research showed that expression of apoptosis-related protein is closely related to the biological characteristics of pancreatic carcinoma.

Bcl-2 gene family is the major regulator of cell apoptosis. Various homogenous or heterogeneous dimers can be formed within this gene family, enhancing or inhibiting apoptosis. For example, Bcl-2 (B-cell lymphoma-2) gene expression inhibits apoptosis, but Bax (Bcl-2 associated-x) promotes it^[1]. As a 25-26 ku polypeptide, Bcl-2 protein is mainly located on nuclear membrane, endocytosolic reticulum and mitochondrial outer membrane. Overexpression of Bcl-2 can inhibit cell apoptosis without any impact on cell proliferation. Bcl-2 associated-x (Bax) shows 40% homology with Bcl-2 and the overexpression of this tumor-inhibiting factor may accelerate cell apoptosis. pBcl-2 and pBax have expression in various cancers but the expression in pancreatic invasive ductal carcinomas (IDCs) is still uncertain. In this experiment, we studied the expression of pBcl-2 and pBax in human IDCs of the pancreas by using immunohistochemical method (Streptavidin-biotinylated-peroxidase staining, SAB method) and investigated the correlation to the clinicopathological parameters.

MATERIALS AND METHODS

Clinical data

Tissue samples: Fifty-nine well-documented surgical

resection cases of pancreatic IDC (from May 1978 to May 1997) were obtained from our hospital. There were 19 male cases and 40 female cases. Ages of the patients ranged from 23 to 76 years (55.1 ± 11.3 years, mean $\pm$ SD). Based on TNM staging standard, 4 patients were in stage I, 8 were in stage II, 42 were in stage III, and 5 were in stage IV. Differentiation degree: 19 cases were well differentiated, 21 cases moderately differentiated and 19 cases poorly differentiated.

Immunohistochemical Staining

Antibodies: Immunostaining was performed using the streptavidin-biotin technique (SAB method). Monoclonal antibody to pBcl-2 (M124) and polyclonal antibody to pBax (A3533) were purchased from Dako Company. According to the manufacturer's instruction, antibody dilutions at various working concentration were prepared: antibody to pBcl-2, 1:100; antibody to pBax, 1:1 000.

Staining procedures

The 4- μ m sections were deparaffinized with xylene thrice for 5 min each, dehydrated in a gradient series of alcohol thrice (100%, 95% and 45% alcohol), and rinsed by PBS. Each section was treated by 0.3% glacial acetic acid, and then microwaved for antigen retrieval (800 W, 5 min), and cooled in the room temperature for 40 min. The slides were covered with 1% peroxyacetic acid for 15 min to block endogenous peroxidase activity. Non-specific binding sites were blocked by 10% normal rabbit serum for 10 min. These sections were first incubated with primary antibody for 2 h at room temperature, and then were rinsed in PBS twice. This is followed by incubation with a secondary antibody for 15 min at 37 °C and then was rinsed in PBS twice. Slides were then treated with streptavidin-peroxidase reagent for 10 min and rinsed in PBS twice. The sections were visualized with 3,3'-diaminobenzidine (DAB) for 5 min, counterstained with hematoxylin and mounted for observation under microscope. There was slight difference between pBcl-2 and pBax staining procedure, the former adopted DAKO Company's signal enhancement system.

Evaluation of immunohistochemical analysis

For pBcl-2, positive result was defined as 5% or more than 5% of the tumor cells showing strong brown cytoplasmic

granules and for pBax, positive result was defined as 10% or more than 10% of the tumor cells showing strong brown cytoplasmic granules. Weak staining was regarded as negative.

Statistical analysis

Qualitative data analysis and correlation analysis were performed by χ^2 test (or Yates' correction test) and Pearson's contingency coefficient, respectively. The survival curves were calculated according to Kaplan-Meier method and were compared using the Mantel-Cox test. Group comparison of univariate analysis was performed first. Furthermore, a multivariate analysis of the maximum likelihood estimate with the Cox proportional hazards model was performed to obtain the conditional risk of death due to IDC of the pancreas. All analyses were performed using the SAS 8.0 Software and a *P*-value of less than 0.05 was considered to be significant.

RESULTS

pBcl-2 and pBax expression in IDCs of the pancreas

pBcl-2 was detected in 21 of 59 (35.6%) and pBax in 29 of 59 (49.2%) patients with IDCs of the pancreas (Figure 1). Contingency coefficient between pBcl-2 and pBax was 0.298, indicating that there was correlation between them ($\chi^2 = 5.74$, $P < 0.05$). The number of pBcl-2(+)pBax(+), pBcl-2(-)pBax(-), pBcl-2(+)pBax(-) and pBcl-2(-)pBax(+) were 14, 23, 7 and 15 cases, respectively. The correlation of pBcl-2 and pBax expression to age, gender, classification, and TNM staging was shown in Table 1.

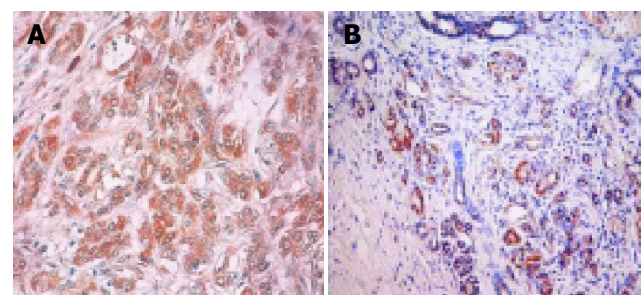


Figure 1 Immunohistochemical staining of pBcl-2 and pBax in pancreatic cancer (original magnification, $\times 200$). **A:** pBcl-2 immunostaining was stained in cytoplasm of tumor cells; **B:** pBax immunostaining was stained in cytoplasm of tumor cells.

Table 1 pBcl-2 and pBax expression and their relationship with clinicopathological parameters for the patients with pancreatic cancer

Parameter	No. of patients	Number of pBcl-2 and pBax expressing (%)			
		pBcl-2 (+)	pBcl-2 (-)	pBax (+)	pBax (-)
Overall	59	21 (35.6)	38 (64.4)	29 (49.2)	30 (50.8)
Age (yr)	<65	17 (36.2)	30 (63.8)	26 (55.3)	21 (44.7)
	≥ 65	4 (33.3)	8 (66.7)	3 (25)	9 (75)
Gender	Male	14 (36.8)	24 (63.2)	18 (47.3)	20 (52.7)
	Female	7 (33.3)	14 (66.7)	11 (52.4)	10 (47.6)
TNM stage	I	1 (25)	3 (75)	3 (75)	1 (25)
	II	5 (62.5)	3 (37.5)	4 (50)	4 (50)
	III	14 (33.3)	28 (66.7)	19 (45.2)	23 (54.8)
	IV	1 (20)	4 (80)	3 (60)	2 (40)
Grade	Well	8 (42.1)	11 (57.9)	11 (57.9)	8 (42.1)
	Moderate	8 (38.1)	13 (61.9)	8 (38.1)	13 (61.9)
	Poor	5 (26.3)	14 (73.7)	10 (52.6)	9 (47.7)

pBcl-2 and pBax expression and their relationship with prognosis

The median post-surgical survival time for pBcl-2(+) and pBcl-2(-) groups were 14.3 (95%CI: 8.1, 26.4) and 7.3 (95%CI: 2.4, 18.5) mo, respectively. Univariate analysis by Cox proportional hazards model indicated that there was a significant difference between these two groups ($\chi^2 = 9.375$, $P = 0.002$), indicating pBcl-2(+) strongly predicted a prolonged survival time. The median post-surgical survival time for pBax(+) and pBax(-) group were 12.9 (95%CI: 3.1, 19.8) and 10.2 (95%CI: 4.6, 14.1) mo, respectively revealing no significant difference between these two groups ($\chi^2 = 0.285$, $P > 0.05$). The median post-surgical survival time for pBcl-2(+)pBax(+), pBcl-2(+)pBax(-), pBcl-2(-)pBax(+), pBcl-2(-)pBax(-) were 14.3 (95%CI: 8.1, 19.8), 14.1 (95%CI: 9.7, 26.4), 5.9 (95%CI: 2.4, 14.8) and 9.9 (95%CI: 4.1, 17.8) mo, respectively. All pair-wise comparisons showed that there was a significant difference between pBcl-2(+)pBax(+) and pBcl-2(-)pBax(+) ($\chi^2 = 5.06$, $P < 0.05$), such was the case for pBcl-2(+)pBax(+) and pBcl-2(-)pBax(-) ($\chi^2 = 7.18$, $P < 0.01$). Survival curves of different groups are shown in Figure 2. However, survival of the pBcl-2(+)pBax(+) group has no significant difference as compared to other three groups together.

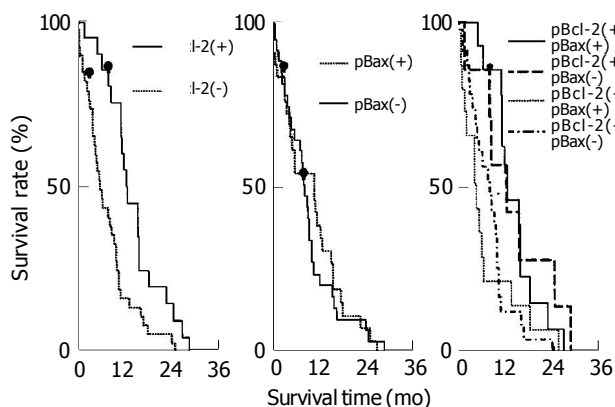


Figure 2 Survival curves with Kaplan-Meier method was applied in analyzing the influence of pBcl-2, pBax and their combined expression on post-surgical survival time.

Multifactor analysis

Cox proportional hazards model was applied in analyzing the correlation of pBcl-2 and pBax expression to clinicopathological factors and post-surgical survival time. Multifactor analysis indicated that pBcl-2, TNM staging, age and pBax were high risk factors, while tumor site, histological grade, and gender might be low risk factors influencing post-surgical survival time (Table 2).

DISCUSSION

pBcl-2 is expressed in various human solid malignancies. Its expression rate is 46-77% in breast cancer, 45-86% colorectal cancer, 12% in adenocarcinoma of lung, 25% in squamous cancer of lung, and 57% in ovarian cancer^[2,3]. However, there is no agreement in pBcl-2 expression of pancreatic IDCs according to different reports. Yuan and

his colleagues reported that the positive rate of Bcl-2 protein was 64% in human pancreatic cancer and significantly higher along with increasing of clinical stage^[4]. Makinen and his colleagues reported that pBcl-2 expression rate was 52% (34/64), and pBcl-2 expression had no correlation with tumor grading or other clinicopathological parameters^[5]. Friess and his colleagues reported that Bcl-2 and Bax expression in pancreatic cancer were 28% and 83%, respectively, which were 3.7 and 5.4 times higher than normal^[6]. Miyamoto and his colleagues reported that Bcl-2 and Bax expression in pancreatic cancer were 23% and 53%, respectively^[7]. In our experiment, pBcl-2 and pBax were detected in 21 of 59 (35.6%) and in 29 of 59 (49.2%) cases, respectively. The disagreement in expression rate might be caused by a difference in antibody itself (different batch), the different immunohistochemical staining procedures and evaluation standard, and racial difference.

Bcl-2 gene can inhibit cell apoptosis. Although it may prevent different cell apoptosis caused by various stimuli, Bcl-2 gene cannot halt DNA injury of these apoptosis-inducing stimuli. Theoretically, pBcl-2 overexpression may shorten the survival time of the cancer patients due to the enhanced tumor cell growth caused by Bcl-2-mediated programmed cell death inhibition. But many clinical trials have proved that pBcl-2 positive patients with colorectal, breast, lung or ovarian cancer had longer survival time than the pBcl-2 negative patients^[2,3,8]. The influence of pBcl-2 expression on prognosis of pancreatic IDCs is still unknown. Friess and his colleagues believed that pBcl-2 expression did not influence survival time^[6]. Makinen and his colleagues reported that pBcl-2 positive patients had longer post-surgical survival time^[5]. In our experiment, univariate analysis revealed that the median post-surgical survival time for pBcl-2(+) and pBcl-2(-) group were 14.3 and 7.3 mo respectively with significant difference between the two groups ($\chi^2 = 9.357$, $P = 0.002$), indicating pBcl-2(+) might be beneficial to assess the survival time. However, survival of the pBcl-2(+)pBax(+) group had no significant difference with the other three groups. The cause for this might be attributed to limited cases. The value of pBax expression in prediction of the prognosis is still controversial. Some scholars reported that pBax positive patient with breast or ovarian cancer had better prognosis than the pBax negative patients. pBax expression in IDCs of the pancreas varied from 53% to 100%. Friess and his colleagues believed that pBax expression played an important role in assessment of the prognosis and pBax positive patient outlasted pBax negative patients

Table 2 Correlation between various factors and survival time by Cox proportional hazards model (dependent variable, mo; censoring variable, death due to pancreatic cancer)

Variable	Parameter estimate (SE)	Conditional risk ratio (95% CI)	P (χ^2)
Overall			
pBcl-2	-1.216 (0.340)	0.297 (0.152-0.577)	0.001
TNM	0.582 (0.246)	1.790 (1.104-2.902)	0.018
Age (yr)	0.037 (0.017)	1.038 (1.004-1.073)	0.026
pBax	0.734 (0.360)	2.084 (1.029-4.222)	0.041
Site	-0.566 (0.487)	0.568 (0.219-1.474)	0.245
Grade	0.169 (0.268)	1.184 (0.701-2.001)	0.528
Gender	0.061 (0.352)	1.063 (0.534-2.118)	0.862

by 12 mo^[6]. The median post-surgical survival time for pBax (+) and pBax(-) group were 12.9 and 10.2 mo, respectively, revealing no significant difference between the two groups ($\chi^2 = 0.285$, $P > 0.05$). Multifactor analysis indicated that pBcl-2, TNM staging, age and pBax were high risk factors influencing post-surgical survival time, indicating that pBcl-2 expression might be related with the prognosis of the patients with pancreatic cancer.

Our further analysis on the combined expression of pBcl-2 and pBax indicated that the median post-surgical survival time for pBcl-2(+)pBax(+) and pBcl-2(-)pBax(-) were 14.3 and 9.9 mo, respectively (the latter had obviously shorter post-surgical survival time). However, there was no significant difference in post-surgical survival time between pBcl-2(-)pBax(-) and pBcl-2(-)pBax(+) group. pBcl-2 and pBax expressions were closely related. *In vitro*, Bcl-2 gene can inhibit cell apoptosis induced by various factors. Theoretically, pBcl-2 overexpression can inhibit cell apoptosis and promote tumor cell proliferation, which has been supported by many experiments. Bold and his colleagues found that the pancreatic cancer cell line with increased Bcl-2 expression had higher incidence of metastasis^[10]. However, our study indicated that pBcl-2 positive patients with pancreatic cancer had a longer survival time. What is the explanation for the discrepancy between medical experiments and clinical practice? *In vivo* study on pBax reveals that Bax protein can regulate Bcl-2 activity by forming Bax-Bcl-2 heterodimers to inhibit apoptosis as well as Bax homodimers to induce apoptosis (Bcl-2 homodimers can also be formed at high concentration to inhibit apoptosis). Thus, the proportional and distributive difference in pBcl-2 and pBax expression may influence the real function of pBcl-2. On the other hand, pancreatic cancer may have distinctive biological behaviors that other cancers do not have. Our previous study has revealed that p53 expression as well as p53 gene mutation may increase the chemotherapy sensitivity of patients with pancreatic carcinoma, which disagrees with the findings in other malignancies^[11].

Our study proved relatively high expression rate of pBcl-2 and pBax in IDCs of the pancreas, indicating their

potential significance in assessment of prognosis. However, further study is necessary due to our limited cases.

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• CLINICAL RESEARCH •

Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines

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cell lines. Results from RT-PCR showed that TS, MRP1, MRP3 and GSTP1 were highly expressed in these five CCA cell lines while DPD and MRP2 were only moderately expressed. It should be noted that MDR1 expression was detected only in KCU-OCA17 cell lines. A strong correlation was only found between the level of MRP3 expression and the IC₅₀ values of etoposide, doxorubicin and pirarubicin ($r = 0.86-0.98$, $P < 0.05$).

CONCLUSION: Sensitivity to chemotherapeutic agents is not associated with the histological type of CCA. Choosing of the appropriate chemotherapeutic regimen for the treatment of CCA requires knowledge of drug sensitivity. MRP3 was correlated with resistance of CCA cell lines to etoposide, doxorubicin and pirarubicin, whereas other chemotherapeutic drugs showed no association. The role of this multidrug resistance-associated protein, MRP3, in chemotherapeutic resistance in CCA patients needs to be further investigated.

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Abstract

AIM: To study the effect of a number of chemotherapeutic drugs on five human intrahepatic cholangiocarcinoma (CCA) cell lines. The expressions of genes that have been proposed to influence the resistance of chemotherapeutic drugs including thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), glutathione-S-transferase P1 (GSTP1), multidrug resistance protein (MDR1) and multidrug resistance-associated proteins (MRPs) were also determined.

METHODS: Five human CCA cell lines (KKU-100, KKU-M055, KKU-M156, KKU-M214 and KKU-OCA17) were treated with various chemotherapeutic drugs and growth inhibition was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Semi-quantitative levels of gene expression were determined by a reverse transcriptase polymerase chain reaction (RT-PCR). Results of IC₅₀ values and the ratios of gene expression were analyzed by linear regression to predict their relationship.

RESULTS: Among five CCA cell lines, KKU-M055 was the most sensitive cell line towards all chemotherapeutic drugs investigated, particularly taxane derivatives with IC₅₀ values of 0.02-3 nmol/L, whereas KKU-100 was apparently the least sensitive cell line. When compared to other chemotherapeutic agents, doxorubicin and pirarubicin showed the lowest IC₅₀ values (<5 µmol/L) in all five CCA

Key words: Cholangiocarcinoma; Chemotherapeutic agents; Drug sensitivity; Drug resistance; Multidrug resistance protein; MRP

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INTRODUCTION

Cholangiocarcinoma (CCA) is a bile duct tumor which accounts for an approximate 15% of liver cancers worldwide^[1]. This cancer can be classified into three major groups including intrahepatic, perihilar and distal extrahepatic CCA. The highest incidence of this cancer has been reported in Laos and Northeastern Thailand^[2]. Intrahepatic type is the most common case of CCA in these countries and infestation of *Opisthorchis viverrini* has been classified as a definite risk factor of the disease^[3]. It has recently been reported that the incidence of CCA in developed countries is now increasing. The incidence of intrahepatic CCA in the USA during 1975-1999 was examined using data from the Surveillance Epidemiology and End Result Program^[4]. The results revealed that there was a major increase in incidence after 1985 by 165% over the entire period. However, the

risk factors of CCA in that population are still unclear. At present, only surgical resection of all detectable tumors is correlated with the improvement in 5-year survival^[5,6]. Evidence from clinical studies demonstrated that the response rate of this cancer to chemotherapy is relatively poor in which partial responses have been observed in only approximately 10–20% of cases^[7–9]. Improvement in the survival of CCA patients will probably not only rely on more aggressive or advanced surgical techniques but also from the improvement in treatment with chemotherapy. When compared to other cancers, data concerning drug sensitivity and drug resistance in CCA are still limited, this may be due to the fact that this cancer has previously been considered as a rare cancer in developed countries^[10–13].

Resistance of cancerous cells to chemotherapeutic drugs is a major cause of the chemotherapeutic failure of chemotherapy^[14–16]. Several factors have been hypothesized to play a role in chemotherapeutic drug resistance in cancers including high expression of drug efflux pumps (e.g. multidrug resistance protein [MDR1] and multidrug resistance-associated proteins [MRPs]^[17]), an increase in detoxification of chemotherapeutic drugs (e.g. glutathione S-transferases [GST]^[18] and dihydropyrimidine dehydrogenase [DPD]^[19]), alteration of drug targets and suppression of drug-induced apoptosis^[20]. Several lines of evidence suggest that both MDR1 and MRP1 are the major contributors of the multidrug resistance phenotypes observed in a number of tumor cells^[21–25].

It has been demonstrated in a CCA cell line, SK-ChA-1, that the resistance of this CCA cell line to 5-FU resulted from an increase in activity of thymidylate synthase (TS), a target enzyme for 5-FU^[26]. DPD, the initial and rate-limiting enzyme in catabolism of 5-FU has also been proposed as a predictable marker of 5-FU treatment in several cancers such as colon cancer and hepatocellular carcinoma^[26–28]. Association between 5-FU resistance and mRNA expression or high activity of DPD in colorectal tumors has been reported in which high activity and high mRNA expression of DPD have resulted in low sensitivity to 5-FU^[19,29,30]. Although 5-FU is the most common chemotherapeutic drug used in the treatment of CCA, the roles of TS and DPD in that cancer are not clear^[26–28]. Moreover, glutathione S-transferase P1 (GSTP1) has been found to be associated with drug resistance in some cancers including CCA^[13,31].

In order to improve the efficacy of chemotherapy in CCA patients, better understanding in drug sensitivity and the mechanism of drug resistance of this cancer is essential. In the present study, we have screened the effect of a number of chemotherapeutic drugs against five intrahepatic CCA cell lines that represent different histological types. Moreover, the expression of genes that have been proposed to influence chemotherapeutic drug resistance was analyzed in order to characterize the molecular mechanism of drug resistance in CCA.

MATERIALS AND METHODS

Reagents

All chemotherapeutic drugs used in this study were obtained as commercial pharmaceutical products. 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), oligo(dT)₁₅, rRNasin ribonucleotide inhibitor and M-MLV reverse transcriptase were obtained from Promega, Madison, WI, USA. Ham's F12, trypsin-EDTA, penicillin G, streptomycin and *Taq* polymerase were obtained from Invitrogen, Carlsbad, CA, USA. Heat-inactivated fetal calf serum was purchased from Seromed Biochrom AG, Berlin, Germany. Other chemicals used were obtained from Sigma Chemical Co. Ltd., St Louis, MO, USA.

Human CCA cell lines

Five human intrahepatic CCA cell lines; namely KKU-100, KKU-M055, KKU-M156, KKU-M214 and KKU-OCA17 were used in this study. All cell lines were established in our institute from CCA patients residing in opisthorchiasis endemic areas in Northeastern Thailand. KKU-100 was developed from a patient with poorly-differentiated adenocarcinoma. KKU-M055 was also from a patient with a primary tumor of poorly differentiated adenocarcinoma while KKU-M156 and KKU-M214, and KKU-OCA17 were derived from patients with moderately and well-differentiated adenocarcinoma, respectively. All cell lines were cultured in either Ham's F12, containing 100 U/mL penicillin and 100 µg/mL streptomycin with 10% fetal bovine serum at 37 °C with 50 mL/L CO₂. The presence of Mycoplasma contamination was periodically checked. These cell lines have been maintained in our laboratory for more than 5 years.

Cytotoxicity assay

Sensitivity of CCA cell lines to chemotherapeutic drugs was determined by MTS assay as described in a previous study^[32]. Briefly, 100 µL of cell suspension (1×10^5 cells/mL) were added to each well of 96-well flat-bottomed microtiter plates and incubated for 24 h at 37 °C in a humidified 50 mL/L CO₂ atmosphere. After incubation, 100 µL of medium containing drug or complete medium for untreated controls were distributed in the 96-well plates and plates were then incubated at 37 °C for 72 h. The culture medium was subsequently removed and 100 µL medium containing 20 µL MTS solution (20 mg/mL) was added to each culture well. The cultures were further incubated for 2 h at 37 °C in a humidified 50 mL/L CO₂ atmosphere. Absorbency was measured at 492 nm using a microplate reader (Tecan Austria GmbH, Salzburg, Austria). The concentration of drug required to inhibit cell proliferation by 50% (IC₅₀) was determined by plotting the percentage of cell growth inhibition *vs* the chemotherapeutic drug concentration as previously described^[33].

RNA extraction and semi-quantitative RT-PCR reaction

Total RNA was extracted from the human CCA cell lines (2×10^6 cells) using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions and quantified by spectrophotometry. Total RNA (5 µg) from each cell line was reverse-transcribed in 50 µL containing 250 pmol/L of oligo(dT)₁₅ primer, 40 units of rRNasin ribonuclease inhibitor and 250 units of M-MLV reverse transcriptase in 50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L DTT, and 0.5 mmol/L

Table 1 Primer sequences used in RT-PCR analysis

Gene	Size of PCR (bp)	Forward primer	Reverse primer
DPD	514	TCCTCCAGGTATGCAGTGCCA	GTTATGGTGGGCAGGTGGGTT
GAPDH	328	CAACAGCCTCAAGATCATCAGC	TTCTAGACGGCAGGTCAGGTC
GSTP1	199	TACGGGCAGTCCCCAAGTT	TGCCCCCTCATAGTTGGTG
MDR1	206	GTCTTTGGTGCCATGGCCGT	ATGTCCGTCGGGTGGGATA
MRP1	262	CTGACAAGCTAGACCATGAATGT	CCTTTGICCAAGACGATCACCC
MRP2	202	GCCAGATTGGCCAGCAAA	AATCTGACCACCGGCAGCCT
MRP3	452	GGGACCCTGCGCATGAACCTG	TAGGCAAGTCCAGCATCTCTGG
TS	579	GAATCACATCGAGCCACTGAAA	GTGTTACTCAGCTCCCTCAGA

dNTPs. Initially, RNA and oligo(dT)₁₅ were mixed together and heated at 70 °C for 10 min and immediately chilled on ice for 5 min. Other reagents were then added and incubated for 15 min at 30 °C. First-strand cDNAs were synthesized at 42 °C for 60 min. PCR amplification was performed using specific primers for the gene of interest. The PCR primer sequences are shown in Table 1. Optimization for the number of PCR cycles and first strand cDNA concentration was determined for linear amplification conditions. The expression levels of mRNA were measured using human GAPDH mRNA as an internal control by being co-amplified with each gene in order to minimize tube-to-tube variation in the efficiency of PCR^[34].

PCR was carried out in a final volume of 25 µL containing first-strand cDNA, 10 pmoL of each primer, 2 pmoL of each GAPDH primer, 1.25 units of *Taq* polymerase in 2.5 µL of 10× *Taq* buffer, 2 mmol/L MgCl₂ and 0.2 mmol/L dNTPs, using a RoboCycler gradient 40 (Stratagene, La Jolla, CA, USA). An initial denaturation step at 94 °C for 3 min was followed by 28 cycles comprising a denaturation step at 94 °C for 1 min, annealing at 62 °C (for GSTP1, MRP1, MRP2 and MDR1) or at 56 °C (for TS, DPD and MRP3) for 1 min and extension at 72 °C for 1 min. The final extension was subsequently performed at 72 °C for 10 min. The PCR products were separated on 2% agarose

gel electrophoresis containing 100 ng/mL of ethidium bromide. Gels were visualized and photographed on an Electronic Dual Light Transilluminator (Ultralum, Carson, CA, USA). The DNA bands were scanned with an image scanner (Canon Electronics Inc., Saitama, Japan) and analyzed with Image Master 1D (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The relative amount of mRNA of the gene of interest was expressed as a ratio to GAPDH mRNA.

Statistical analysis

The IC₅₀ values for each chemotherapeutic agent as tested in each of the five CCA cell lines were expressed as mean±SD. Student's *t*-test was used for statistical analysis. Association between gene expression profiles and the IC₅₀ for the five CCA cell lines was examined using the linear regression analysis. A *P* value <0.05 was considered as statistically significant.

RESULTS

Cytotoxic effect

The results for cytotoxic effects of 13 chemotherapeutic agents on five human intrahepatic CCA cell lines are shown in Table 2. Among these cell lines, KKU-M055 showed the

Table 2 IC₅₀ values of chemotherapeutic agents on CCA cell lines

Drug	IC ₅₀ (µmol/L)				
	Poorly differentiated		Moderately differentiated		Well differentiated
	KKU-100	KKU-M055	KKU-M156	KKU-M214	KKU-OCA17
Anthracyclins					
Doxorubicin	2.3±0.8 ^b	0.04±0.02	1.8±1.0 ^b	0.25±0.05	0.84±0.83
Pirarubicin	0.27±0.22 ^a	0.01±0.01	0.35±0.06 ^b	0.17±0.03 ^b	0.13±0.05 ^b
Platinum derivatives					
Cisplatin	37±3 ^b	0.05±0.01	222±38 ^b	130±12 ^b	6.22±0.19 ^b
Carboplatin	13±3 ^b	0.26±0.01	229±36 ^b	107±4 ^b	10.4±2.7 ^a
Oxaliplatin	71.7±38.7 ^a	0.11±0.00	7.0±4.7	38.0±6.7 ^b	6.8±1.3 ^b
Pyrimidine analog					
5-Fluorouracil	1 018±326 ^d	46±42 ^c	144±55 ^d	29.1±17.6 ^d	3.46±0.91
Taxanes					
Paclitaxel	39.0±6.8 ^b	0.0030±0.0001	10.9±5.8 ^a	2.62±0.75 ^a	12.5±1.9 ^b
Docetaxel	41.2±7.2 ^b	0.00002±0.00002	0.80±0.36 ^a	12.4±3.7 ^a	1.3±0.3 ^b
Vinca-alkaloids					
Vincristine	216.2±52.1 ^d	2.05±0.68 ^c	0.97±0.64	85±19 ^d	0.82±0.17
Vinorelbine	2.1±0.2 ^b	0.13±0.01	0.23±0.01 ^a	164±120	0.64±0.17 ^a
Others					
Etoposide	416±131 ^b	0.04±0.01	274±217 ^b	54±26 ^a	126±88 ^a
Irinotecan	23±6 ^a	3.41±0.51	13.1±2.1 ^b	43±9 ^b	3.60±1.16
Mitomycin C	45±2 ^b	0.01±0.00	187±0 ^b	78±32 ^a	5.07±0.17 ^b

Data represents mean±SD of at least three experiments. ^a*P*<0.05, ^b*P*<0.01 vs KKU-M055; ^c*P*<0.05, ^d*P*<0.01 vs KKU-OCA17.

highest sensitivity to all of the chemotherapeutic drugs investigated with the IC_{50} values $<5 \mu\text{mol/L}$ (except for 5-FU). In addition, KKKU-100 appeared to be the most resistant cell line to 5-FU (with the IC_{50} value $>1\,000 \mu\text{mol/L}$) and to taxane compounds (with IC_{50} values of $40 \mu\text{mol/L}$). All cell lines were sensitive to anthracycline compounds, doxorubicin and pirarubicin with IC_{50} values ranging from 0.04 – $2.3 \mu\text{mol/L}$. In the case of platinum derivatives, KKKU-M055 was highly sensitive to cisplatin, carboplatin and oxaliplatin (IC_{50} values $<0.3 \mu\text{mol/L}$) whereas KKKU-M156 appeared to be resistant to cisplatin and carboplatin (IC_{50} values $>200 \mu\text{mol/L}$). With respect to vinca alkaloids, KKKU-M055, KKKU-M156 and KKKU-OCA17 were sensitive to both vincristine and vinorelbine (IC_{50} values $<2 \mu\text{mol/L}$) whereas KKKU-M214 was appeared to be the least sensitive cell line (IC_{50} values ranging from 85 to $164 \mu\text{mol/L}$). Most of the cell lines except for KKKU-M214 were apparently sensitive to vinorelbine, a new vinca alkaloid. In contrast to KKKU-M055, the other four cell lines were apparently resistant to etoposide and mitomycin C. With regards to irinotecan, most of the cell lines were moderately sensitive to this agent (IC_{50} values ranging from 3 to $43 \mu\text{mol/L}$).

Expression of mRNAs for TS, DPD, GSTP1, MDR1 and MRPs in CCA cell lines

Expression of TS, DPD, GSTP1, MDR1 and MRPs in five CCA cell lines were investigated by semi-quantitative RT-PCR using specific primers. As shown in Figure 1, mRNA for TS, DPD, GSTP1, and MRP1-MRP3 were detected in all CCA cell lines. High levels of MDR1 expression were observed only in KKKU-OCA17. All CCA cell lines expressed low levels of MRP2 as compared to MRP1 and MRP3. Strong correlations were found between MRP3 mRNA expression and the IC_{50} value of etoposide ($r = 0.98$, $P < 0.05$), doxorubicin ($r = 0.96$, $P < 0.05$) and pirarubicin ($r = 0.86$, $P < 0.05$) (Figure 2). On the other hand, no significant correlations were observed between the levels of GSTP1, MRP1 and MRP2 expression and IC_{50} values of all chemotherapeutic drugs investigated. Neither the expression levels of TS nor DPD in the CCA cell lines were significantly correlated with IC_{50} values of 5-FU.

DISCUSSION

We have investigated the sensitivity of five CCA cell lines in response to a number of different classes of chemotherapeutic

agents including the drugs that are commonly used in CCA patients (i.e. 5-FU, mitomycin C and doxorubicin)^[7,35], as well as new chemotherapeutic drugs in order to provide a preclinical rationale for treatment of this cancer. Our results demonstrated that variations in the sensitivity to chemotherapeutic drugs were observed among these five intrahepatic CCA cell lines and sensitivity to chemotherapeutic drugs was not associated with the histological type of CCA. However, all CCA cell lines investigated in this study were apparently sensitive to new chemotherapeutic drugs including anthracyclins (pirarubicin and doxorubicin), vinca-alkaloid (vinorelbine), taxanes (docetaxel, paclitaxel) and the camptothecin derivative (irinotecan). Although 5-FU is the most common drug used for treatment of CCA, poor response rates have been reported in CCA patients^[7,36]. It should be noted that the IC_{50} values of 5-FU for CCA cell lines, particularly KKKU-100 and KKKU-M156, were much higher than those previously reported in colon carcinoma cell lines (i.e. HCC-48 and COLO20 with IC_{50} values of 8.6 and $16 \mu\text{mol/L}$, respectively^[28]) and in the cervical squamous carcinoma cell lines (i.e. SiHa and HeLa with IC_{50} values of $11.4 \mu\text{mol/L}$ ^[37] and $2.8 \mu\text{mol/L}$ ^[38], respectively).

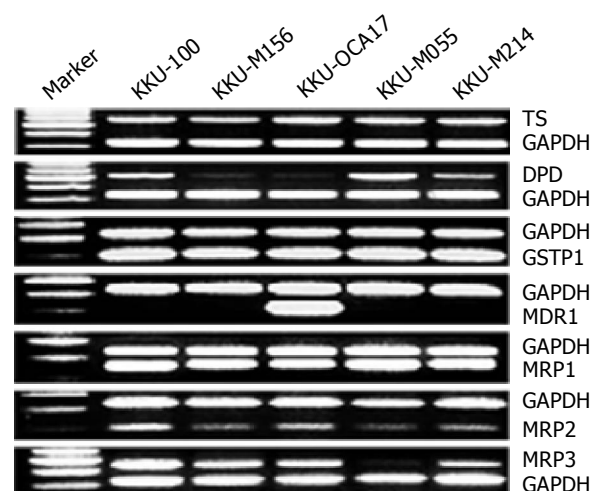


Figure 1 Expressions of TS, DPD, GSTP1, MDR1 and MRPs mRNA in five CCA cell lines determined by semi-quantitative RT-PCR analysis. Total RNA was extracted and used for RT-PCR. Each lane was loaded with $25 \mu\text{L}$ of PCR product. mRNA of the gene of interest was co-amplified with GAPDH mRNA as an internal control. Lane 1 is 100 bp DNA marker, lane 2-6 are PCR products from KKKU-100, KKKU-M156, KKKU-OCA17, KKKU-M055 and KKKU-M214, respectively.

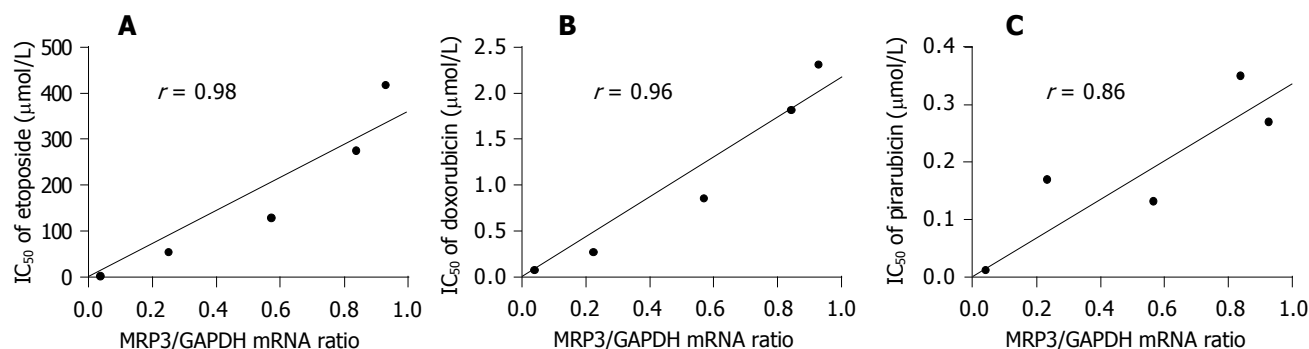


Figure 2 Relationships between the relative mRNA expression of MRP3 and the IC_{50} value of etoposide. (A) doxorubicin; (B) and pirarubicin; (C) in five CCA cell lines.

The expression of genes that have been reported to be involved in chemotherapeutic drug resistance has also been investigated in the present study (Figure 2). TS, DPD, GSTP1, MRP1, MRP2 and MRP3 were expressed in all CCA cell lines albeit at different levels while MDR1 expression was detected only in one cell line. To determine whether these genes could be candidates for determining drug-resistance-phenotypes in CCA, linear regression analysis was performed and results demonstrated that the expression of MRP3 was significantly correlated with the resistance of these CCA cell lines to etoposide, doxorubicin and pirarubicin. Consistent with our finding, a strong association between MRP3 mRNA levels and response to doxorubicin has been reported in lung cancer^[39].

In contrast to the previous report in a metastatic CCA cell line, SK-ChA-1^[26], we did not find any association between the sensitivity to 5-FU and the levels of TS and DPD expression among the CCA cell lines investigated. The level of expression of a detoxifying enzyme, GSTP1 mRNA, was markedly elevated in all CCA cell lines and this may explain why CCA is quite resistant to chemotherapeutic drugs. The MDR1 gene was highly expressed only in KKU-OCA17 but not observed in other cell lines suggesting that MDR1 is not constitutively expressed in most of CCA cells.

The levels of MDR1 expression in these five CCA cell lines were not correlated with the IC₅₀ value of any anticancer drug tested. These findings were inconsistent with those previously reported in 60 diverse cancer cell lines^[24]. Unlike MDR1, all three MRPs investigated were expressed in these five CCA cell lines. MRP1 which presents in non-expressing MDR1 tumor cells^[40] was also detected in our CCA cell lines that lacked MDR1 expression. The levels of MRP2 expression observed in these five CCA cell lines were markedly lower than the level of MRP1 expression. Consistent with those expressions found in proliferative cholangiocytes^[41], MRP3 was also expressed in CCA cell lines. The level of MRP3 expression was significantly correlated with IC₅₀ values of etoposide, doxorubicin and pirarubicin. These drugs have previously been reported to induce MDR1 and MRP1 expression in several tumor cell lines^[38,42-47].

In conclusion, the data obtained from this *in vitro* drug screening will be useful for selection of chemotherapeutic treatment in CCA patients. The role of MRP3 in the mechanism of chemotherapeutic drug resistance in CCA patients needs to be further investigated.

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• CLINICAL RESEARCH •

Blood platelet and monocyte activations and relation to stages of liver cirrhosis

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Abstract

AIM: Blood platelets (plt) and monocytes are the cells that play a crucial role in the pathogenesis of liver damage and liver cirrhosis (LC). In this paper, the analysis of mutual relationship between platelets and monocytes activation in LC was conducted.

METHODS: Immunofluorescent flow cytometry was used to measure the percentage of activated platelet populations (CD62P, CD63), the percentage of plt-monocyte aggregates (pma) (CD41/CD45), and activated monocytes (CD11b, CD14, CD16) in the blood of 20 volunteers and 40 patients with LC. Platelet activation markers: sP-selectin, platelet factor 4 (PF4), beta-thromboglobulin (β TG) and monocyte chemotactic peptide-1 (MCP-1) were measured and compared in different stages of LC.

RESULTS: Platelet activation with the increase in both β TG serum concentration and elevation of plt population (CD62P and CD63 as well as MIF CD62P and CD63) is elevated as LC develops and thrombocytopenia rises. There is a positive correlation between medial intensity of fluorescence (MIF) CD62P and MIF CD63 in LC. We did not show any relationship between monocyte activation and pma level. SP-selectin concentration correlates positively with plt count and pma, and negatively with stage of plt activation and MIF CD62P and MIF CD63. There was no correlation between MCP-1 concentration and plt, monocyte activation as well as pma level in LC. CD16 monocytes and MIF CD16 populations are significantly higher in the end stage of LC. A positive correlation occurs between the value of CD11b monocyte population and MIF CD14 and MIF CD16 on monocytes in LC.

CONCLUSION: Platelet and monocyte activation plays an important role in LC. Platelet activation stage does not influence monocyte activation and production of plt aggregates with monocytes in LC. With LC development, thrombocytopenia may be the result of plt consumption in platelet-monocyte aggregates.

INTRODUCTION

There are many reports presenting multipotential properties of blood plt, e.g. in inflammatory and immunological processes. It is connected with the plt secretory reaction, interaction with inflammatory cells, immunological cells and with plt abilities to phagocytosis. Thrombotic and inflammatory processes have common biochemical links and the same cellular elements, which are the part of the immune system, are engaged in them^[1,2]. Platelet co-operation with neutrophils and monocytes can be observed in many inflammatory, thrombotic processes and autoimmune diseases^[3]. Two mechanisms of plt activation are taken into consideration. One of them is the release of soluble substances capable of plt activation (such as proteases, thromboxan A₂, arachidonic acid metabolites, peroxides, PAF, and cathepsin G) in pathological conditions. The second one is a direct contact of inflammatory cells with plt. The result is the increase in expression of superficial adhesive molecules^[4]. Such interaction is possible thanks to membrane receptors such as GPIIb/IIIa on plt and CD11/CD18 on leukocytes^[5]. In inflammatory processes, activated plt can module functions of cells that participate in inflammation^[6]. Platelet agonists (e.g. collagen from extracellular matrix) lead to the rise of expression of CD11b antigen on leukocytes and vice versa, leukocytes incubation with CD11b agonists decreases P-selectin expression on plt. GPIIb/IIIa or P-selectin blockage on plt decreases leukocyte activation with drop of CD11b expression (Li 2000). Substances that activate binding of plt and monocytes originate from damaged vessel endothelium, activated leukocytes, macrophages or thrombocytes. These are thrombin, collagen, thromboxan A₂, serotonin, plt activating factor, catecholamines, and others^[7,8]. Recent studies have proven that plt have a significant role in pathogenesis of liver damage. Blood plt while activated in inflammatory and immune processes and in the hemostatic disorders in the liver, release active compounds (PF4, β TG, PDGF, *etc*)^[9]. Biological significance of these proteins is stimulating influence on fibrinogenesis

and mitogenesis of Ito cells in the liver. *In vivo* studies prove that after selective decrease in plt count, liver tissue damage is smaller depending on neutrophils. The influence of activated neutrophils is significant, at the time of “oxygen outburst”; active substances activating plt are released, such as cathepsin G, which has stronger activating properties than thrombin^[10]. Platelet activating factor (PAF) is an important link mediating intercellular contacts. It is produced in acute and chronic inflammatory conditions by e.g. plt, monocytes, and plays a key role in plt leukocyte-dependent activation^[11]. P-selectin, present in activated plt, and soluble (s) P-selectin in the blood play a role in plt-blood cells interactions^[12,13]. Platelet activation promotes binding with leukocytes, granulocytes, and monocytes producing aggregate complexes with these cells^[14,15]. P-selectin, a component of alpha-granules internal membrane, appears on plt surface after its activation and is also released as soluble to the surrounding microenvironment. In dense granules there are proteins specific for plt: Plt factor 4 (PF4) and β -thromboglobulin (β TG), secreted outside from activated plt as a consequence of release reaction. PF4 has chemotactic properties towards neutrophils, influences fibroblasts and inhibits endothelial cell proliferation^[16,17]. High concentrations of PF4 occur in conditions with increased plt activation, such as after myocardial infarction, operations exertion, and in chronic hepatitis. β TG is a sensitive marker of plt activation, comparable to P-selectin^[18]. After thrombocyte activation, other receptors (CD63, CD107b (LAMP-2)), originating from lysosomal granules, are transported to the surface^[19,20].

There are not many studies concerning the relationship between plt and monocytes activation in liver cirrhosis (LC) and therefore an attempt to solve this problem is justified. Platelet activation was analyzed by estimating their surface receptors and molecules soluble in peripheral blood. Cytometric estimation of circulating plt-monocyte complexes in patients with various stadiums of LC was performed.

MATERIALS AND METHODS

Patients

The studies were conducted in the group of 40 patients with advanced LC (28 men and 12 women), aged 50 ± 13 years and in 20 healthy volunteers (12 women and 8 men), aged 40 ± 7 years. Liver cirrhosis was confirmed clinically and histologically. Patients in the course of immunosuppression, anti- or pro-coagulant therapy and with neoplastic diseases were excluded from the study. None of the patients suffered from coronary heart disease or heart infarction. Patients with LC were divided into groups (B and C) in accordance with the classification of liver failure according to Child-Pugh^[21]. Ethical approval for research was obtained from local ethics committee in Medical University.

Blood preparations for cytometric examination

The blood was collected before breakfast. Pressure bandage was not used and massage was avoided not to stimulate blood plt. We used needles of the diameter bigger than 0.8 mm, blood was let due to venal pressure, hypotension was not used neither were syringes. The first 2 mL of blood was

used for biochemical examination, next 2 mL for plt examination. Platelets were prepared for cytometric studies according to recommendations of the European Working Group on Clinical Cell Analysis protocol^[22].

Resting platelets

Venal blood was collected in plastic tubes, which contained EDTA K₂ 1.5 mg/mL of blood. Immediately after collection, 1% solution of paraformaldehyde in phosphate buffered saline (PBS) deprived of Ca²⁺ and Mg²⁺ ions was added to 100 μ L of blood. Monoclonal antibodies (mAbs) were used against CD61 and CD41 marked by fluorescein isothiocyanate isomer 1 (FITC) and against CD62P marked by phycoerythrin (PE), CD63PE (Becton Dickinson). Plastic tubes were filled with 20 μ L of whole blood with EDTA K₂ each. Ten microliters of mAbs CD61/FITC and CD62P/PE or CD63PE and 0.5 mL of PBS (without Ca²⁺ and Mg²⁺ ions) were added to each tube. After mixing (vortex), the suspension was incubated for 30 min in dark place and at room temperature. Blood plt were examined in flow cytometer Coulter EPICS XL (Coulter Electronics Corporation, FL, USA). We analyzed per sample approximately 10 000 blood plt. by dividing the population into normal plt, microplatelets and macroplatelets. Parameters obtained from the gate of normal plts were taken for the analysis.

Monocyte population

Peripheral blood monocyte and plt population were determined using cytometric method with the flow cytometer equipped with an argon ion laser. Monocytes were identified by their characteristic forward and orthogonal light scatter properties. Circulating monocytes with adherent plt were identified by positivity for RED670 with 670-nm band-pass filter and FIC with a 525-nm band-pass filter. Monoclonal antibodies stained with fluoresceine (FITC) or phycoerythrin (PE), (Dako, Denmark) were used. The following monocytes populations were tested: CD11b, CD14, CD16 and CD45. Venal blood was collected in the mornings in plastic tubes containing EDTA K₂ of 1.5 mg/mL of blood. Cytometric analysis was performed within 2 h after blood collection. The amount of 5 μ L of stained monoclonal antibodies was added to 50 μ L of whole blood. Afterwards, it was incubated in room temperature for 15 min. Erythrocytes were eliminated by addition of diluted lysis fluid (ImmunoPrep-Reagenziensystem ABC, Coulter). We analyzed per sample approximately about 2 000 monocytes. Platelet-monocyte complexes were determined using mAb CD41 PE (blood plt) and CD45 FITC (monocyte population) as the percentage of double positive cells. Platelet and monocyte count were carried out in hematological analyzer Sysmex (Japan).

MCP-1, sP-selectin

Using ELISA method we measured the level of sP-selectin (sP-selectin, R&D System, Oxon, UK), MCP-1 (R&D System, Oxon, UK). The blood was centrifuged at 2 000 r/min for 15 min. at 4 °C and all sera were stored at a temperature of -76 °C. Immunoenzymatic studies were carried out simultaneously.

β-Thromboglobulin, platelet factor 4

A sample of 4.5 mL of venal blood were taken, in the manner described above (in blood preparation for flow cytometry), to plastic tubes in ice containing heparin (Vacutainer CTAD, Becton Dickinson, UK). Tubes were centrifuged in 3 500 r/min for 30 min at the temperature of 4 °C. Plasma was examined and βTG and PF4 were examined by means of immunospectrophotometric method (Asserachrom, Diagnostica Stago).

Statistical analysis

The results were presented as mean±SD and medians. Statistical analysis was performed by non-parametrical Mann-Whitney *U*-test. Result correlation was calculated with the use of Spearman correlation test. Statistically significant values were considered for $P<0.05$.

RESULTS

Platelet count drops and plt secretion activity can be observed together with the stage of liver failure advancement in LC (Table 1). There is a negative correlation between plt amount and PF4 concentration in stadium B and C according to Child-Pugh classification as well as βTG in patients in stadium C (Table 2). LC reveals the increase in plt activation with the rise in population of CD62P and CD63 phenotypes despite thrombocytopenia intensification. Moreover, there is a higher expression on plt of receptors MIF (medial

intensity of fluorescence) CD62P and CD63 (statistically significant) in cases of LC. A negative correlation between MIF CD62P and MIF CD63 and plt count was observed in the stadium Child-Pugh C. A positive correlation between MIF CD62P and MIF CD63 ($r = 0.51$, $P<0.01$) occurs in LC, there was no such relationship in the control group. There is also a positive correlation between plt activation stage and PF4 concentration in blood sera in LC. PF4 level correlates positively with plt CD62P population ($r = 0.53$, $P<0.02$), CD63 ($r = 0.66$, $P<0.04$), MIF CD62P ($r = 0.54$, $P<0.02$), and MIF CD63 ($r = 0.46$, $P<0.005$) in patients with LC.

In LC, monocyte percentage increases in peripheral blood. Cytometric analysis shows that monocytes CD16 population is raised significantly with LC advancement. MIF CD16 on monocytes also increases (statistically significant). The population of CD11b monocytes significantly drops with cirrhosis advancement. However, a significant increase in MIF CD11b receptor expression on monocytes (MIF) can be observed.

Circulating plt-monocyte complexes tend to increase in LC. In C stage of LC, CD45 receptor expression on monocytes in plt-monocyte aggregates is markedly higher. A strongly positive correlation ($r = 0.9$) between MIF CD45 on plt aggregates and monocytes is observed in LC as in healthy liver. There is a positive correlation between plt count and the level of aggregates plt-monocytes. Platelet activation stage (CD62P, CD63, PF4, βTG) correlates negatively with plt-monocytes aggregates (Table 3). Monocyte CD16 population is significantly higher in LC as compared to the healthy group and depends on liver failure stage. There is a positive correlation between monocyte CD16 percentage and receptor (MIF) CD45 expression in plt-monocyte aggregates ($r = 0.56$, $P<0.02$). A positive correlation also occurs between MIF CD16 and MIF CD45 on monocytes in aggregates plt-monocytes ($r = 0.53$, $P<0.03$). There is a positive correlation between CD11b monocyte population and MIF CD14 receptor expression on monocytes ($r = 0.45$, $P<0.05$) and MIF CD16 ($r = 0.35$, $P<0.04$) in LC. It is also observed that MIF CD14 values on monocytes are comparable in B and C stage of LC and healthy subjects.

The level of MCP-1 chemokine, released by activated monocytes, is much lower in LC than in healthy subjects. There is a negative correlation between MCP-1 concentration

Table 1 Blood plt, monocyte serum and cytometric parameters in clinical stages of LC according to Child-Pugh classification

	Healthy	Cirrhosis hepatitis stage (Child-Pugh)	
		B	C
Blood plt			
Blood plt ($1 \times 10^3 / \mu\text{L}$)	219±25	124±77 ^a	115±66 ^a
sP-selectin (pg/mL)	133±70	74±42 ^a	69±35 ^a
PF4 (IU/mL)	36±7	30±9	26±16
βTG (IU/mL)	128±45	137±40	101±62
CD62P (%)	6±1.8	10±4 ^a	9.3±3 ^a
MIF CD62	0.47±0.08	0.66±0.2 ^a	0.66±0.2 ^a
CD63 (%)	0.7±0.3	2.2±1.5	1.5±0.9
MIF CD63	0.3±0.04	0.4±0.09 ^a	0.4±0.07 ^a
Monocyte			
Monocyte (%)	5.7±3.1	6.8±3.3	7.0±2.9
MCP-1 (pg/mL)	218±129	150±93	153±81
MIF CD14	26±2	27±7	26±4
CD16 (%)	2.9±1.6	7.9±7.8 ^a	8.1±5.2 ^a
MIF CD16	1.5±0.5	1.9±1.1	2.9±1.5 ^a
CD11b (%)	100±0.1	99.5±0.8	99.5±2.0 ^a
MIF CD11	17.4±5.8	18.5±4.8	21.2±5.9 ^a
Aggregates			
Plt-monocytes (%)	13.5±4.0	10.7±5.6	13.6±16.8
MIF CD45 on plt-monocyte aggregates	11.8±3.6	11.6±3.5	15.1±4.5

^a $P<0.05$ vs Healthy statistical significance in healthy subjects, Mann-Whitney *U*-test. MIF-medial intensity of fluorescence.

Table 2 Correlations (*r*) of blood plt count and their activation markers in clinical stages of LC

	Cirrhosis hepatitis stage (Child-Pugh)	
	B	C
sP-selectin	0.61	0.58
MCP-1	0.41	0.39
PF4	-0.42	-0.62
βTG	NS	-0.45
CD62P	-0.53	-0.52
MIF CD62P	NS	-0.52
CD63	-0.58	-0.41
MIF CD63	-0.56	-0.3
Plt-monocyte aggregates	0.77	0.81

There was no correlation in the controls. Spearman's test, NS - no statistical differences.

and monocyte count in peripheral blood ($r = -0.56$, $P < 0.0001$), MIF CD14 on monocytes ($r = -0.4$, $P < 0.01$), and albumin concentrations ($r = -0.58$, $P < 0.01$) in LC.

Soluble P-selectin concentration in LC correlates positively with plt count, plt-monocyte aggregates and biochemical liver injury stage (ALT, AST, ALP, GGT). However, it correlates negatively with plt activation and receptors CD62P and CD63 expression (Table 4). A negative correlation of sP-selectin concentration and monocyte (MIF CD14 and MIF CD16) activation can be also observed.

Table 3 Correlations (r) of plt-monocytes aggregate level and markers of plt activation (Spearman's test)

	Healthy	Cirrhosis hepatitis stage (Child-Pugh)	
		B	C
Plt CD62P (%)	NS	-0.47 ^b	-0.58 ^b
MIF CD62P	NS	-0.45 ^b	-0.58 ^b
Plt CD63 (%)	NS	-0.3 ^a	-0.49 ^b
SP-selectin	NS	0.6 ^b	0.51 ^b
PF4	NS	NS	-0.68 ^b
βTG	NS	NS	-0.44 ^a

^a $P < 0.05$, ^b $P < 0.01$ vs Healthy NS – no statistical correlations.

Table 4 Correlations (r) of sP-selectin value with monocyte and plt activation markers and other parameters in LC

	Cirrhosis hepatitis stage (Child-Pugh)	
	B	C
AST (U/L)	NS	0.56 ^b
ALT (U/L)	NS	0.41 ^a
ALP (U/L)	NS	0.42 ^a
GGT (U/L)	NS	0.54 ^b
Leukocyte ($1 \times 10^3 / \mu\text{L}$)	0.6 ^a	0.58 ^b
Monocyte (%)	NS	-0.37 ^a
MIF CD14	-0.73 ^b	NS
MIF CD16	-0.3 ^a	NS
CD62P (%)	-0.57 ^b	NS
MIF CD62P	-0.53 ^b	NS
CD63 (%)	-0.54 ^b	NS
MIF CD63	-0.5 ^a	NS

In healthy subjects we observed no statistical correlations (Spearman's test).

^a $P < 0.05$, ^b $P < 0.01$ vs other groups NS – no statistical correlations.

DISCUSSION

Blood plt, by connecting hemostasis and inflammatory processes, participate in pathogenesis of chronic liver diseases. Thrombocytes play an important role in intensification of damage and fibrosis of liver tissues. The level of plt damage depends on severity of the course of liver disease, and injury is reversible with liver efficiency improvement. The causes of plt activation in LC are complex and not yet fully understood. In LC, thrombocytopenia is connected with shorter life span of plt and is a result of constant plt activation by cytokines (IL2, IL6, TNF α), use up in subclinical DIC, intensified elimination by the reticulo-endothelial system of the spleen and liver^[23]. Hyperdynamic portal circulation and retention in the spleen microcirculation in LC act to stimulate plt. Immunological and inflammatory phenomena

in liver tissues and their influence are the other causes of plt activation. P-selectin expression on plt is elevated in the microcirculation at inflammation sites^[24]. Endotoxemia often accompanies severe LC. In experimental liver injury with lipopolysaccharides (LPS), plt and neutrophil counts in peripheral blood are significantly lower and are increased in the liver tissue^[25].

Endotoxemia activates plt and causes their migration from blood to sinusoidal and perisinusoidal spaces^[26]. Platelets removed under experimental conditions protect from focal liver necrosis^[27]. Platelets with P-selectin, undergoing adhesion to vascular endothelial cells, facilitate the stop of circulating neutrophils in small vessels and initiate inflammatory and thrombotic processes. P-selectin may represent a possible link between inflammation and thrombosis. P-selectin correlated with plt counts, but not with endothelial injury and tissue factor or coagulation factors in chronic liver disease^[28].

The results of our investigations showed that the more developed thrombocytopenia, the stronger plt activation in LC. With developed thrombocytopenia in LC, plt CD63+ population increases, which reflects strong plt activation with elevated MIF CD62P and CD63. As cirrhosis develops and plt count diminishes, plt aggregates with monocytes increase. Our studies showed a negative correlation between the stage of plt activation (CD62P, CD63, PF4, βTG) and plt aggregates creation with monocytes in LC. It proves that plt activation influence binding with monocytes. It seems that thrombocytopenia in LC may be the result of plt consumption of plt in aggregates with other cells. There was no relationship between monocyte activation and the level of plt-monocyte aggregates.

CD62P and CD63 receptor expression on plt (both MIF and population percentage) as well as PF4 and βTG release are sensitive markers of plt activation. It is significant that plt activation was connected with the increase of plt aggregates with monocytes. There was a negative correlation between plt activation (MIF CD62P, MIF CD63) with sP-selectin concentration in blood serum. Our studies suggest that in LC plt are not the main source of the soluble form of P-selectin and it does not reflect plt activation.

Monocyte activation is connected with CD11b population elevation that presents β2-integrin, which enable binding with ICAM-1, fibrinogen, and factor α . Activated CD14/16 monocyte population has proinflammatory properties and produces large amount of TNF- α . Activated plt are bound to PMN with the involvement of P-selectin and activated β2-integrin CD11b/CD18^[29]. Monocytes adhere to activated plt, mediated by P-selectin. Thrombin-activated plt induce secretion of monocyte chemotactic protein-1 by monocytes. It is suggested that activated plt regulate chemokine secretion by monocytes in inflammatory sites^[30]. Hyperdynamic condition in portal hypertension may be important in triggering the monocyte and plt activation. Platelet-monocyte complexes have been implicated in inflammatory processes. Microenvironment conditions in liver tissue regulate monocyte differentiation to become dendritic cells despite the presence of fibroblasts. Monocyte chemoattractant protein 1 (MCP-1) has chemotactic properties, and recent evidence suggests that they might participate in the fibrotic process by inducing the secretion of extracellular

matrix components.

Our studies revealed that MCP-1 level did not reflect inflammatory activity in LC, as it was lower in blood serum than in healthy subjects. There was no relation observed between MCP-1 concentration and activation of plt, monocytes, (population CD16, CD11b) and predisposition for plt-monocyte complexes creating. Thus, MCP-1 is not a mediator in plt-monocyte contacts in LC. Secretion of MCP-1 correlated with intensity of the inflammatory infiltrate in chronic liver disease. In active cirrhosis, MCP-1 expression was present in the portal tract, in regenerating bile ducts, and in inflammatory infiltration sites. In the group of patients in Child-Pugh C stage, CD45 receptor expression on monocytes in plt-monocyte aggregates is much higher which proves that complex creation happens due to CD45⁺ monocyte activation and/or there is an increase in CD45 receptors on monocytes (difference statistically insignificant). It seems that although thrombocytopenia is a non-desired effect in LC, it is at the same time, a protective phenomenon against intensification of inflammatory processes in the liver. Monocyte and plt activation in LC may contribute to progressive liver injury. Thus, further investigations are needed to check pharmacological effect on plt and monocyte activation blockade and intensification of liver regeneration.

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• BRIEF REPORTS •

Association of *Helicobacter pylori* infection and giardiasis: Results from a study of surrogate markers for fecal exposure among children

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Abstract

AIM: To investigate whether *Helicobacter pylori* (*H pylori*) infection is associated with hepatitis A virus (HAV) infection, presence of enteroparasites, and other surrogates of fecal exposure.

METHODS: We conducted a cross-sectional study in 121 children consecutively admitted at a pediatric hospital in Salvador, Brazil. *H pylori* and HAV infection were identified by the presence of serum antibodies. Stool specimens were examined for the presence of ova and parasites. A structured questionnaire inquiring about sanitary conditions and life style was applied to each subject.

RESULTS: Fifty-one of the 121 children (42.1%) were found to be seropositive for *H pylori*, and 45 (37.2%) for HAV. The seroprevalence of *H pylori* and HAV both increased significantly with age. Cross-tabulation of data showed that 26 (21.5%) were seropositive and 51 (42.1%) were negative for both *H pylori* and HAV antibodies ($\chi^2 = 7.18$, OR = 2.8, CI 1.30-5.97). The age adjusted OR for an HAV-infected child being *H pylori* positive was 2.3 (CI 1.02-5.03). The agreement between *H pylori* and HAV seropositivity was fair ($\kappa = 0.24$). After controlling for possible confounding, the variables remaining independently associated with seropositivity to *H pylori* were age, presence of *Giardia lamblia* in feces (OR = 3.2, 95%CI, 1.1-9.5) and poor garbage disposal quality (OR = 2.4, 95%CI, 1.1-5.1).

CONCLUSION: Our data suggest that *H pylori* infection

is associated with surrogate markers of fecal exposure. Thus, we conclude that the fecal-oral route is relevant in the transmission of HP among children in an urban setting of a developing country. The association observed between *G. lamblia* and *H pylori* infection may have several explanations. Further studies to investigate this relationship are warranted.

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Key words: *H pylori*; Hepatitis A virus; Epidemiology; Transmission; Enteroparasites; Brazil

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INTRODUCTION

Helicobacter pylori (*H pylori*) is now thought to be one of the most important factors in the pathogenesis of upper gastrointestinal diseases such as gastritis and peptic ulcer disease^[1,2]. Several mechanisms of *H pylori* transmission involving the fecal-oral or oral-oral route have been proposed^[3-5]. Yet the design of prevention measures is hampered by uncertainties about the exact mode and route of *H pylori* transmission. Earlier studies have indicated that person-to-person transmission is a major mode of spread of *H pylori*^[6-8]. However, even when *H pylori* is transmitted from person to person, it is still controversial whether the transmission route is via an oral-oral, fecal-oral or some other route^[9,10].

The isolation of *H pylori* from feces has been used to argue for the fecal-oral route of transmission^[11]. The consumption of uncooked vegetables contaminated by raw sewage in the water supply has also been suggested to be a key factor in the transmission of *H pylori*^[12]. Despite such evidence supporting fecal-oral transmission, *H pylori* has also been isolated from dental plaque and saliva^[4,13,14], suggesting that the bacterium may be acquired by the oral-oral route. Thus, as an attempt to clarify this issue, we directly compared the seroprevalence of *H pylori* with the prevalence of intestinal parasites known to be acquired by the fecal-oral route, and with the seroprevalence of antibody to hepatitis

A virus (HAV) also known to be a sensitive marker of fecal-oral exposure^[15], in a population of children admitted to a pediatric hospital in Salvador, Brazil.

MATERIALS AND METHODS

This study was conducted in a non-profit, 90-bed pediatric hospital (Hospital da Criança), which serves uninsured children of low socioeconomic status in the city of Salvador, in northeastern Brazil. Salvador has an ethnically diverse population of 2.3 million, and is the third largest city in Brazil.

Subject selection

We enrolled 121 out of 132 eligible children consecutively admitted at the Hospital da Criança over a 6-month period (from August 2001 to February 2002). Thus, the response rate was 92%. Children with life-threatening diseases, living outside the metropolitan area of Salvador or who had been vaccinated against hepatitis A virus (HAV) were excluded from the study.

Data collection

A structured questionnaire was administered in person by trained and certified interviewers to either parent of each child. On an average, each interview lasted 30-45 min. Neither the interviewers nor the children's parents were aware of the *H pylori* infection status of the study subject at the time the interview took place. The protocol was approved by the Hospital Ethical Committee, and written informed consent was obtained from the parents of all children, before enrollment in the study.

The data collection instrument contained approximately 88 items, including questions on demographic and household characteristics. A crowding index was calculated by dividing the number of household members by the number of rooms. Garbage disposal system was classified as good or poor. Good systems included scheduled garbage collection and absence of garbage in children's playgrounds (household vicinity). Also, poor systems included non-scheduled garbage collection or presence of garbage in children's playgrounds. Household excreta disposal system was also classified as good or poor. Good systems included either the municipal sewer or a septic tank with leach field, and poor systems included cesspools and portables.

Serology

Serum was separated, frozen, and stored at -20 °C until assayed. *H pylori* status was determined by the presence of anti-*H pylori* IgG antibodies in the ELISA using a commercially available kit (Enzignost® Anti-*H pylori* II/IgG; Dade Behring, Marburg, Germany). All testing was performed according to the manufacturer's instructions. ELISA test uses inactivated *H pylori* antigen (detergent extract from a CagA- and VacA positive strain isolate) absorbed to reaction wells. A sample was considered positive for IgG antibodies to *H pylori* if the corrected absorbance reading of the serum sample was 0.250 or more (cut-off). The specificity and sensitivity are estimated to be 98.8% and 93.4%, respectively (Dade Behring, data on file). It has been

shown that the ELISA test and other serological tests distinguish those infected with *H pylori* from those who have not been infected^[16]. All sera were tested blindly.

Serum Hepatitis A antibodies were analyzed by a commercially available microparticle enzyme immunoassay (AxSYM HAVAB 2.0, Abbott Laboratories, Abbott Park, IL) on an AxSYM analyzer. Serum samples with absorbance levels ranging from 0 to 1.4 were considered reactive for hepatitis A antibodies. The sensitivity of this enzyme immunoassay is 98.9%, and the specificity is 99.7% (data provided by Abbott Laboratories, Abbott Park, IL).

Stool examination

Stool specimens were examined using the spontaneous sedimentation method. Fifty grams of feces were mixed with about 100 mL of tap water and sieved through a 2 mL mesh sieve, washed with 50 mL of water and pressed with a spatula to recover as much of the water as possible. After 40 min, the supernatant was decanted until 50 mL remained. The beaker was refilled to 200 mL with tap water and the suspension allowed standing. After 40 min, the supernatant was decanted until 30 mL remained, and 1 mL thereof was examined (in about 200 µL aliquots) microscopically at 100× magnification for the presence of ova and parasites.

Statistical analysis

The seroprevalence of *H pylori* infection and HAV was separately analyzed in relation to all considered variables. Odds ratio (OR) with 95% CI for *H pylori* or HAV infection, given the presence of a particular characteristic, was used as the measure of association. In addition, because we wished to study the unconfounded effects of the variables examined, we entered these terms into a model and used logistic regression in order to estimate OR and CI adjusted for selected covariates. In this multivariate analysis, full models were fitted, and the nonsignificant ($P > 0.1$) variables were eliminated in a stepwise, backward elimination algorithm, least significant first, to determine the final model. P values less than 0.05 were considered statistically significant. The relation between seroprevalence of *H pylori* infection and HAV was evaluated by means of the χ^2 test with continuity correction, OR, and κ statistical analysis (calculated according to Landis and Koch)^[17]. The κ statistic, a measure of the agreement between two observers or tests, ranges from -1 to 1 with 1 indicating perfect agreement, 0 indicating the agreement expected on the basis of chance alone, and values between 0 and 0.4 a poor to fair agreement^[18]. All analyses were conducted with the SPSS statistical program (SPSS, Inc., Chicago, Illinois, USA).

RESULTS

Table 1 presents selected sociodemographic characteristics, sanitary conditions and the prevalence of intestinal parasites in the 121 patients. The sample comprised mainly of Black/mixed children with a mean age of 4.8 years, and more than two thirds of family supporters who had attended less than middle school. The presence of intestinal parasites was common: 76 of 121 patients (63%) had at least one parasite.

The frequency distribution of parasites detected is shown in Table 1. The most common and chief complaints on hospital admission included respiratory tract diseases (33.9%), urinary tract diseases (12.4%), skin/muscle infection (10.7%) and gastrointestinal tract diseases (7.4%).

Of the 121 patients, 51 (42.1%) children were found to be seropositive for *H pylori*, and 45 (37.2%) for HAV. The seroprevalence of *H pylori* and HAV both increased significantly with age (Table 2) and did not differ according

Table 1 Selected characteristics of 121 children admitted to a public hospital in Salvador, Brazil, 2001-2002

	N (%)
Age group (in yr)	
1-3	43 (35.5)
4-6	50 (41.3)
7-9	16 (13.2)
10-12	12 (9.9)
Gender	
Male	66 (54.5)
Female	55 (45.5)
Ethnicity	
Black	56 (46.3)
Mixed	59 (48.8)
White	6 (5.0)
Educational attainment of family supporter	
Less than middle school	87 (71.9)
Complete middle and less than high school	21 (17.4)
High school or more	13 (10.7)
Household monthly income ¹	
Less than \$US 100.00	38 (31.4)
\$US 100.00 to \$US 190.00	63 (52.1)
More than \$US 190.00	20 (16.5)
Crowding index	
≤1.0	50 (41.3)
1.1-2.0	45 (37.2)
2.1-3.0	15 (12.4)
>3.0	11 (9.1)
Presence of enteroparasites	
Endolimax nana	61 (50.4)
Ascaris lumbricoides	48 (39.7)
Trichiurus trichiura	23 (19.0)
Giardia lamblia	19 (15.7)
Entamoeba histolytica	6 (5.0)
Iodamoeba butschlii	6 (5.0)

¹In \$US.

Table 2 Seropositivity for *H pylori* and HAV in the study population, Salvador, Brazil, 2001-2002

Age group (in yr)	Number of subjects	Seropositive for <i>H pylori</i> n (%)	Seropositive for HAV n (%)
1-3	43	12 (27.9)	7 (16.3)
4-6	50	25 (50.0)	19 (38.0)
7-9	16	7 (43.8)	12 (75.0)
10-12	12	7 (58.3)	7 (58.3)
Total	121	51 (42.2)	45 (37.2)

χ^2 for linear trend: 4.01 ($P = 0.045$) and 16.07 ($P < 10^{-4}$) for age in the *H pylori* and HAV groups, respectively.

to gender (data not shown). Cross-tabulation of data showed that 26 (21.5%) were seropositive, and 51 (42.1%) were negative for both *H pylori* and HAV antibodies, whereas 25 (20.7%) and 19 (15.7%) were seropositive only for *H pylori* or HAV, respectively ($\chi^2 = 7.18$, OR = 2.8, CI = 1.30-5.97). The age adjusted OR for an HAV-infected child being *H pylori* positive was 2.3 (CI 1.02-5.03). The agreement between *H pylori* and HAV seropositivity, as measured by κ statistic, was fair ($\kappa = 0.24$).

The associations between selected characteristics and *H pylori* or HAV infection are depicted in Table 3. Gender and ethnicity were not significantly associated with either *H pylori* or HAV infection. Children at the highest category of the crowding index had an increased prevalence of HAV infection. Having a poor garbage disposal system significantly increased the odds of both *H pylori* and HAV infection by 2.5 and 2.3, respectively. Our results also suggested that having a poor household excreta disposal system increased the likelihood of *H pylori* and HAV seropositivity, but only the latter association was statistically significant. Patients living in homes with a restroom outside the household were found to have significantly higher prevalences of *H pylori* and HAV infection (Table 3). In regard to enteroparasites, the presence of *G lamblia* significantly increased the odds of *H pylori* and HAV infection by 3.6 and 4.3, respectively. There was no significant association between the other intestinal parasites investigated and either *H pylori* or HAV infection (Table 3).

Table 3 Age-adjusted OR for the association between *H pylori* or hepatitis A infection (HAV) and selected characteristics in 121 children, Salvador, Brazil, 2001-2002

Sociodemographic characteristics	Seropositivity for <i>H pylori</i>	Seropositivity for HAV
	OR (95% CI) ¹	OR (95% CI) ¹
Gender		
Male	1 (referent)	1 (referent)
Female	1.0 (0.5-2.0)	0.8 (0.4-1.7)
Ethnicity		
Non-black	1 (referent)	1 (referent)
Black	2.1 (0.9-4.5)	0.7 (0.3-1.6)
Crowding index		
≤1.0	1 (referent)	1 (referent)
1.1-2.0	0.8 (0.4-1.6)	1.0 (0.5-2.2)
2.1-3.0	1.0 (0.3-3.2)	1.0 (0.3-3.4)
>3.0	3.3 (0.9-12.4)	9.6 (2.0-46.1) ^b
Sanitary conditions		
Poor garbage disposal quality (vs good)	2.5 (1.1-5.3) ^a	2.3 (1.0-5.2) ^a
Poor household excreta disposal system (vs good)	1.7 (0.8-3.6)	2.8 (1.2-6.1) ^a
Home restroom outside the household (vs inside)	2.6 (1.0-6.3) ^a	3.8 (1.4-9.9) ^b
Presence of intestinal parasites		
None	1 (referent)	1 (referent)
Ascaris lumbricoides	1.3 (0.6-3.1)	2.1 (0.8-5.4)
Trichiurus trichiura	0.9 (0.3-2.7)	1.4 (0.4-4.7)
<i>G lamblia</i>	3.6 (1.1-11.8) ^a	4.3 (1.3-13.9) ^a
Endolimax nana	1.7 (0.8-3.8)	2.1 (0.9-4.9)
Entamoeba histolytica	0.7 (0.1-4.6)	0.3 (0.0-3.4)
Iodamoeba butschlii	1.5 (0.3-8.6)	0.4 (0.0-3.5)

¹OR (95% CI); ^a $P < 0.05$, ^b $P < 0.01$ other groups.

Multivariate analysis

After adjusting for selected covariates using logistic regression, the variables that remained independently associated with seropositivity for both *H pylori* and HAV were age and the presence of *G lamblia* in feces. Poor garbage disposal quality remained significantly associated with *H pylori* infection only, as shown in Table 4.

Table 4 Results of the multivariate analysis of selected variables and their association with *H pylori* or HAV infection in 121 children, Salvador, Brazil, 2001-2002

	Seropositivity for <i>H pylori</i>	Seropositivity for HAV
	OR (95% CI) ¹	OR (95% CI) ¹
Age group (in yr)		
1-3	1 (referent)	1 (referent)
4-9	2.6 (1.1-6.1) ^a	5.1 (1.9-13.9) ^b
10-12	4.2 (1.0-16.7) ^a	9.0 (2.0-39.5) ^b
Sanitary conditions		
Poor garbage disposal quality (vs good)	2.4 (1.1-5.1) ^a	2.2 (0.9-4.9)
Presence of intestinal parasites		
<i>G. lamblia</i>	3.2 (1.1-9.5) ^a	3.7 (1.2-11.7) ^b

¹OR (95% CI); ^aP<0.05, ^bP<0.01 vs others.

DISCUSSION

The sources and routes of transmission of *H pylori* infection are still a topic of debate. This study was designed to investigate putative risk factors for *H pylori* infection, focusing on those operative in childhood. HAV is a fecally transmitted agent with a high incidence of infection in areas with poor sanitation practices. If the fecal-oral route is significant in the transmission of *H pylori*, we would expect the seroprevalence status of this bacterium to correlate with that of HAV (allowing for differences in infectivity). Primary analysis of our data showed that there is a significant correlation between the serostatus of *H pylori* and HAV, with an overall concordance rate of seropositive and seronegative results of 63.6% ($\chi^2 = 7.18$). However, the χ^2 statistic is not a good measure of the degree of association, particularly when dealing with highly prevalent variables. The κ statistic is a more suitable method for ascertaining the strength of agreement in such situations, and it revealed only a fair association between these two infections. These results, thus, offer only a weak support to the hypothesis of a common mode of transmission of those two infections. They agree with previous studies that found the correlation between *H pylori* and HAV to be only a result of the high age-related seroprevalence of these two infections^[19-21].

In our data, poor garbage disposal was associated with both *H pylori* and HAV infection. It has been shown that houseflies have the potential for mechanical transmission of *H pylori*^[22]. The accumulation of garbage with organic matter may result in larger numbers of this vector and increase the likelihood of food contamination by flies. The same can be argued for HAV infection. There was also a strong association of poor household excreta disposal system with HAV infection, but not with *H pylori* infection; even though our estimates suggested an association in the same

direction. Lastly, having a restroom outside the household was significantly associated with both *H pylori* and HAV infection. In these communities, restrooms outside the household have no sink or faucet, and typically lack toilet paper. Therefore, subjects using such facilities are more likely to have poor hygiene practices and to be at increased risk for acquiring infections transmitted by the fecal-oral route. The variables we used as surrogates for the fecal-oral mode of transmission were correlated with *H pylori* infection and have been cross validated with HAV infection. Thus, suggesting the fecal-oral route may play a significant role in the transmission of *H pylori*.

We found an association between *H pylori* infection and the presence of *G. lamblia* in feces. It is well known that *G. lamblia* is transmitted by the fecal-oral route, and thus, this association may indicate that both microorganisms share this common route of transmission. Consistently with that, *G. lamblia* infection was also correlated to HAV infection, a sensitive marker of fecal exposure. One may also argue that gastric giardiasis increases the susceptibility to *H pylori* infection. In a study in Italy, Doglioni *et al*^[23], found *H pylori* infection in 37 of 41 (90.2%) patients with gastric giardiasis. Alternatively, *H pylori*-induced chronic gastritis may increase the susceptibility to *G. lamblia* infection. In both scenarios, the association observed between *H pylori* and *G. lamblia* infection could occur independently of a common mode of transmission.

Methodological merits and limitations

In this study, a consecutive sample of subjects with a high participation rate has made it unlikely that selection bias might have occurred. Parents and interviewers were both blinded to the serostatus of the children, thus preventing information and observational bias, respectively. A limitation of our study is related to the difficulties in measuring variables reflecting sanitary and environmental conditions. As we had to rely on self-report when assessing these characteristics, this might have posed an additional source of inaccuracy to our data, which is likely to result in nondifferential classification error and attenuation of the associations measured.

In conclusion, Our results suggest that *H pylori* infection is associated with some surrogate markers of fecal exposure which were cross-validated by HAV infection, a known indicator of such exposure. Therefore, we conclude that the fecal-oral route may be relevant in the transmission of HP among children in an urban setting of a developing country. The association between *G. lamblia* and *H pylori* infection also described here can have several explanations. Further studies to investigate this relationship in more detail are warranted.

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• BRIEF REPORTS •

***Helicobacter pylori* eradication lowers serum homocysteine level in patients without gastric atrophy**

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Abstract

AIM: To determine whether *Helicobacter pylori* (*H. pylori*) infection caused hyperhomocysteinemia by altering serum vitamin B₁₂, serum folate and erythrocyte folate levels and whether eradication of this organism decreased serum homocysteine level.

METHODS: The study involved 73 dyspeptic *H. pylori*-positive patients, none of them had gastric mucosal atrophy based on rapid urease test and histology. Out of 73 patients, 41 (56.2%) showed a successful eradication of *H. pylori* 4 wk after the end of treatment. In these 41 patients, fasting serum vitamin B₁₂, folate and homocysteine levels, and erythrocyte folate levels before and 4 wk after *H. pylori* eradication therapy were compared.

RESULTS: The group with a successful eradication of *H. pylori* had significantly higher serum vitamin B₁₂ and erythrocyte folate levels in the post-treatment period compared to those in pre-treatment period (210±97 pg/mL vs 237±94 pg/mL, $P < 0.001$ and 442±212 ng/mL vs 539±304 ng/mL, $P = 0.024$, respectively), but showed no significant change in serum folate levels (5.6±2.6 ng/mL vs 6.0±2.4 ng/mL, $P = 0.341$). Also, the serum homocysteine levels in this group were significantly lower after therapy (13.1±5.2 µmol/L vs 11.9±6.2 µmol/L, $P = 0.002$). Regression analysis showed that serum homocysteine level was positively correlated with age ($P = 0.01$) and negatively with serum folate level before therapy ($P = 0.003$).

CONCLUSION: Eradication of *H. pylori* decreases serum homocysteine even in patients who do not exhibit gastric mucosal atrophy. It appears that the level of homocysteine in serum is related to a complex interaction among serum

vitamin B₁₂, serum folate and erythrocyte folate levels.

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Key words: *H. pylori*; Gastritis; Vitamin B₁₂; Folate; Erythrocyte folate; Homocysteine

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped bacterium that causes chronic infection in human stomachs, and often leads to gastritis and peptic ulcers^[1]. Recent data indicate a possible correlation between *H. pylori* infection and coronary heart disease^[2,3]. The connection between *H. pylori* infection and hyperhomocysteinemia is one way in which this organism may be linked to the development of coronary diseases. Researches have shown strong associations between hyperhomocysteinemia and inadequate vitamin intake and insufficient vitamin concentrations in plasma, particularly vitamin B₆, vitamin B₁₂ and folate levels^[4,5]. Several studies have demonstrated that *H. pylori* infection has negative effects on serum levels of vitamin B₁₂ and folate^[6-8].

Homocysteine metabolism involves a complex interaction between folate and vitamin B₁₂^[9]. Our aims in this study were to examine whether *H. pylori* infection affected serum homocysteine, serum vitamin B₁₂, serum folate, and erythrocyte folate levels in non-ulcer dyspeptic patients without gastric mucosal atrophy, and to evaluate the effect of eradication of *H. pylori* on serum homocysteine level.

MATERIALS AND METHODS

Patients

This study included 73 dyspeptic patients (24 men and 49 women; mean age 41±12 years) and was conducted between May 2002 and February 2003. The protocol was approved by the Human Research Ethics Committee of Baskent University, and informed consent was obtained from all subjects. Each individual was referred to our center for endoscopic examination, and diagnosed with *H. pylori* infection by rapid urease test (Pronto Dry, Medical Instr., Solothurn, Switzerland) and histopathologic evaluation. In each case, two biopsy specimens from the gastric antrum

and two from the corpus were examined. The tissues were stained with hematoxylin and eosin, and Giemsa stain. Gastritis was defined using the Sydney classification^[10]. The same pathologist, who was blinded to the clinical conditions of the patients, performed all histological examinations. None of these dyspeptic patients exhibited gastric mucosal atrophy. All patients underwent *H. pylori* eradication therapy (2 wk of a combination regimen of lansoprazole 30 mg twice daily, amoxicillin 1 000 mg twice daily, and clarithromycin 500 mg twice daily). Repeat endoscopy was done 4 wk after the completion of treatment to assess the eradication status of *H. pylori* in each patient.

Before and at the time of the investigation, none of the 73 patients was taking medication known to alter serum homocysteine levels, such as methotrexate, theophylline, anticonvulsants or antidepressants. The other exclusion criteria were chronic renal failure, hypothyroidism, previous gastric surgery, smoking habit, and use of proton pump inhibitors, antibiotics or vitamin supplementation in the 4 wk prior to enrollment in the study.

Determination of serum homocysteine, vitamin B₁₂, folate, and erythrocyte folate levels

A blood sample was drawn from each patient before and 4 wk after the completion of eradication therapy. Each sample was collected after overnight fasting, and serum homocysteine, serum vitamin B₁₂, serum folate, and erythrocyte folate levels were measured. Serum homocysteine level was determined using a commercial fluorescence polarization immunoassay (AXSYM Homocysteine, Abbott Laboratories, Abbott Park, IL, reference ranges for male and females were 5.9–16.0 and 3.36–20.44 $\mu\text{mol/L}$, respectively). Serum vitamin B₁₂ was measured by electrochemiluminescence immunoassay (Elecsys Vitamin B₁₂, Roche, IN, reference range: 197–866 pg/mL), and serum folate and erythrocyte folate levels were determined using a binding assay technique (Elecsys folate, Roche, IN, reference range: 3–17 and 93–641 ng/mL, respectively).

Table 1 Serum vitamin B₁₂, folate, erythrocyte folate, and homocysteine levels before and after *H. pylori* eradication treatment (mean $\pm$ SD)

	Patients with successful eradication (<i>n</i> = 41)		<i>P</i>
	Before treatment	After treatment	
Serum vitamin B ₁₂ (pg/mL)	210 $\pm$ 97	237 $\pm$ 94	<0.001
Serum folate (ng/mL)	5.6 $\pm$ 2.6	6.0 $\pm$ 2.4	0.341
Erythrocyte folate (ng/mL)	442 $\pm$ 212	539 $\pm$ 304	0.024
Serum homocysteine ($\mu\text{mol/L}$)	13.1 $\pm$ 5.2	11.9 $\pm$ 6.2	0.002

Table 2 Relationships between serum homocysteine level and serum or erythrocyte levels of vitamins, and patient age and sex before *H. pylori* eradication

	Pearson's <i>p</i>	<i>P</i>
Homocysteine–age	0.272	0.020
Homocysteine–sex	–0.201	0.088
Homocysteine–B ₁₂	–0.267	0.023
Homocysteine–serum folate	–0.367	0.001
Homocysteine–erythrocyte folate	–0.336	0.004
Serum folate–erythrocyte folate	0.654	<0.001

Statistical analysis

All analyses were performed using the statistical package for the social sciences (SPSS) for Windows, version 9.05. Normality of the distribution of the results for the four variables (serum vitamin B₁₂, serum folate, serum homocysteine, erythrocyte folate) were tested using the Kolmogorov-Smirnov test, and all were found to be normally distributed. Data were presented as mean $\pm$ SD. *P* values <0.05 were considered statistically significant. The paired *t*-test was used to compare pre- and post-treatment serum levels of homocysteine, vitamin B₁₂, folate, and erythrocyte folate levels in all patients. Univariate analysis using Pearson's correlation test was done to evaluate the relationship between serum homocysteine and age, sex, serum folate, and erythrocyte folate levels. Logistic regression analysis was done to identify which parameters independently influenced serum homocysteine level. The independent variables tested in this model were age, sex, serum folate and vitamin B₁₂ levels.

RESULTS

Of the 73 patients, 41 (56.2%) showed a successful eradication of *H. pylori* 4 wk after the end of treatment. The group with a successful eradication of *H. pylori* had significantly higher serum vitamin B₁₂ and erythrocyte folate levels in the post-treatment period compared to those in pre-treatment period (210 $\pm$ 97 pg/mL *vs* 237 $\pm$ 94 pg/mL, *P* < 0.001 and 442 $\pm$ 212 ng/mL *vs* 539 $\pm$ 304 ng/mL, *P* = 0.024, respectively), but showed no significant change in serum folate levels (5.6 $\pm$ 2.6 ng/mL *vs* 6.0 $\pm$ 2.4 ng/mL, *P* = 0.341) (Table 1). Also, the serum homocysteine levels in this group were significantly lower after the therapy (13.1 $\pm$ 5.2 $\mu\text{mol/L}$ *vs* 11.9 $\pm$ 6.2 $\mu\text{mol/L}$, *P* = 0.002) (Table 1 and Figure 1).

Univariate analysis of the data collected before eradication treatment revealed that serum homocysteine level was positively correlated with the age of the patient (*r* = 0.272; *P* = 0.02), and negatively with serum folate level (*r* = –0.367; *P* = 0.001), serum vitamin B₁₂ level (*r* = –0.267; *P* = 0.023), and erythrocyte folate level (*r* = –0.336; *P* = 0.004) (Table 2). Regression analysis identified age (*P* = 0.01) and serum folate level (*P* = 0.003) as the only two factors independently associated with serum homocysteine level. The erythrocyte folate level, which was strongly correlated with serum folate level (Table 2), was not included in multivariate analysis.

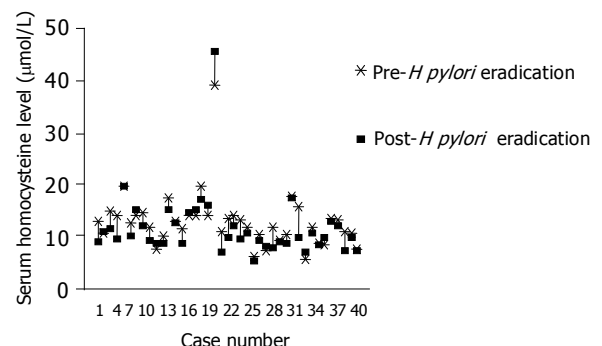


Figure 1 Changes in serum homocysteine level before and after *H. pylori* eradication (*P* = 0.002).

DISCUSSION

Several recent studies have investigated the relationship between coronary artery disease and *H pylori* infection, and the results are controversial^[2,3,11-13]. Hyperhomocysteinemia is a factor that is suggested to be responsible for the development of atherosclerosis in the setting of chronic *H pylori* infection. In recent years, homocysteine has been shown to be an important contributor to atherosclerosis^[14-16]. One meta-analysis involving 10 000 patients revealed no meaningful correlations between *H pylori* and vascular risk factors^[17]. Research has shown that homocysteine can directly cause endothelial damage^[18], affect platelet function and coagulation factors^[19], and increase the oxidation of low-density lipoproteins^[20]. In the light of these findings, a number of investigators have focused on *H pylori* infection as a possible cause of hyperhomocysteinemia. However, these findings are also inconsistent^[12,13,21,22].

It has been well established that chronic *H pylori* infection causes atrophic gastritis^[1], and decreased absorption of both vitamin B₁₂ and folic acid has been documented in patients with this condition^[9]. Furthermore, a recent study done at our clinic showed that even patients with non-atrophic *H pylori* gastritis exhibited low vitamin B₁₂ levels^[8,23-25]. This is supported by investigations that demonstrated food-cobalamin malabsorption in patients with *H pylori* gastritis who did not have mucosal atrophy^[8]. As noted above, in the present study we found that serum vitamin B₁₂ level was significantly higher after treatment, regardless of the patient's eradication status. This indicates that the degree of malabsorption, or perhaps consumption of this vitamin by the organism, decreases when *H pylori* density in the gastric mucosa is reduced by eradication therapy. Patients with chronic *H pylori* infection exhibited decreased secretion of ascorbic acid by the gastric mucosa and elevated gastric pH^[26,27]. It has been demonstrated that low levels of ascorbic acid in gastric juice or high pH of gastric juice could cause less folate absorption from the diet^[28]. In the 41 dyspeptic *H pylori*-positive patients we studied, baseline serum folate levels were in the normal range and there was no significant change in this parameter after therapy (5.6 ± 2.6 ng/mL *vs* 6.0 ± 2.4 ng/mL, $P = 0.341$). We suspect that this may be because the levels were normal before treatment, and because the typical Turkish diet contains high levels of folate. Our patients also showed significantly higher erythrocyte folate levels after *H pylori* eradication therapy. This is also likely linked to vitamin B₁₂ levels, since one important reaction in erythrocytes involves vitamin B₁₂-dependent transfer of the methyl group from N⁵methylenetetrahydrofolate to homocysteine. Lack of adequate vitamin B₁₂ impedes this reaction and leads to leakage of unconjugated folate from cells, whereas correction of the deficiency could restore erythrocyte folate levels^[29].

Our analysis identified the age of the patient and serum folate level as independent determinants of serum homocysteine level. As described above, folate re-methylation of homocysteine to methionine required vitamin B₁₂. However, this vitamin had less influence on serum homocysteine concentration than serum folate^[30]. Some authors have stressed that it is incorrect to state that vitamin B₁₂ plays no role in homocysteine metabolism, and that

the effect of this vitamin is often masked by the role of folate^[31]. Also, research showed that when oral folic acid supplementation provided a certain serum level of folate (10 µg/L was considered the approximate cut-off), serum folate had less influence on homocysteine levels^[31]. Our results are in line with these findings and statements. We conclude that serum folate level is a primary determinant of serum homocysteine level in dyspeptic *H pylori*-positive patients, even though infection with this organism is known to reduce serum B₁₂ levels. Our study did not show that increased serum vitamin B₁₂ after *H pylori* eradication had a positive effect on serum homocysteine levels, but like other authors, we believe that the impact of vitamin B₁₂ may be indirect or masked.

One report in the literature states that each 1-µmol/L drop in serum homocysteine level represents a 10% decrease in the risk of vascular disease^[14]. We found that the mean serum homocysteine level in our patients with complete *H pylori* eradication was decreased by slightly more than 1 µmol/L. Although homocysteine has not been considered to be as important as other risk factors such as hypercholesterolemia, smoking, diabetes mellitus, and hypertension, we suggest that prolonged hyperhomocysteinemia possibly due to *H pylori* infection since childhood, especially in developing countries may play a contributing role in the pathogenesis of atherosclerosis. This suggests that eradication of this microorganism can lower the risk of vascular diseases in dyspeptic *H pylori*-positive patients. It also indicates that there is a significant benefit to prescribing *H pylori* eradication even in the absence of mucosal atrophy or other severe gastroduodenal lesions. Such a treatment may be more important in countries where the rates of nutritional folic acid and/or vitamin B₁₂ deficiency are particularly high. The property of diet consumed in a population is certainly very important to achieve an acceptable serum and tissue levels of many nutrients including folate and cobalamin. As we have suggested in a recent paper, even some patients with a high *H pylori* load in their gastric mucosa may show normal serum levels of these vitamins probably because of consuming foods and drinks containing a high level of these vitamins^[8].

The main etiologic factors thought to underlie the high prevalence of vitamin B₁₂ deficiency in the elderly population are dietary deficiency and malabsorption due to atrophic gastritis. A recent study conducted at our center has confirmed that older age is an independent factor in vitamin B₁₂ deficiency, but disproved the malabsorption-atrophic gastritis link since only patients without gastric mucosal atrophy were investigated^[32]. The findings of our present study support the positive correlation between age and serum homocysteine level that has been reported previously^[33,34]. We suggest that this connection may be explained by a complex interaction among serum vitamin B₁₂, serum folate and erythrocyte folate levels.

In conclusion, even in dyspeptic *H pylori*-positive patients who do not exhibit gastric mucosal atrophy, complete eradication of *H pylori* is associated with a significant drop in serum homocysteine. In countries where *H pylori* infection is highly prevalent, it may be beneficial to implement widespread dietary fortification with folic acid and vitamin

B₁₂, and/or to provide eradication treatment for all infected patients. Further research is needed to determine whether these approaches offer significant clinical benefits in terms of lower cardiovascular risk.

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• BRIEF REPORTS •

Genetic association of cytokines polymorphisms with autoimmune hepatitis and primary biliary cirrhosis in the Chinese

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CONCLUSION: The polymorphisms of IL-1RN and IL-6 -174G/C appear to be associated with PBC in Chinese patients.

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Key words: Autoimmune hepatitis; Primary biliary cirrhosis; Cytokine; Polymorphisms; Gene susceptibility

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Abstract

AIM: To characterize gene polymorphism of several cytokine gene in-patients with AIH and PBC and to analyze the difference of the polymorphism distribution between Chinese patients and healthy controls.

METHODS: The study population consisted of 62 patients with AIH, and 77 patients with PBC. The genetic profile of four cytokines was analyzed by restriction fragment length polymorphism after specific PCR amplification (PCR-RFLP) or sequence-specific primers PCR (SSP-PCR). The analyzed gene polymorphism included interleukin-1 (IL-1) (at position +3 953 and IL-1RN intron 2), IL-6 (at position -174), IL-10 promoter (at position -1 082, -819, and -592). The control group consisted of 160 healthy blood donors.

RESULTS: The majority of Chinese people including patients and healthy controls exhibited IL-1B 1,1 genotype, and there was no significant difference in AIH, PBC patients and controls. There were highly statistically significant differences in the distribution of the IL-1RN gene polymorphism between the patients with PBC compared with controls. The frequency of IL-1RN 1,1 was significantly higher (90.9% vs 79.4%, $P = 0.03$) and the frequency of IL-1RN 1,2 was significantly lower in PBC patients (6.5% vs 17.5%, $P = 0.01$). No statistical difference was observed between AIH patients and controls. All of the 160 healthy controls and 62 cases of AIH patients exhibited IL-6-174GG genotype, and there were four cases, which expressed IL-6-174GC genotype in 77 cases of PBC patients. The frequency of IL-6-174GC was markedly significantly higher in PBC patients compared with controls (5.2% vs 0%, $P = 0.004$). No statistically significant difference was found in the distribution of IL-10 promoter genotype in AIH and PBC patients compared with controls.

INTRODUCTION

Autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are two groups of important autoimmune liver diseases. AIH is characterized by elevated serum transaminase levels, hypergammaglobulinemia, serum autoantibodies, and a good response to immunosuppressive therapy. PBC exhibits progressive destruction of the bile ducts leading to fibrosis and cirrhosis. Specific autoantibodies and disorder of liver function also characterize PBC^[1,2]. Although their etiologies are unknown, genetic factors have been implicated to be involved in the pathogenesis of AIH and PBC^[3-7]. In previous studies human HLA DRB1*0301, DRB*0401 (in Caucasians) and DRB1*0405 (in the Chinese) have been identified as independent determinants of susceptibility to AIH. In addition, the genetic typing of HLA class II and III alleles revealed a highly significant increase of HLA DR8, DPB1*0501 and 0301, and C4A*B2 and C4A*Q0 complement alleles in patients with PBC compared with controls, and the HLA DRB1*0801-DQA1*0401-DQB1*0402 haplotype was considered to represent a marker of disease progression.

A number of genes outside the MHC locus may play a role in susceptibility to autoimmune liver diseases^[8-11]. We have recently shown that the polymorphism of cytotoxic T lymphocyte-associated antigen-4 and vitamin D receptor genes have been associated with Chinese patients with AIH and PBC, and there is no association between the polymorphism of tumor necrosis factor (TNF)-alpha promoter and the same group patients with AIH and PBC^[13]. It has been well established that interleukin-1 (IL-1), IL-6 play important roles in developing chronic inflammation, and IL-10 is an important cytokine with anti-inflammatory, anti-immune, and anti-fibrotic functions. The aim of

this study is to investigate, the relationship between both diseases susceptibility in the Chinese population and the one polymorphic site in IL-6 promoter -174, the three polymorphisms sites in the IL-10 gene promoter -1082, -819, -592), as well as the IL-1 gene family: IL-1B and IL-1RN.

MATERIALS AND METHODS

Study population

A total of 139 patients who satisfied international criteria for AIH and PBC were obtained from Eastern China, including Shanghai, Zhejiang province and Jiangsu province. Sixty-two patients with AIH fulfilled the following criteria: (1) Absence of epidemiological risk factors and serological markers for hepatitis B and hepatitis C virus infection by immunoassays; (2) Presence of ANA, SMA or LKM-1, anti-soluble liver antigen antibody (SLA); (3) Absence of drug history; (4) Seven patients had concurrent nonhepatic immunologic diseases, including four with chronic ulcerative colitis and three with autoimmune thyroid disease; (5) Thirty patients were seropositive for other defined autoantibodies, including 17 atypical pANCA, 11 parietal cell antibody, 4 thyroglobulin autoantibody or thyromicrosomal autoantibody. The clinical and laboratory features of the 62 patients are shown in Table 1. The diagnosis of AIH was based on the revised criteria defined by the International Autoimmune Hepatitis Group^[1].

The PBC group consisted of 77 well-characterized patients, which met the diagnostic criteria for PBC in the practice guideline written by AASLD^[2]: (1) presence of cholestatic liver disease including jaundice, fatigue and pruritus with abnormally high levels of cholestatic parameters such as serum alkaline phosphatase (ALP), serum bilirubin and gamma glutamyl transpeptidase; (2) Absence of biliary

obstruction as assessed by ultrasound or endoscopic retrograde cholangiopancreatography; (3) Serum positive AMA titer $\geq 1:1\ 000$, and type M2 AMA positive; (4) Absence of serological markers for hepatitis B and C virus infection by immunoassays. There were 10 patients who had serum aspartate transaminase 30-50 U/L (normal value 10-40 U/L) underwent liver biopsy in PBC group, and the result showed that the histological change was compatible with PBC. Other patients have elevated serum aspartate transaminase (>60 U/L). There were 41 patients who had ANA positive in their serum (titer $\geq 1:100$). Sixty-eight were females, median age at diagnosis of PBC was 51.3 years (range 32 to 79), and seven were males, median age was 47.0 (range 38 to 60). Controls from the same geographic area, were blood donors.

ANA were sought by indirect immunofluorescence (IIF) on Hep-2 cells and rat liver tissue sections in all patients. SMA was similarly checked by IIF on rat stomach tissue sections in all patients. LKM-1 and AMA were detected on rat stomach and liver tissues sections in all patients. SLA and type M2 AMA were sought by ELISA. The IIF and ELISA kits were bought from Euroimmun Company, Germany.

DNA extraction

Genomic DNA was isolated from fresh peripheral blood leukocytes using the Qiagen DNA isolation kit (Qiagen, Germany). DNA samples were quantified and subjected to specific PCR reaction as described.

Cytokines gene polymorphisms

Genomic DNA (100 ng) was amplified in reaction mixtures containing 200 $\mu\text{mol/L}$ each of dATP, 1 pmol each primer, 1.5 $\mu\text{mol/L}$ MgCl_2 , 2 U Taq polymerase (MBI), 1 $\times$ PCR buffer with NH_4SO_4 (MBI) on a MJ PTC-200 Peltier Thermal Cycler, according to the following protocols (Table 2).

IL-1B +3953 C/T polymorphism

For detection of IL-1B +3 953 C/T polymorphism, we used PCR-restriction fragment length polymorphism (PCR-RFLP). Amplification was performed at 94 °C for 2 min followed by 40 cycles at 94 °C for 30 s, 50 °C 45 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min. The 249-bp product was digested with Taq I and electrophoreses on 3% agarose. The presence of a base-exchange substitution at position +3 953 creates the Taq I restriction site in allele 1, but not allele 2. Therefore, Taq I digestion of the 249-bp IL-1B amplicon results in fragments of 114- and 135-bp allele 1) and/or intact amplicon allele 2).

IL-1 receptor antagonist gene 86-bp mini-satellite

Genotyping for IL-1 receptor antagonist gene (IL-1RN) was performed by PCR. There is a penta-allelic polymorphic site containing variable numbers of an 86-bp tandem repeat sequence in intron 2 of IL-1RN. Briefly, the conditions for amplification were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 67 °C 45 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min. Following amplification, the amplicon was visualized on a 2% agarose gel electrophoresis. Five alleles were assigned based on amplicon size: allele 1 four repeats (410 bp), allele 2 two repeats (240 bp), allele 3

Table 1 Clinical features in patients with AIH

Clinical features	Female (n = 44)	Male (n = 18)
Age (yr)	50 (16-79)	47 (21-62)
Duration symptoms (mo)	36.4 (6-134)	38.8 (18-72)
Concurrent immunologic diseases	5	2
ALP:AST <1.5	44	11
1.5-3.0	5	2
Bilirubin (3.4-17.1 $\mu\text{mol/L}$)	62.4 (40-84.7)	63.5 (42-79.6)
Immunoglobulin G (7.6-16.6 g/L)	30.9 (18.1-34)	32.6 (18-33.4)
ANA $\geq 1:100$	30	11
SMA $\geq 1:100$	3	1
ANA and SMA $\geq 1:100$	8	3
LKM-1 $\geq 1:100$	1	1
SLA $\geq 1:100$	2	2
Average alcohol intake	<25 g/d	<25 g/d
Other autoantibodies		
atypical pANCA $\geq 1:32$	13	4
PCA	7	4
TG or TM	4	0
AIH Score ¹	>15	>15

TG, thyroglobulin autoantibody; TM, thyromicrosomal autoantibody; PCA, parietal cell antibody. ¹AIH score: revised scoring system for diagnosis of AIH.

three repeats (325 bp), allele 4 five repeats (500 bp) and allele 5 six repeats (595 bp).

IL-6 promoter-174 G/C polymorphism

IL-6 promoter-174 genotype was determined in an allele-specific PCR as previously described^[14]. The DNA template was denaturated at 95 °C for 2 min. A total of 40 cycles of PCR were performed, consisting of a denaturation step for 45 s at 94 °C, an annealing step for 45 s at 59.5 °C, and an extension reaction for 1 min at 72 °C. A final extension step at 72 °C for 2 min was added after the last PCR cycle. PCR products were resolved by agarose gel electrophoresis (2%) and visualized by ethidium bromide.

Polymorphisms of IL-10-1082G/A, -819C/T and -592A/C

Polymorphisms at two positions of the IL-10 promoter gene sequence -1 082 and -592 were determined by PCR-RFLP. Briefly, a 139-bp fragment (for -1 082C/T) and a 412-bp fragment (for -592G/A) were separated and amplified. PCR conditions: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, annealing step for 45 s at 58 °C (61.8 °C, for -592G/A), and 72 °C for 40 s, and a final extension at 72 °C for 5 min. The presence of nucleotides G at position -1 082 creates the Mn1 I restriction site, therefore, Mn1 I digestion of the 139 bp IL-10 amplicon result in fragments of 106 and 33 bp. The presence of nucleotides A at position -592 creates the Afa I restriction site, Afa I digestion of the 412-bp IL-10 amplicon result in fragments of 176 and 236 bp. After the digestion reactions, products were resolved by agarose gel electrophoresis (3%) and visualized by ethidium bromide.

Statistical analysis

Statistical analyses (two-tailed Fisher's exact test, odds ratios, confidence intervals) was calculated using the NOSA software package.

Table 2 PCR primer pairs for genotyping in cytokines gene

Gene	Position	Primers	Product size (bp)
IL-1B	+3 953	5'-GTGTGTCATCAGACTTTGACC-3'	249
		5'-TTCAGTTCATATGCACCAGA-3'	
IL-1RN	exon2	5'-GCCCTCAGCAACACTCTCTAT-3'	
		5'-CATCTTCCTGGTCTGTCAGC-3'	1
IL-6	-174G/C	5'-CATCTTCCTGGTCTGTCAGC-3'	
		5'-CCCTAGTTGTGTCCTGGC-3'	190
IL-10	-1 082G/A	5'-CCCTAGTTGTGTCCTGGC-3'	190
		5'-GAGCTTCTCTTTCGTTCC-3'	
	-819T/C	5'-TCTTACCTATCCCTACTTCC-3'	139
		5'-CTCGTGCACCAACCTGGC-3'	
	-592A/C	5'-AACTGAGGCACAGAGATG-3'	402
		5'-AACTGAGGCACAGAGATA-3'	402
	-592A/C	5'-AGCAACACTCTCGCCGAAC-3'	
		5'-GGTGAGCACTACCTGACTAGC-3'	412
HLA-DRB1	5c	5'-CCTAGGTCACAGTGACGTGG-3'	
	3c	5'-TGC CAA GTG GAG CAC CCA A-3'	796 ²
		5'-GCA TCT TGC TCT GTG CAG AT-3'	

¹Variable numbers of an 86 bp tandem repeat sequence. ²The inter-reference of allele-specific PCR.

RESULTS

Table 3 summarizes the phenotypic expression deduced from the genetic polymorphism in the four selected cytokines in the patients with AIH and PBC, in healthy individuals who were blood donors. The majority of Chinese people including patients and healthy controls exhibited IL-1B 1,1 genotype, and there was no significant difference in AIH, PBC patients and controls. There were highly statistically significant differences in the distribution of the IL-1RN gene polymorphism between the patients with PBC compared with controls. The frequency of IL-1RN 1, 1 was significantly higher (90.9% *vs* 79.4%, *P* = 0.03) and the frequency of IL-1RN 1, 2 was significantly lower in PBC patients (6.5% *vs* 17.5%, *P* = 0.01). No statistical difference was observed between AIH patients and controls.

All of the 160 healthy controls and 62 cases of AIH patients exhibited IL-6 -174GG genotype, and there were four cases, which expressed IL-6-174GC genotype in 77 cases of PBC patients. The frequency of IL-6-174GC was markedly significantly higher in PBC patients compared with controls (5.2% *vs* 0%, *P* = 0.0036). No statistically significant difference was found in the distribution of IL-10 promoter in AIH and PBC patients compared with controls.

Table 3 Genotype distributions of cytokines in AIH, PBC patients *vs* controls

Cytokine	Genotype	Controls (%)	AIH (%)	<i>P</i>	PBC (%)	<i>P</i>
IL-1B	+3 953					
	1,1	155 (96.9)	61 (98.4)	0.53	75 (97.4)	0.82
	1,2	4 (2.5)	1 (1.6)	0.69	2 (2.6)	0.96
	2,2	1 (0.0)	0 (0)	0.53	0 (0)	0.46
IL-1RN	Intron 2					
	1,1	127 (79.4)	51 (82.3)	0.63	70 (90.9)	0.026
	1,2	30 (17.5)	10 (16.1)	0.64	5 (6.5)	0.013
	2,2	3 (1.9)	1 (1.6)	0.90	2 (2.6)	0.75
	others	0 (0)	0 (0)	-	0 (0)	-
IL-6	-174 G/C ¹					
	GG	160 (100)	62 (100)	-	73 (94.8)	0.0036
	GC	0 (0)	0 (0)	-	4 (5.2)	0.0036
	CC	0 (0)	0 (0)	-	0 (0)	-
IL-10	-1082G/A					
	AA	144 (90.0)	54 (87.1)	0.53	64 (83.1)	0.13
	GA	16 (10.0)	8 (12.9)	0.53	13 (16.9)	0.13
	GG	0 (0)	0 (0)	-	0 (0)	-
	-819T/C					
	CC	13 (8.1)	7 (11.3)	0.46	7 (9.1)	0.80
	CT	72 (45.0)	27 (43.6)	0.84	41 (53.3)	0.23
	TT	75 (46.9)	28 (45.2)	0.81	29 (37.7)	0.18
	-592A/C					
	CC	16 (10.0)	10 (16.1)	0.2	11 (14.3)	0.33
	CA	83 (51.9)	32 (51.6)	0.97	41 (53.3)	0.84
	AA	61 (38.1)	20 (32.3)	0.41	25 (32.5)	0.40

¹The frequency of IL-6 -174C allele in PBC group *vs* control, *P* = 0.0038.

DISCUSSION

The etiology of autoimmune liver diseases remains unidentified. However it is well established that a complex genetic background contributes to disease susceptibility

and severity. The previous studies showed that HLA alleles, and the polymorphisms of TNF- α promoter, CTLA-4, C4A were associated with an increasing prevalence of autoimmune liver diseases in Caucasians. Recently we found a significant association of CTLA-4 and vitamin D receptor gene polymorphism in Chinese AIH and PBC patients' comparison with controls^[12]. In order to search more correlative gene with autoimmune liver diseases in the Chinese population, we extended study objects in this study.

Our study was designed to analyze the potential association between cytokine gene polymorphism and the patients with AIH, PBC. We found a statistically significant difference in IL-1RN phenotype between the patients with PBC and the healthy controls. The frequency of IL-1RN 1, 1 was significantly higher and the frequency of IL-1RN 1, 2 was significantly lower in PBC patients. This result was partly coincident with Donaldson's, but we did not find any significant difference in the distribution of IL-1B genotype^[11]. IL-1 is a proinflammatory cytokine, which interacts with both tumor necrosis factor and IL-6, and may be important in both inflammation and fibrosis. The IL-1 gene family on chromosome 2q13, encodes three proteins, including IL-1 α , IL-1 β , and the IL-1 receptor antagonist (IL-1RA). IL-1RA competes with IL-1 β (and IL-1 α) for binding to the IL-1 receptors and is a potent inhibitor of IL-1 activity^[15]. IL-1RN is the second association with PBC to be described by our group, which maps to chromosome 2. The other associated gene is CTLA-4 (2q33) which appears to be associated with AIH and PBC. These two studies based on the same series of patients identify chromosome 2q as an important location for further genetic studies in PBC. The IL-1B*2 allele is associated with high IL-1 β production, the IL-1RN*2 allele is associated with high IL-1RN production. There is a genetic link between IL-1RN*2 and IL-1B*1, they both contribute to lower IL-1 β production^[16-18].

IL-6 is a pleiotropic cytokine capable of regulating proliferation, differentiation and activity of a variety of cell types, and plays a pivotal role in acute phase response and in the balancing of the pro-inflammatory/anti-inflammatory pathways^[14]. IL-6 gene maps to chromosome 7p21, there is G>C single nucleotide polymorphism at the promoter -174, and this base transition is associated with different IL-6 plasma levels in healthy subjects, and affects the rate of IL-6 gene transcription^[19,20]. Previous studies showed that IL-6 -174G/C polymorphism was associated with multiple diseases, including juvenile chronic arthritis, atherosclerosis and diabetes. The IL-6 -174C allele contributes to lower production *in vitro* and *in vivo*, the IL-6 -174CC homozygosity expressed in the renal transplant donor and the IL-6 -174GG homozygosity expressed in receptor had been shown to associate significantly with the incidence and severity of graft-versus-host disease (GVHD), both acute GVHD and chronic GVHD^[21,22]. In this investigation, we also found that frequency of IL-6 -174C was strong and significantly increased in PBC patients compared with controls among the Chinese. This result supported the IL-6 -174G/C polymorphism contributing to the change of susceptibility to PBC for some people.

IL-10 is an important suppressor factor for both

immunoproliferative active and inflammatory responses^[23,24]. It is produced by numerous cell types including activated CD4⁺ T cells, cytotoxic CD8⁺ T cells, monocytes, Kupffer cells, hepatocytes, and hepatic stellate cells. IL-10 inhibits antigen specific activation, proliferation, and cytokine production by reducing the antigen presenting capacity of monocytes, associated with downregulation of class II HLA molecules and B7 expression on their surface. In contrast, in B lymphocytes, IL-10 stimulates proliferation, immunoglobulin secretion, and isotype class switching from IgM to IgA. IL-10 also has potent anti-inflammatory effect. It downregulates the synthesis of proinflammatory cytokines and chemokines by monocytes and Kupffer cells stimulated by endotoxin, including IL-1, TNF- α , IL-6, IL-8, and IL-12, and upregulates the synthesis of the IL-1R antagonist. Finally, IL-10 may also exert antifibrotic effects in the liver through inhibition of collagen gene transcription and increased collagenase expression by hepatic stellate cells^[25,26].

The IL-10 gene is located on the long arm of chromosome 1 in the 1q32 band and has been shown to contain three distinct SNPs within its promoter region, at nucleotide position -1082 (G→A), -819 (C→T), and -592 (C→A), with the SNP at position -1082 giving rise to differential IL-10 production. These three polymorphisms have been shown to be in strong linkage disequilibrium, and three main haplotypes (GCC, ACC, and ATA) are known to segregate in most populations^[27]. In this study we have shown the haplotypes distribution of three SNPs in IL-10 promoter region in controls were all quite different from that of the European Caucasians, and similar with that of the Singapore Chinese and Japanese population^[28,29]. In Italian PBC patients, the frequency of homozygosity for G/G at position -1082 was significantly higher than that of local controls, and the frequencies of haplotype GCC possibly linked to higher IL-10 production, were also significantly higher in PBC patients than local controls. However, in Japanese population, there were no significant difference in the three SNPs and haplotypes between PBC patients and controls. Our research has also shown that there were no significant differences in the three SNPs and haplotypes among AIH, PBC patients and controls in the Chinese population. Excessive production of IL-10 may play an important role in some populations in modulating the onset of PBC. Further, immunogenetic studies of AIH, PBC should take into account ethnic and geographic variations, this knowledge can then be utilized to clarify their etiologies, and develop new treatment options including manipulation of the cytokine network, an approach which is currently being applied to several autoimmune diseases with some success^[30].

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• BRIEF REPORTS •

Assessment of small intestinal bacterial overgrowth in uncomplicated acute diverticulitis of the colon

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Abstract

AIM: Small intestinal bacterial overgrowth (SIBO) may contribute to the appearance of several gastrointestinal nonspecific symptoms. Acute diverticulitis is affected by some similar symptoms and bacterial colonic overgrowth. We assessed the prevalence of SIBO in acute uncomplicated diverticulitis and evaluated its influence on the clinical course of the disease.

METHODS: We studied 90 consecutive patients (39 males, 51 females, mean age 67.2 years, range 32-91 years). Sixty-one patients (67.78%) and 29 patients (32.22%) were affected by constipation-or diarrhea-prevalent diverticulitis respectively. All subjects were investigated by lactulose H₂-breath test at the entry and at the end of treatment. We also studied a control group of 20 healthy subjects (13 males, 7 females, mean age 53 years, range 22-71 years).

RESULTS: Oro-cecal transit time (OCTT) was delayed in 67/90 patients (74.44%) (range 115-210 min, mean 120 min). Fifty-three of ninety patients (58.88%) showed SIBO, while OCTT was normal in 23/90 patients (25, 56%). In the control group, the mean OCTT was 88.2 min (range 75-135 min). The difference between diverticulitic patients and healthy subjects was statistically significant ($P < 0.01$). OCTT was longer in constipation-prevalent disease than in diarrhea-prevalent disease [180.7 min (range 150-210 min) vs 121 min (range 75-180 min) ($P < 0.001$)], but no difference in bacterial overgrowth was found between the two forms of diverticulitis. After treatment with rifaximin plus mesalazine for 10 d, followed by mesalazine alone for 8 wk, 70 patients (81.49%) were completely asymptomatic, while 16 patients (18.60%) showed only slight symptoms. Two patients (2.22%) had recurrence of diverticulitis, and two other patients (2.22%) were withdrawn from the study due to side-effects. Seventy-nine of eighty-six patients (91.86%) showed normal OCTT (range 75-105 min, mean 83 min),

while OCTT was longer, but it was shorter in the remaining seven (8.14%) patients (range 105-115 min, mean of 110 min). SIBO was eradicated in all patients, while it persisted in one patient with recurrence of diverticulitis.

CONCLUSION: SIBO affects most of the patients with acute diverticulitis. SIBO may worsen the symptoms of patients and prolong the clinical course of the disease, as confirmed in the case of persistence of SIBO and diverticulitis recurrence. In this case, we can hypothesize that bacteria from small bowel may re-colonize in the colon and provoke recurrence of symptoms.

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Key words: Small intestinal bacterial overgrowth; Oro-cecal transit time

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INTRODUCTION

Diverticular disease of the colon is the most common disease affecting the large bowel in the Western world^[1]. This disease is correlated with the aging process and a low-fiber diet and bears a considerable amount of morbidity^[2]. The continuous aging process of the population also leads to an increase of this disease. To refer to an acquired deformity present in perhaps two-thirds of the elderly as a "disease" may be inaccurate, particularly as a large majority of those affected will remain entirely asymptomatic. Nonetheless, 20% of patients may manifest clinical illness^[3].

The true incidence of colonic diverticulosis is difficult to measure, mainly because most patients are asymptomatic. The incidence clearly increases with age, varying from less than 10% in those under 40 years, to 50-66% of patients over 80 years^[4-6]. Only a small proportion (5-10%) of patients who develop diverticulitis are younger than 50 years^[7], and the course of the disease in these patients does not seem more aggressive than that in older patients^[8].

It is commonly thought that multiple factors (anatomic features intrinsic to the colon, alterations in colonic wall with aging, dietary fiber, motor dysfunction, abnormal intraluminal pressure, and possible genetic influences) including altered motility of the large bowel play a great

role in the genesis of colonic diverticula. Motility disorders of the gut are a predisposing factor in development of small intestinal bacterial overgrowth (SIBO)^[9] that may contribute to the appearance of several gastrointestinal nonspecific symptoms, such as bloating, abdominal pain, flatulence and diarrhea^[10]. Since acute diverticulitis is affected by some similar symptoms^[3], and diverticulitis is affected by bacterial colonic overgrowth, the aim of this study was to assess the prevalence of SIBO in acute uncomplicated diverticulitis and its influence on the clinical course of the disease.

MATERIALS AND METHODS

A prospective study was conducted on 90 consecutive patients (39 males, 51 females, mean age 67.2 years, range 32-91 years) with acute uncomplicated diverticulitis of the colon to assess the oro-cecal transit time (OCTT) and SIBO. Diagnosis of diverticulitis, defined as inflammation and/or infection associated with diverticula of the colon^[11], was performed by colonoscopy.

We assessed the following symptoms in all the enrolled patients: constipation, diarrhea, abdominal pain, rectal bleeding, and mucus passage with the stools. The intensity of the symptoms was quantified with a quantitative scale (0-10 according to increasing worsening of symptoms): 0: absence; 1-2: slight; 3-5: mild; 6-7: moderate; 8-10: severe.

In order to assess the prevalence of SIBO, all subjects were studied after an overnight fast for 24 h before the test. They were requested not to smoke on the morning of the test. End expiratory samples were collected before the patients drank the test solution (10 g of lactulose suspended in 100 mL of tap water) and at 15 min-intervals thereafter, for 240 min. Hydrogen concentrations in each collected sample were measured with a breath-hydrogen analyzer (EC60 gastrolyzer breath hydrogen monitor, Bedfont Scientific Ltd, Upchurch, Kent, UK). OCTT was defined as the time elapsing between lactulose ingestion and a sustained increase of 10 ppm or more of H₂ excretion above the baseline value, which was about 75±15 min^[12]. We also evaluated the presence of bacterial overgrowth, which was defined by the presence of a peak >20 ppm occurring >15 min before the colonic peak. The patients with an elevated fasting H₂ combined with an early increase in H₂ after lactulose ingestion were considered positive for bacterial overgrowth^[13]. We compared the results obtained in a control

group, comprising of 20 healthy subjects (13 males, 7 females, and mean age 53 years, range 22-71 years).

All patients were treated with rifaximin (Rifacol®, Formenti SpA, Milano, Italy) 800 mg/d plus mesalazine (Pentacol® 800, Sofar SpA, Trezzano Rosa [MI], Italy) 2.4 g/d for 10 d, followed by mesalazine 1.6 g/d for 8 wk.

Medical control visit and a new lactulose H₂-breath test (H₂-BT) were performed at the end of the 8th wk of treatment with mesalazine alone, and the presence of possible side-effects was evaluated. Moreover, the patients were invited to a control visit whenever they considered necessary.

Statistical analysis

Data were analyzed by χ^2 test. $P<0.05$ was considered statistically significant.

RESULTS

Diverticulitis was localized in the overall colon of 10 patients (11.11%), in the transverse-descending-sigma of 13 patients (14.45%), in the left-sided colon of 12 patients (13.33%), in the descending colon-sigma of 45 cases (50%), in the sigma-rectum of 7 cases (7.78%) and in the rectum of 3 patients (3.33%). Sixty-one patients (67.78%) and 29 patients (32.22%) were affected by constipation-or diarrhea-prevalent diverticulitis respectively.

OCTT was delayed in 67/90 patients (74.44%), ranging from 115 to 210 min, averaged 120 min (Figure 1). Fifty-three of ninety patients (58.88%) showed bacterial overgrowth, while OCTT was normal in 23/90 CD patients (25, 56%), ranging from 75 to 90 min averaged 82.5 min.

In the control group, the mean OCTT was 88.2 min, ranging from 75 to 135 min. The difference between patients with diverticulitis and healthy subjects was statistically significant ($P<0.01$).

When we subdivided the studied population according to bowel movements (constipation-or diarrhea-prevalent colonic diverticulitis), we could note that OCTT was longer in constipation-prevalent disease than in diarrhea-prevalent disease. OCTT was 180.7 min (range 150-210 min), 121 min (range 75-180 min) in constipation- and diarrhea-prevalent colonic diverticulitis, respectively with a statistically significant difference ($P<0.001$, Figure 2).

Interestingly, we did not find any difference in bacterial overgrowth between patients. In fact, 26/53 (49.05%) patients and 27/53 (50.95%) patients with SIBO were affected

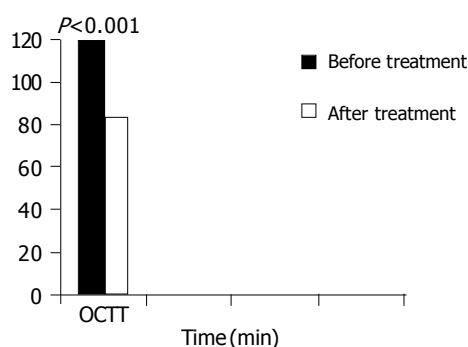


Figure 1 OCTT before and after treatment.

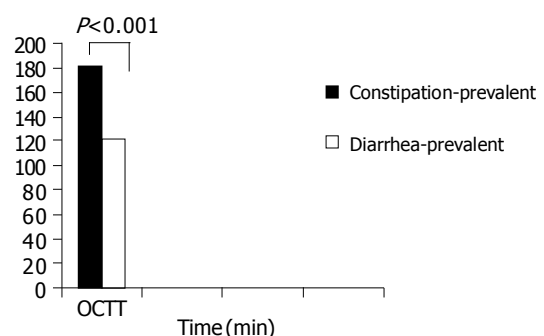


Figure 2 OCTT in constipation-or diarrhea-prevalent diverticulitis.

by constipation-prevalent and diarrhea- prevalent colonic diverticulitis, respectively.

Seventy patients (per-protocol: 81.49% [CI: 67-94%]; on intention-to-treat: 77.78% [CI: 60-85%]) were completely asymptomatic after the 8th wk of treatment with mesalazine alone (overall symptomatic score: 0), while 16 patients (per-protocol: 18.60%; on intention-to-treat: 17.77%) showed only mild symptoms (overall symptomatic score: 44). Two patients (2.22%) had recurrence of diverticulitis (abdominal pain, constipation, and fever) after 4 and 6 wk of treatment with mesalazine alone, and two other patients (2.22%) showed severe side-effects (severe diarrhea [more than 8 bowel movements/day]), and they were withdrawn from the study. The overall score of patients decreased from 1 439 to 44 ($P<0.001$).

Lactulose H₂-BT was re-evaluated at the end of the 8th wk of treatment with mesalazine alone. Seventy-nine of eighty-six (91.86%) patients showed normal OCTT, ranging from 75 to 105 min, averaged 83 min, while OCTT was longer but it was shorter in the remaining seven (8.14%) patients, ranging from 105 to 115 min, averaged 110 min. SIBO was eradicated in all patients. In contrast, a new lactulose H₂-BT showed persistence of SIBO in one patient with recurrence of diverticulitis.

DISCUSSION

Abnormal intraluminal pressure and disordered colonic motility have been implicated as pathogenetic factors in diverticulosis. In particular, alteration of colonic motility has been described as the pathophysiological mechanism in diverticular disease. It has been reported that colonic motility is influenced by the aging process, as shown by the decrease of high-amplitude propagated contraction frequency with the age, whereas segmental contractile activity increases^[14].

This study showed clearly that most of the patients with acute diverticulitis showed a high prevalence of SIBO. The mechanisms underlying the genesis of SIBO in uncomplicated diverticulitis of the colon may be as follows. The first involved mechanism may be the fecal stasis. In fact, the muscle thickening observed in affected bowel segments was thought to be obstructive, and to contribute to the delayed transit of feces^[15]. Studies with intracolonic displacement tools suggested that an accentuation of segmentary motor activity (as observed in diverticular disease) could abolish oro-aboral progression of contents^[16], thereby facilitating retropulsion and drying of the semiliquid fecal matter. These findings have led to the second mechanism causing SIBO that may be the colonic bacterial overgrowth. Changes in intestinal microflorae may be one of the putative mechanisms responsible for colonic inflammation, and could be the key point in the development of symptoms in acute uncomplicated diverticulitis. Acute diverticulitis is a condition characterized by abnormal bacterial colonic overgrowth. This last condition, associated with the reverse peristalsis affecting the colon in diverticular disease, may then favor a small bowel colonization by colonic bacterial florae, provoking SIBO. This hypothesis was confirmed by this study as most patients with acute diverticulitis of the colon showed delayed OCTT (74.44%) and SIBO (58.88%).

The reason why SIBO evaluation is important in acute uncomplicated diverticulitis of the colon is that SIBO is a small bowel disease that has never been investigated in patients with acute uncomplicated diverticulitis, but it may play a role in the genesis of the symptoms experienced by these patients. SIBO is characterized by nonspecific GI complaints, ranging from mild symptoms such as bloating, abdominal pain and flatulence to a severe malabsorption syndrome with diarrhea, steatorrhea and weight loss, and small intestinal mucosal lesions in some cases^[10]. Bacteria are responsible for intraluminal sugar fermentation with the production of a great amount of H₂ and CH₄ (causing bloating, abdominal pain and flatulence). Furthermore, they could cause bile salt deconjugation and dehydroxylation of fatty acids with consequent fat malabsorption and impaired ileal resorption of bile acids, which have irritative and cathartic effects on colonic mucosa. The final consequence of these events is the development of diarrhea, abdominal pain and mucus passage with stools. These symptoms are indistinguishable from those of acute uncomplicated diverticulitis, and may then worsen patient symptoms. Moreover, it may prolong the clinical course of the disease. This last hypothesis has been confirmed by the patients with diverticulitis recurrence and persistence of SIBO. In this case we can hypothesize that bacteria from the small bowel may re-colonize in the colon and provoke recurrence of symptoms.

Fortunately, the main treatment for SIBO and acute uncomplicated diverticulitis is the same. In fact, rifaximin has been effectively used both in the treatment of diverticular disease^[17] and in the treatment of SIBO^[18]. On the other hand, delayed colonic motility persisted despite resolution of acute episodes. This colonic alteration may then persist as a risk factor for SIBO recurrence in patients with colonic diverticulitis. Persistence or recurrence of symptoms often indistinguishable from those of diverticulitis, may lead to unnecessary diagnostic examinations to exclude recurrence of diverticulitis. However, a correct exclusion of SIBO by lactulose breath test may contribute to the correct diagnosis and exclusion of diverticulitis recurrence in most of the cases.

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• BRIEF REPORTS •

Diagnostic value of fine motor deficits in patients with low-grade hepatic encephalopathy

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Abstract

AIM: The role of motor dysfunction in early diagnosis of low-grade hepatic encephalopathy remains uncertain. We performed a pilot study to comparatively investigate the kinematic characteristics of small and large rapid alternating movements in patients with liver cirrhosis and low-grade hepatic encephalopathy.

METHODS: A kinematic analysis of alternating handwriting (7.5 mm) and large drawing movements (DM, 175 mm) was performed in 30 patients with liver cirrhosis (no hepatic encephalopathy: $n = 10$; minimal hepatic encephalopathy: $n = 9$; grade I hepatic encephalopathy: $n = 11$; healthy controls: $n = 12$). The correlation between kinematic parameters, clinical neuro-psychiatric symptoms of cerebral dysfunction and the grade of encephalopathy was investigated.

RESULTS: Both movement types, handwriting and drawing, were significantly slower in cirrhotic patients. In contrast to large DM, the deterioration of handwriting movements significantly correlated with the increase of symptoms of motor dysfunction and differentiated significantly within the group of cirrhosis patients corresponding to the degree of hepatic encephalopathy.

CONCLUSION: The deterioration of fine motor control is an important symptom of low-grade hepatic encephalopathy. The kinematic analysis of handwriting allows the quantitative analysis of alterations of motor function and is a possible tool for diagnostics and monitoring of motor dysfunction in patients with low-grade hepatic encephalopathy.

Key words: Hepatic encephalopathy; Liver cirrhosis; Motor dysfunction; Handwriting; Kinematic analysis

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INTRODUCTION

The majority of patients with liver cirrhosis suffer from mild cerebral dysfunction which is characterized by a variety of clinical symptoms and psychometric deficits^[1]. This dysfunction is partly related to the current ammonia level^[2,3] and, as recently suggested, could to a certain degree be caused by neurodegenerative processes triggered by recurrent hyperammonemia frequently associated with liver cirrhosis^[4].

Mild forms of cerebral dysfunction in liver cirrhosis include minimal hepatic encephalopathy (MHE) and grade I hepatic encephalopathy (grade I HE). Patients with grade I HE manifest with minor signs of motor dysfunction, such as asterixis and tremor, as well as with attention and concentration deficits^[5-7]. Minimal hepatic encephalopathy is diagnosed in patients that demonstrate deficits in attention and visuo-motor co-ordination but otherwise show no evident or only transient symptoms of cerebral dysfunction^[1,6,8]. The exact differentiation between these forms of low-grade hepatic encephalopathy still remains difficult.

Symptoms of motor dysfunction are an important finding in patients with liver cirrhosis and can be detected in different movement types^[9]. Although alterations of handwriting have been earlier observed in patients with liver cirrhosis^[10,11] a quantitative analysis of handwriting in these patients has not yet been performed. Meanwhile, handwriting has been applied for a quantitative and qualitative analysis of movement deficits in a variety of movement disorders^[12-14].

The purpose of this study was to comparatively investigate the alteration of two types of rapid alternating movements large drawing and small handwriting movements in patients with liver cirrhosis and to assess the feasibility of kinematic analysis of handwriting for diagnosis of motor dysfunction in low-grade hepatic encephalopathy.

MATERIALS AND METHODS

Subjects

Thirty patients (w/m: 10/20, mean age 55.53 years, range:

27-72 years) with liver cirrhosis at the out-patient Clinic of the Department of Gastroenterology and Hepatology of Innsbruck Medical University participated in this study, which was approved by the Ethics Committee of Innsbruck University. Prior to inclusion, patients underwent a detailed hepatologic workup and gave informed consent. At the time of testing patients did not show any clinical or laboratory signs of infection, gastrointestinal bleeding, anemia, electrolyte abnormalities or renal insufficiency. The following exclusion criteria were applied in this study: (1) hepatic encephalopathy, clinical grade II-IV^[15], as revealed by clinical neurological investigation; (2) abuse of psychotropic substances; (3) known major neuropsychiatric disorder, as defined by ICD-10; (4) less than 6 mo of complete alcohol abstinence. The patients were diagnosed as follows: cirrhosis associated with hepatitis C ($n = 12$); alcoholic steatohepatitis ($n = 10$); non-alcoholic steatohepatitis ($n = 2$); primary biliary cirrhosis ($n = 1$); primary sclerosing cholangitis ($n = 1$); autoimmune hepatitis ($n = 1$); cirrhosis associated with hepatitis B ($n = 2$); polycystic liver disease ($n = 1$). The severity of cirrhosis was assessed as follows: Child A ($n = 4$); Child B ($n = 25$); Child C ($n = 1$). A group of 12 healthy subjects (w/m: 7/5, mean age 56.08 years, range: 49-66 years) served as controls.

Movement analysis

A commercially available system CS (CS, Munich, Germany) for the analysis of planar movements was applied in this study. The system consists of a digitizing graphic tablet (WACOM IV, UltraPad A4 serial tablet, model DU-1212, WACOM Europe GmbH, Krefeld, Germany) with a sampling rate of 200 Hz and a spatial resolution of 0.05 mm and software for signal processing and data analysis. Processing and kinematic analysis were performed using a CSWin 1.0 software, which allows processing of raw digital data using an established filtering algorithm for kinematic analysis based on kernel functions^[16]. The following basic kinematic parameters were analyzed: mean frequency of movements (number of strokes per second)-the measure of ability to perform a sequence of alternating movements (FREQ), the mean duration of the movement cycle (CYCD; ms/cycle) and the number of velocity inversions per movement (NIV). Patients performed two types of rapid alternating movements: a block of handwriting movements (HW), performed as a series of connected letters "λλλ" (7.5 mm high) and a block of drawing movements (DM), performed as a repeated drawing of a connection line between two points (about 175 mm distance). In both tests subjects were instructed to perform movement as fast as possible. In order to make the subjects familiar with the digital board and the experimental set-up a series of standardized training attempts were performed by all subjects prior to the main tests.

Clinical neuro-psychiatric rating and psychometric tests

The clinical investigation focused on the symptoms which are frequently found in patients with liver cirrhosis and low-grade hepatic encephalopathy^[17,17] and was performed by an experienced neuropsychiatrist prior to the motor tests. Neurological examination included the assessment of asterixis, postural tremor, rigidity, adiadochokinesia (pronation-supination of both forearms), slowing of upper extremities,

impaired concentration and attention deficits. The intensity of symptoms was defined as a combination of their frequency of appearance and their severity. The symptoms were assessed as follows: absent, slight, moderate and severe and were assigned with 0, 1, 2, and 3 scores, respectively. The cumulative clinical score was calculated as a sum of scores of the seven symptoms listed above. The psychometric test battery included trail-making tests A and B (TMT A and TMT B)^[18,19] as well as the digit symbol test (DST). A cumulative psychometric index was calculated as the sum of age-adjusted percentile scores for TMT A, TMT B and DST^[18,19] while one score point was assigned for every 10 percentiles. Patients without obvious symptoms of hepatic encephalopathy and normal psychometric performance (cumulative visuo-motor index >9.0) were assigned with absent HE ($n = 10$). Patients without clinical symptoms but with reduced psychometric performance (cumulative index below 9.0) were diagnosed with minimal hepatic encephalopathy ($n = 9$). Patients with apparent clinical symptoms of cerebral dysfunction but without somnolence and disorientation were diagnosed with grade I hepatic encephalopathy ($n = 11$).

Statistical analysis

The analysis of the difference between patients and controls was performed using *t*-test statistics. ANOVA was applied for the analysis of the association between the degree of cerebral dysfunction and movement parameters. The analysis of the association between kinematic parameters and clinical symptoms was performed using Spearman's correlation analysis. For data processing and statistical analysis the SPSS 11.5 software package was applied.

RESULTS

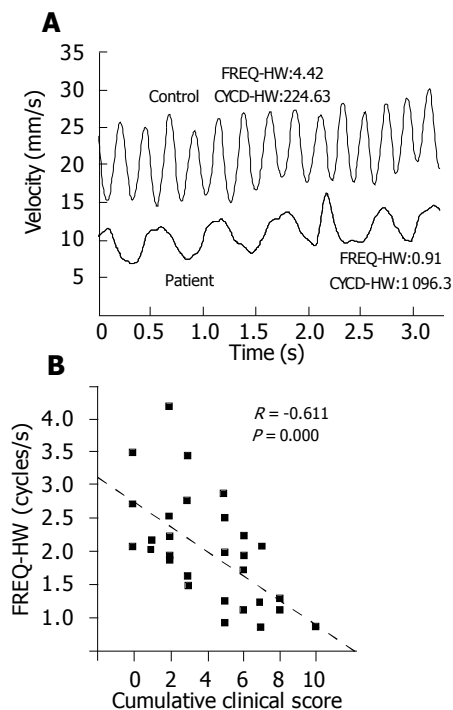
The kinematic parameters of both investigated movement types were substantially altered in patients with liver cirrhosis as compared to controls (Figure 1A). In particular, the frequency of handwriting movements (FREQ-HW) was significantly lower in cirrhotic patients (mean FREQ-HW = 2.03) than in controls (mean FREQ-HW = 3.76, *t*-test: $P < 0.001$). Similarly, the frequency of DM was significantly reduced in patients (mean FREQ-DM = 0.84) as compared to controls (mean FREQ-DM = 1.85; *t*-test: $P < 0.001$). Cycle duration (CYCD) was significantly increased in cirrhotic patients with respect to both movement types (*t*-test: $P < 0.001$ for HW and DM).

The kinematic parameters of handwriting movements significantly differed within the group of patients depending on the degree of hepatic encephalopathy. The results of the one-way ANOVA analysis presented in Table 1 show that, in contrast to DM, for all parameters of handwriting the difference between the subgroups of patients (no encephalopathy, MHE, and grade I) was highly significant. The Spearman's correlation analysis showed that FREQ-HW and CYCD-HW correlates significantly with the visuo-motor index (Table 1) and the cumulative clinical score (Table 1 and Figure 1B). In contrast, the corresponding parameters of DM (FREQ-DM and CYCD-DM) did not show any association with the decrease of visuo-motor index nor with the increase of the cumulative clinical score (Table 1).

Table 1 Correlation between kinematic parameters and symptoms of low-grade hepatic encephalopathy and analysis of the association between the degree of hepatic encephalopathy and kinematic parameters within the group of patients with liver cirrhosis (ANOVA)

Kinematic parameters	One-way ANOVA	Spearman correlation analysis: <i>P</i> -value and (<i>P</i>)	
	grade 0 HE, MHE and grade I HE	Visuo-motor index	Cumulative clinical score
FREQ-HW	0.003 ^b	0.594 (0.000) ^b	-0.611 (0.000) ^b
FREQ-DM	0.592	0.136 (0.245)	-0.203 (0.150)
CYCD-HW	0.010 ^a	-0.504 (0.003) ^b	0.537 (0.002) ^b
CYCD-DM	0.699	-0.128 (0.258)	0.220 (0.131)
NIV-HW	0.002 ^b	-0.618 (0.000) ^b	0.672 (0.000) ^b
NIV-DM	0.934	-0.370 (0.053)	0.310 (0.108)

^a $P < 0.05$, ^b $P < 0.01$ vs others HW-handwriting; DM-drawing movements; FREQ-frequency; CYCD-cycle duration; NIV-number of velocity inversions per stroke; HE-hepatic encephalopathy; MHE-minimal hepatic encephalopathy.

**Figure 1** A: Examples of velocity curves of handwriting movements; a patient (man, 66 years) with liver cirrhosis (Child B, hepatitis C) with minimal hepatic encephalopathy (curve below) and a control subject (man, 65 years; curve above); B: Correlation between frequency of handwriting movements (FREQ-HW) and cumulative clinical score.

The analysis of impairment of movement automatization was performed using the NIV which indicates the degree of decomposition of velocity profile. Values of NIV were significantly increased in patients with liver cirrhosis as compared to controls for both movement types. Again, the degree of decomposition of handwriting movements (NIV-HW) increased significantly within the group of patients depending on the degree of hepatic encephalopathy (Table 1). In contrast, Spearman's correlation analysis showed no association between the deficits in DM (NIV-DM) and the degree of hepatic encephalopathy (Table 1).

DISCUSSION

The main finding of this pilot study is that the impairment of handwriting correlated significantly with the grade of hepatic encephalopathy, clinical neuro-psychiatric symptoms

and visuo-motor psychometric tests. These findings are consistent with the previous studies, which have shown motor deficits in hepatic encephalopathy^[9] and suggest that deficits in fine motor control are common in cirrhotic patients with low-grade encephalopathy. Furthermore, the comparison between handwriting and DM show that the former are strongly associated with the degree of hepatic encephalopathy, whereas the latter showed no correlation within the group of patients.

Low-grade hepatic encephalopathy and in particular motor deficits in patients with liver cirrhosis are associated with alterations in basal ganglia, different cortical regions and dysfunction of cortico-cerebellar pathways^[20-23]. Similarly, several motor control subsystems, such as frontal and parietal cortical regions, cortico-cerebellar pathways as well as basal ganglia are involved in handwriting^[24]. The dysfunction of one or several of these subsystems in patients with liver cirrhosis may result in motor deficits during the course of disease. Early detection of motor deficits would provide the possibility to reveal cirrhosis-associated cerebral dysfunctions in an early stage. Our study shows that the analysis of fine motor control is more sensitive in diagnostics of low-grade hepatic encephalopathy than the analysis of large-scaled movements. We suggest that the impairment of handwriting can be reliably detected and monitored because of the high level of automatization of handwriting movements. On the other hand, the availability of established tools for the kinematic analysis of handwriting allows a quantitative assessment of movement parameters^[16,25]. Our study suggests the use of handwriting analysis for the early detection and assessment of minor motor deficits in diagnostics and monitoring of low-grade hepatic encephalopathy in patients with liver cirrhosis. This would lead to substantial improvement of the clinical management of these patients.

Additionally, our findings point towards another important practical aspect of motor impairment in patients with liver cirrhosis. Patients with low-grade hepatic encephalopathy and even those without apparent signs of cerebral dysfunction may suffer from impairment of basic fine motor skills which are important for every day functioning and could be easily overseen in a standard clinical investigation.

We conclude that further studies of low-grade hepatic encephalopathy in patients with liver cirrhosis should address the detailed evaluation of motor function and that the analysis of handwriting may provide the basis for studies in this context.

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• BRIEF REPORTS •

Dinucleotide microsatellite repeats are essential for the diagnosis of microsatellite instability in colorectal cancer in Asian patients

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Abstract

AIM: The molecular diagnosis of microsatellite instability (MSI) in colorectal cancer (CRC) is based on the analysis of five microsatellite markers. Among them, the two mononucleotide microsatellite repeats are considered more informative for this analysis than the three dinucleotide ones. The aim of this study is to establish the most relevant markers for MSI analysis in colorectal cancers from Asian patients.

METHODS: The MSI analysis of 143 CRC cases in a routine molecular diagnostic laboratory was reviewed. Analysis by fluorescence-based PCR of the five recommended microsatellites was performed, followed by data interpretation according to internationally accepted guidelines. The results were analyzed to address (1) the rate of success in the analysis of histopathological samples not specifically prepared for molecular analysis; (2) the relative importance of individual markers in the diagnosis of high-MSI (H-MSI).

RESULTS: MSI analysis was unsuccessful in 34 cases (24%), but for tissues archived in recent years the unsuccessful rate was 5%. We found the D2S123 marker the most vulnerable to inadequate tissue preservation, failing to amplify in 58 instances. Approximately 30% (32/109) of the cases were H-MSI, while 7/109 (6%) were low-MSI. A detailed analysis of the H-MSI cases revealed that the dinucleotide repeats (and D5S346 in particular) were more relevant than the mononucleotide repeats in assigning the correct MSI status.

CONCLUSION: The analysis of dinucleotide repeats is essential for the establishment of MSI status in Asian CRC patients.

INTRODUCTION

The diagnosis of microsatellite instability (MSI) in colorectal cancer (CRC) is based on the molecular analysis of five microsatellite markers, including two mononucleotide repeats (Bat-25 and Bat-26) and three dinucleotide repeats (D2S123, D5S346 and D17S250)^[1]. However, it has been argued that the analysis of a single mononucleotide repeat such as Bat-26^[2] may be sufficient for this purpose. Furthermore, the comparison of the five microsatellites in hereditary non-polyposis colorectal cancer (HNPCC)-proven cases has highlighted the higher *informativity*^[3] of mononucleotide repeats for this analysis.

In a recent report^[4], based on a limited number of 27 patients, we suggested that the dinucleotide markers were the most frequently unstable and the most relevant ones for the diagnosis of MSI. To fully confirm or refute this fact, we reviewed all the MSI analyses on CRC cases performed in the Molecular Diagnosis Centre at the National University Hospital of Singapore since the establishment of the test 3 years ago.

MATERIALS AND METHODS

A total of 143 cases were analyzed. The mean age of the patients was 46 years. These were cases referred to our laboratory because of clinical features that required the consideration of the HNPCC syndrome, following the Amsterdam criteria.

MSI analysis

The analysis was done as reported elsewhere^[3,4]. Briefly, CRC tissue and normal colonic mucosa were manually microdissected. DNA was extracted with the DNeasyTM Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. This was followed by a capillary electrophoresis, fluorescence-based PCR analysis of the five microsatellite repeats mentioned above, namely

Bat-25 (4q12-*c-kit*), Bat-26 (2p16.3-*bMSH2*), D2S123 (2p16-*bMSH2*), D5S346 (5q21-*APC*), and D17S250 (17q11.2-*BRC4*). A subset of those cases with positive results was subjected to immunohistochemical staining with hMSH2 and hMLH1 antibodies, as well as sequencing of the *bMSH2* and *bMLH1* genes^[4]. An example of microsatellite analysis and mismatch repair gene immunohistochemistry is shown in Figure 1.

MSI diagnostic interpretation

The criterion for high MSI status (H-MSI) is based on instability in at least two microsatellite markers. Instability of a single marker represents low microsatellite instability (L-MSI). Cases with no evidence of instability in any of the markers are considered microsatellite stable (MSS). In view of this, cases with successful PCR amplification in only 2, 3 or 4 markers, and with instability in at least two of those markers, were considered H-MSI; cases with unsuccessful PCR amplification in one or more microsatellites and one or no unstable markers in the successful ones, were regarded as “unsuccessful” analyses, as in these cases we would not be able to exclude L-MSI or H-MSI status.

RESULTS

Success rate and tissue adequacy

With these criteria in mind, a total of 109 successful cases were analyzed (76%), while 34 (24%) unsuccessful cases were excluded. This apparently high percentage of unsuccessful cases was directly related to the age of the paraffin blocks: in fact, the bulk of the unsuccessful cases had paraffin blocks of more than 5 years of age (and sometimes up to 19 years old), at a time when fixation and embedding conditions were not so critically controlled to maintain full nucleic acid preservation. In those cases from recent years,

from our own laboratories, where an adequate control in fixation was exercised (10% buffered formalin during a tightly controlled fixation period of 12-24 h), the success rate was 95% (61/64 cases).

Approximately 30% (32/109) were H-MSI, while 7/109 (6%) were L-MSI. Lack of PCR amplification was noted with the following frequency: Bat-25-10/143 (7%); Bat-26-28/143 (19%); D2S123-58/143 (40%); D5S246-13/143 (9%); and D17S250-26/143 (18%). As unsuccessful amplification was noted predominantly in those cases with older blocks and poor DNA preservation, this analysis gives an indication of the resistance or vulnerability of specific microsatellites to DNA degradation. It appears clear that D2S123 is the most vulnerable, while Bat-25 and D5S346 are the most resistant. This can only be explained in part by the size of the amplicon; indeed, D2S123 is vulnerable as it has the largest PCR product, but does not explain the fact that Bat-26 and Bat-25, with a narrow base-pair difference of 4 units, have a significant difference in outcome. As suggested elsewhere^[5], PCR re-design may be important in narrowing down these differences when looking at paraffin-embedded samples with potential DNA degradation.

Importance of individual markers

To analyze the “diagnostic importance” of each individual marker, we looked at the 13 H-MSI cases in which the diagnosis was made because the minimum number of markers (2) was unstable (Table 1). In these cases, the exclusion of one of those markers would have rendered a different diagnosis altogether. The frequency of instability in these cases for each individual marker is: Bat-25-7 cases; Bat-26-3 cases; D2S123-3 cases; D5S346-8 cases; D17S250-5 cases. Thus, it appears that the dinucleotide repeats are at least as relevant as the mononucleotide repeats for the analysis of H-MSI in these cases; in particular, D5S346

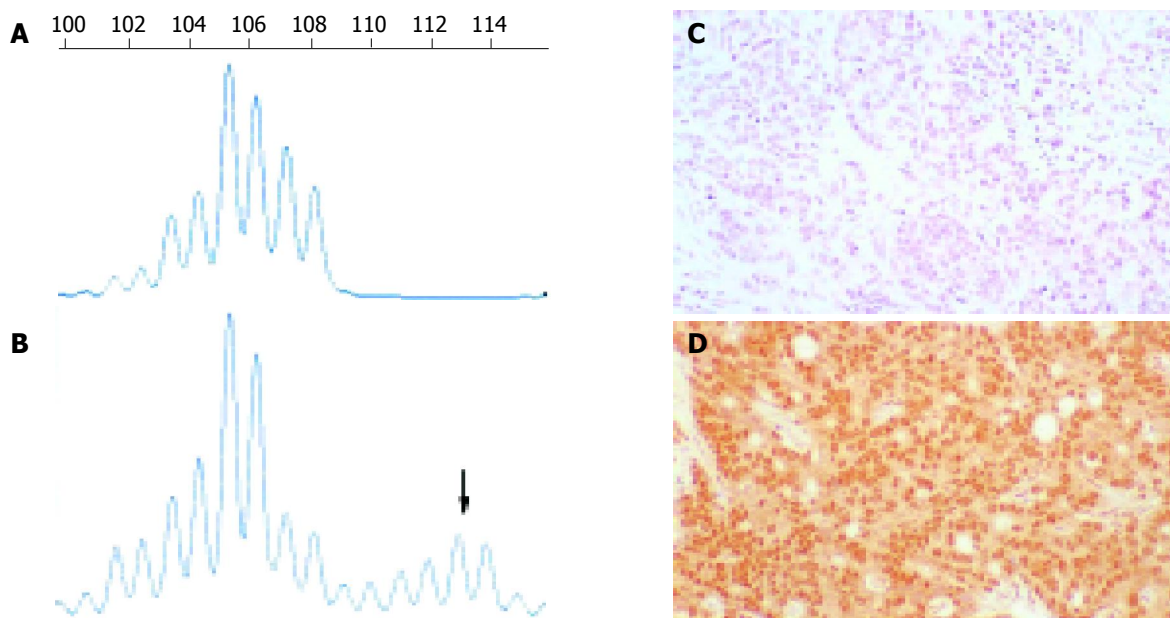


Figure 1 Illustration of MSI and loss of mismatch repair protein hMLH1. **A:** Microsatellite instability analysis of normal mucosa for dinucleotide repeat microsatellite D5S346; **B:** MSI analysis of CRC for dinucleotide repeat microsatellite D5S346 in the same patient, with arrow indicating the area of

instability; **C:** Analysis of hMLH1 protein expression by immunohistochemistry in the same CRC case, showing loss of protein expression; **D:** Analysis of hMLH1 protein expression by immunohistochemistry in a positive control case.

seems to be the most frequently unstable marker. Furthermore, 10/13 cases in this series show no Bat-26 instability.

Table 1 Results of MSI analyses in cases with a minimum number of two unstable microsatellites. S/N: individual case code; +: evidence of instability for a given marker; -: no evidence of instability for a given marker

S/N	Results					Status
	Bat-25	Bat-26	D2S123	D5S346	D17S250	
1	+	-	-	+	-	H-MSI
7	-	-	-	+	+	H-MSI
31	-	-	+	-	+	H-MSI
34	-	-	+	-	+	H-MSI
41	-	-	-	+	+	H-MSI
56	+	+	-	-	-	H-MSI
58	-	-	+	+	-	H-MSI
87	-	+	-	+	-	H-MSI
95	+	-	-	-	+	H-MSI
103	+	-	-	+	-	H-MSI
106	+	-	-	+	-	H-MSI
110	+	-	-	+	-	H-MSI
140	+	+	-	-	-	H-MSI

DISCUSSION

This analysis allows some conclusions in the application of MSI analysis in Asian patients. Firstly, they confirm that analysis of Bat-26 alone may not be sufficient to establish the MSI status in CRC^[6]. Secondly, it highlights the importance of the inclusion of dinucleotide repeats in the MSI analysis of Asian patients. The relevance of dinucleotide repeats in our patient population is a fact difficult to explain. Comparative studies of microsatellite polymorphisms in different ethnic populations have shown a difference^[7] that perhaps may be applicable to Asian patients as well. Also, the relevance of mononucleotide repeats in MSI analysis was generally calculated taking HNPCC-proven patients as the gold standard. With the increasing evidence that the MSI status may be useful in establishing the prognosis of H-MSI, sporadic CRC patients^[8,9], the clinical importance of individual MSI markers may have to be redefined.

In conclusion, dinucleotide repeats are essential for the

establishment of MSI status in Asian CRC patients. In general, a comparative analysis of the relevance of microsatellites in different ethnic populations, and in both HNPCC and H-MSI sporadic CRC patients, may be of importance when applying this test in the future for both HNPCC diagnosis and CRC prognostication.

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• BRIEF REPORTS •

Antral exfoliative cytology for the detection of *Helicobacter pylori* in the stomach

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Abstract

AIM: To evaluate the efficacy of antral exfoliative cytology method in the diagnosis of *Helicobacter pylori* (*H. pylori*) infection in the stomach.

METHODS: Fifty patients were submitted to upper digestive tract endoscopy due to complaints of dyspepsia. The material for exfoliative cytology was obtained by extensive brushing of the gastric antral mucosa and Papanicolaou stain was used to identify the bacteria. The authors also performed gastric biopsies to collect material for urease tests and histologic studies, with hematoxylin-eosin and fucsin stains in order to identify the microorganism. The gold standard used to detect the presence of *H. pylori* was an analysis of the combined results from the gastric biopsies by urease test and histological method.

RESULTS: Antral exfoliative cytology method exhibited 90.3% sensitivity, 66.6% specificity, accuracy of 81.6%, positive predictive value of 82.3% and negative predictive value of 80.0%, in this population with a prevalence of 63.3%.

CONCLUSION: Antral exfoliative cytology was demonstrated to be a sensitive, accurate and easy to perform method for investigating *H. pylori* infection in the stomach.

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Key words: *H. pylori*; Exfoliative cytology; Stomach

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is among the pathogens that most

frequently colonize the gastrointestinal tract and is found in approximately 60% of the world's population^[1].

Its distribution in the entire gastric mucosa can be focal, segmental or diffuse, and it is found within or under the layer of mucus that envelops the epithelium of gastric cells, especially in the antrum^[2-4].

In broad terms, the ideal method to study *H. pylori* infection should have high sensitivity and specificity, besides being cost-effective, easy to perform, rapid and well tolerated by patients, so that it can be repeated as necessary^[5,6].

The heterogeneous distribution of *H. pylori* in the gastric mucosa is a possible source of false-negative results^[7,8]. Exfoliative cytology method utilizes a brush to collect material from the broad surface of the gastric mucosa and may prove to be a better option than other techniques that use only a small quantity of gastric fragments, collected for the urease test and histological analysis.

In view of the controversy between the various studies carried out in diverse regions of the world^[6,9,10], there is still a need for continued investigations to compare endoscopic methods that are easy to perform, cost-effective and routinely used to detect the presence of *H. pylori* in the gastric environment.

The objective of this study was to evaluate the efficacy of antral exfoliative cytology method for the diagnosis of *H. pylori* infection of the stomach.

MATERIALS AND METHODS

From May to August 2002, the authors examined 50 patients suffering from peptic gastroduodenal illness. The sample comprised seven (14%) females and 43 (86%) males, with a mean age of 42.0 years (SD 15.7; range 15-79; and median 40.0).

The inclusion criterion included free and informed consent from the patients. The exclusion criteria were: use of oral or parenteral medication for peptic disease in the previous three months; prior treatment for *H. pylori*; pediatric patients; presence of immunodeficiency; active upper digestive bleeding; and the presence of non-peptic diseases of the upper digestive tract diagnosed during the endoscopic examination.

This investigation was conducted according to the ethical standards accepted by the Helsinki Declaration of the World Medical Association, adopted in 1964 and amended in 1996.

All patients were submitted to upper digestive endoscopies performed by the principal author. The biopsy specimens were collected using conventional biopsy forceps and the gastric brushings were obtained with protected cytological brushes (Wiltek, Medical Inc., USA).

During the endoscopic examinations, material was collected firstly by brushing the antral mucosa, after which

fragments were collected from the gastric mucosa using conventional biopsy forceps for histopathological analysis. Four fragments were collected from the region of the small curvature of the antrum, at approximately 2 cm from the pylorus, and close to the *incisura angularis*^[3,4], separating two fragments for direct detection of the presence of *H pylori* and histopathological analysis, and the remaining two fragments for indirect detection of the presence of *H pylori* by urease test. In addition, two fragments were obtained from the gastric structure, in the region of the small curvature, close to the *incisura angularis*, for direct detection of the presence of *H pylori* and histopathological analysis.

An experienced pathologist, blind to the urease test results, firstly performed the cytologic analysis and then the histological exam. The study identified *H pylori* bacteria with curved morphology, and S-shaped micro organisms, measuring approximately 1-3 μm .

The biopsy material was placed in bottles with buffered 10% formaldehyde for fixation and then forwarded for analysis.

The gastric biopsies were stained with fucsin dye and the semi-quantitative evaluation of *H pylori* was performed according to the criteria proposed by the Sydney System^[11] (Figure 1). The material from the gastric brushings was submitted to cytologic evaluation and testing for *H pylori* using Papanicolaou stain^[12].

Cytologic screening of the gastric brushings was performed in high power fields (HPF), with 400 $\times$ enlargement in an optical microscope, with semi-quantitative evaluation of the following parameters: cellularity; density of inflammatory cells (polymorphonuclear neutrophils); and density of *H pylori*.

The cellularity of the smears was classified according to the presence of groupings of epithelial cells (GEC), in accordance with the following criteria: 0 (no GEC); + (1-3 GEC); ++ (4-6 GEC); and +++ (over 6 GEC)^[13].

The analysis of *H pylori* was based on the following criteria: 0 (no bacteria detected); + (presence of sporadic bacteria); ++ (numerous bacteria in most of the fields); and +++ (clusters of bacteria in all the fields examined)^[14] (Figure 2).

The density of the polymorphonuclear neutrophils (PMN) was evaluated by the examiner, according to the following parameters: absent; +/+++ (rare isolated PMNs in the

smear); ++/+++ (presence of PMNs distributed among epithelial cells without forming groupings); and +++/+++ (presence of PMNs forming groupings among epithelial cells).

The urease test (Probac, Sao Paulo, Brazil) was performed by Claudio A.R. Gomes Jr. The test was recorded as positive when the color around the area of the specimen inserted changed to pink within 24 h.

To verify the agreement between the quantity of bacteria observed in the histological analysis and in the gastric brushing, and the time of any color change in the urease test, the following levels were established: level 1-characterized as no observation of bacteria in the histology and cytology, and negative urease test at 24 h; level 2-positive histology result (+), positive cytology result (+) and positive urease test at 24 h; level 3-positive histology result (++), positive cytology result (++) and positive urease test at 6 h; and level 4-positive histology result (+++), positive cytology result (+++) and positive urease test at 2 h.

The following levels were established for verifying agreement between the presence or absence of *H pylori*, the presence and intensity of cellularity, and the presence and intensity of inflammatory cells in the gastric brushings: level 1-neither *H pylori* nor inflammatory cells, absent or rare cellularity; level 2-*H pylori*, with discrete presence of inflammatory cells and discrete cellularity (+); level 3-*H pylori*, with moderate presence of inflammatory cells and moderate cellularity (++); and level 4-*H pylori*, with intense presence of inflammatory cells and intense cellularity (+++).

To establish the gold standard for comparison of the exfoliative gastric cytology method, the study considered patients affected by *H pylori* to be only those in whom the bacteria were detected by both the histology method and the urease test. The patients considered to be unaffected by the bacteria were those that tested negative in both the histology method and the urease test.

The quantitative variables were represented by their absolute (*n*) and relative (%) frequencies. The arithmetic mean, the median and the standard deviation of the ages were also calculated. The agreement between the different tests for the investigation of *H pylori* was measured by the kappa statistic (κ), which measures nonrandom agreement, with accompanying *p* values referring to rejection of the null hypothesis that $\kappa = 0$ (i.e., agreement due to chance alone).

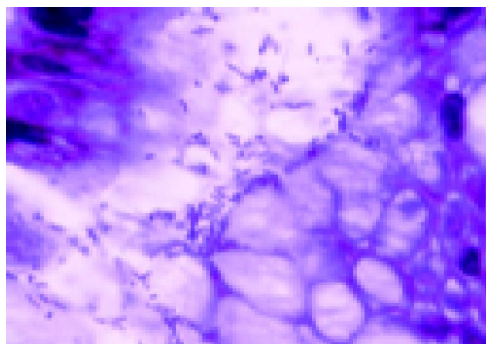


Figure 1 Photomicrography: antral biopsy (Fucsin stain, 1 000 $\times$). Presence of numerous (+++ / +++) *H pylori* bacteria, forming clusters over the epithelial foveolar surface.

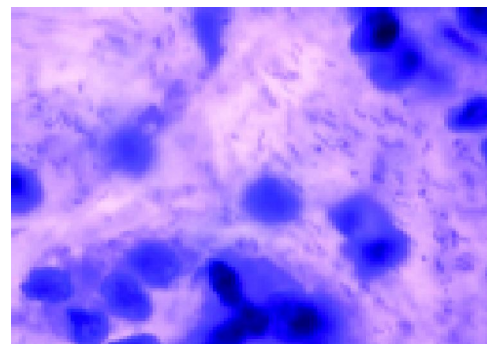


Figure 2 Photomicrography: antral exfoliative cytology (Papanicolaou stain, 1 000 $\times$). Presence of numerous (+++ / +++) *H pylori* bacteria distributed among the epithelial cells, forming clusters.

It was considered that values of $\kappa > 0.75$ represented excellent agreement beyond chance, values of $\kappa < 0.40$ showed poor agreement beyond chance, and fair to good beyond chance ("reasonable") when $0.40 \leq \kappa \leq 0.75$. The study also measured sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the exfoliative cytology in relation to the gold standard, constituted by combined analyses of both the urease test and histology method results.

The calculations of the sensitivity, specificity, PPV, NPV and accuracy were based on the gold standard.

According to the criteria adopted, 49 patients were included in the make-up of the gold standard, as one patient presented a discrepancy between the urease test and histology method results. For the other agreement tests, the study used the results from all 50 patients.

In all tests, $\alpha = 0.05$ was set as the rejection level for the null hypothesis, according to the standards currently adopted in biological studies.

RESULTS

The following illnesses were diagnosed by endoscopy among the patients included in this study: gastritis ($n = 49$); duodenitis ($n = 9$); active or healed duodenal ulcer ($n = 6$); hiatal hernia ($n = 5$); esophagitis ($n = 6$); gastric ulcer ($n = 1$); and esophageal varices ($n = 1$).

With regard to the histopathological analysis of the biopsies collected, this study revealed chronic gastritis in all the patients. There was active chronic gastritis in 34 patients (68%) and inactive in 16 (32%). As to the intensity of the gastritis, 11 patients (22%) presented mild gastritis, 31 (62%) moderate gastritis, and 8 (16%) severe gastritis.

Among the patients, 31 (63.26%) were infected by *H. pylori* and 18 (36.73%) were considered uninfected, according to the criteria adopted for the study.

The cytology method confirmed 32 patients (64%) to be affected by *H. pylori*, with 29 patients (58%) diagnosed by histological method, and 30 affected patients (60%) detected by the urease test.

When the results obtained by exfoliative cytology method were compared with those from the histology method and the urease test combined (gold standard), it was verified that exfoliative cytology presented 90.3% sensitivity, 66.6% specificity, 81.6% accuracy, PPV of 82.3% and NPV 80.0%. Accuracy represents the overall percentage of results from the exfoliative cytology tests that agree with the gold standard. Sensitivity shows the percentage of patients infected with *H. pylori*, correctly identified as positives by the exfoliative cytology, whereas specificity indicates the percentage of patients not infected correctly identified as negatives by cytology. The predictive positive value shows the percentage of individuals with positive cytology tests that were truly infected with *H. pylori*, whereas the negative predictive value shows the percentage of patients with negative cytology that were not in fact infected. As the positive and negative predictive values are dependent on the prevalence of the disease in the studied population, our results should be taken considering a local *H. pylori* prevalence of 63.3% (Table 1).

Table 1 Comparison of the results from the exfoliative cytology method against those obtained from the combined histology and urease test

Exfoliative cytology	Histology and urease both positive	Histology and urease both negative
Positive	$n = 28$	$n = 6$
Negative	$n = 3$	$n = 12$

n = number of patients. Accuracy: $81.6\% = (28+12/28+3+6+12)$; Sensitivity: $90.3\% = (28/28+3)$; Specificity: $66.6\% = (12/12+6)$; PPV: $82.3\% = (28/28+6)$; NPV: $80.0\% = (12/12+3)$; PPV: positive predictive value; NPV: negative predictive value.

When the three methods were analyzed together, the agreement between the positive and negative results of the exfoliative cytology method, urease test and histology method, as measured by the kappa statistic (κ), was reasonable ($\kappa = 0.70$) and statistically significant ($P < 0.001$).

When the agreement was analyzed by the kappa statistic, taking two of the methods into consideration, the agreement between the urease test and the histology method was excellent ($\kappa = 0.95$); while the agreement between the urease test and the exfoliative cytology method was only reasonable ($\kappa = 0.55$). Likewise, the agreement between the exfoliative cytology method and the histology method was also considered reasonable ($\kappa = 0.60$).

According to the kappa statistic, the measurements of agreement for the quantification of *H. pylori* by the histology and exfoliative cytology methods, and time of the color change in the urease test, were reasonable at levels 1 and 4, but no better than chance at levels 2 and 3, although the agreement was statistically significant at all levels ($P < 0.05$) (Table 2).

Table 2 Agreement between the quantification of *H. pylori* by the histology and exfoliative cytology methods and the time of the color change in the urease test

Level	Kappa value (κ)	Kappa value (P)
1	0.70	< 0.000001
2	0.25	$= 0.0009$
3	0.28	$= 0.0002$
4	0.49	< 0.000001

According to the kappa statistic, the results were no better than chance agreement at all levels for the presence of *H. pylori*, inflammatory cells and cellularity in the gastric brushing.

According to the kappa statistic, there was no better than chance agreement between cellularity and presence of *H. pylori* in the gastric brushings at levels 1-3. While at level 4, the rate of agreement was poor.

According to the kappa statistic, the agreement between the presence of inflammatory cells and *H. pylori* in the gastric brushings was poor at all levels.

DISCUSSION

There is still no consensus regarding the diagnostic method that has to be considered as the gold standard^[14], despite which histological method of analysis has been used worldwide due to its high sensitivity and the possibility it

also offers for evaluating alterations in the gastric mucosa that result from the presence of these bacteria^[3]. Nevertheless, urease test may be considered as the method of choice for diagnosis of *H pylori* in the stomach^[15]. In the present study, urease test and histology were demonstrated to have practically the same efficacy regarding the detection of *H pylori* in the stomach, thereby reinforcing the choice of these two exams as the gold standard for comparison with exfoliative cytology method.

Bacteria in the gastric mucosa are often present in small quantities and their irregular distribution can influence the results of exams using fragments from that site^[7]. In the exfoliative cytology method used to detect *H pylori* in the stomach, an appropriate brush is used to obtain material from a broad area of the gastric mucosa, thereby presenting greater potential for efficacy among those cases in which the bacteria has a focalized distribution^[10,16].

The present study adopted as its gold standard, the agreement between urease test and histology diagnostic methods. The exfoliative cytology method presented sensitivity of 90.3% and specificity of 66.6%. Rodríguez *et al.*^[10], compared the cytology method (Papanicolaou stain) with the histology method (hematoxylin-eosin and Warthin-Starry stain) and concluded that the former was superior for the detection of *H pylori*.

Paniagua *et al.*^[6], using histology as the gold standard, studied the exfoliative cytology method and found 72.0% sensitivity, 100% specificity and 77.0% accuracy, where the prevalence was 85.0%, giving an agreement of 0.76, according to the kappa statistic. In the present study, in the comparison between the results from the exfoliative cytology method and those from the histology method combined with those from the urease test, the accuracy reached 81.6%, with an underlying prevalence of 63.6%, and the kappa statistic revealed an agreement of 0.60, between the histology and cytology methods.

Dalla Libera *et al.*^[16], taking the culture of fragments from the gastric mucosa as the gold standard, demonstrated the advantages of cytology over histology for the detection of this micro-organism. In addition to greater sensitivity and specificity (100% and 92.5%, respectively) they reported that the cost and time spent by the pathologist for analyzing the material were markedly lower.

Other studies that have compared cytologic and histologic analyses for the detection of these bacteria have yielded conflicting results. De Francesco *et al.*^[17] has stated that the cytology method is more sensitive, reliable and faster than histological analysis. Ghossoub and Lachman^[13], in a study with 21 patients, concluded that histological method was superior for detection of *H pylori*. Mendoza *et al.*^[18], using cultures of fragments from the antral mucosa as their gold standard, obtained sensitivity of 74.4% and specificity of 92.0% with cytology, concluding that "Diff-Quik" stain makes identification of the bacteria easier than Papanicolaou technique, since it stains *H pylori* blue, thereby facilitating its differentiation from other bacteria and residues found on the imprint.

Carmona *et al.*^[19] compared several diagnostic methods for the detection of bacteria using biopsy and gastric brush. They concluded that exfoliative cytology, analyzed with the use of "Diff-Quik" stain, should be considered the method

of choice for identification of the microorganism. The values obtained by Carmona *et al.*^[19] for sensitivity (95.23%); specificity (94.87%); PPV (96.77%), and NPV (92.50%), were higher than those found in the present study. However, when Gram's stain was used, their values were similar to those obtained in the present study, suggesting that the Papanicolaou stain, used in this investigation, could be considered valid, with the added advantages of low cost and ease of use.

Sentürk *et al.*^[14], considering histology method as the gold standard, compared different methods for the detection of *H pylori* and used May-Grünwald-Giemsa stain to analyze the gastric brushings. That study revealed, using the exfoliative cytology method, a sensitivity of 87.7%, specificity of 60.7%, PPV of 85.3% and NPV of 65.4%. The results obtained by the present study showed slightly higher values than those reported by Sentürk *et al.*^[14], however, the gold standard adopted in this investigation comprised two diagnostic tests in conjunction, as opposed to the single test used by Sentürk *et al.*^[14].

This study demonstrated reasonable agreement, when none of the three methods detected the presence of *H pylori* or when there was a large quantity of bacteria in the specimens collected from the stomach. Using the same method (kappa statistic), for the agreement between the quantity of bacteria found in histology, cytology of the gastric brushings and the speed of the color change in the urease test. At levels 2 and 3, when the quantity of *H pylori* in the gastric brushing and histology was interpreted as low and moderate, respectively, the agreement between the three tests was no better than chance, demonstrating that these are cases that present evidence of greater diagnostic difficulty and disparity between the results offered by the methods used in the present study.

The low quantity of bacteria found in the gastric fragments and the prior reading could explain the false-negative results in the urease test^[5]. Midolo and Marshall^[8] considered that a positive urease test result requires the presence of at least 1000 bacteria in the specimens obtained from the stomach. The irregular distribution and low quantity of the microorganism in the stomach could lead to false-negative results when using the histology method^[16]. The staining methods used in histologic and cytologic studies could also influence the detection of *H pylori*, especially in specimens with low quantities of the bacteria.

According to Debonnie *et al.*^[20], the main condition for cytology method to provide good results lies in the adequate cellularity of the gastric brushing, a factor which cannot be appropriately evaluated at the time material is being collected by cytological brush. Ghossoub and Lachmann^[13] have affirmed that the cytology method could be carried out with the collection of a sufficient amount of the mucus layer, even without adequate representation of cellularity. In this study, the kappa statistic revealed a no more than reasonable agreement only at level 4 (intense cellularity and greater density of the bacteria) in the analysis of the association between findings of cellularity and presence of *H pylori*. Rodríguez *et al.*^[10] have noted that the greater the cellularity of the gastric brushing, the easier it will be to detect *H pylori* in the material studied, a finding that was not observed in this study.

In the conditions under which this study was carried out,

the results from gastric exfoliative cytology for the detection of *H pylori* enabled the conclusion that this method is reliable for the detection of the bacteria in the stomach, when compared with the association between the urease test and the histology method. Furthermore, exfoliative cytology, in an isolated comparison with the urease test and histology method results, presented reasonable agreement. When analyzed simultaneously for the quantification of *H pylori*, the exfoliative cytology method, the urease test and the histology method results were demonstrated to be reliable for confirming the absence of the microorganism in the stomach, without relation between the presence or absence of the *H pylori* and the intensity of the presence or absence of inflammatory and gastric epithelial cells in the exfoliative cytology.

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• BRIEF REPORTS •

Dextrose in the banked blood products does not seem to affect the blood glucose levels in patients undergoing liver transplantation

Kwok-Wai Cheng, Chao-Long Chen, Yu-Fan Cheng, Chia-Chih Tseng, Chih-Hsien Wang, Yaw-Sen Chen, Chih-Chi Wang, Tung-Liang Huang, Hock-Liew Eng, King-Wah Chiu, Shih-Hor Wang, Chih-Che Lin, Tsan-Shiun Lin, Yueh-Wei Liu, Bruno Jawan

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Abstract

AIM: Hyperglycemia commonly seen in liver transplantation (LT) has often been attributed to the dextrose in the storage solution of blood transfusion products. The purpose of the study is to compare the changes of the blood glucose levels in transfused and non-transfused patients during LT.

METHODS: A retrospective study on 60 biliary pediatric patients and 16 adult patients undergoing LT was carried out. Transfused pediatric patients were included in Group I (GI), those not transfused in Group II (GII). Twelve adult patients were not given transfusion and assigned to Group III (GIII); whereas, four adult patients who received massive transfusion were assigned to Group IV (GIV). The blood glucose levels, volume of blood transfused, and the volume of crystalloid infused were recorded, compared and analyzed.

RESULTS: Results showed that the changes in blood glucose levels during LT for both non-transfused and minimally transfused pediatric groups and non-transfused and massively-transfused adult groups were almost the same.

CONCLUSION: We conclude that blood transfusion does not cause significant changes in the blood glucose levels in this study.

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Key words: Organ; Liver surgery; Transplantation; Anesthesia; General monitoring; Blood glucose transfusion; Bank blood component; Infusion; 5% dextrose in 1/4 saline.

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INTRODUCTION

Contrary to earlier reports^[1], hyperglycemia during liver transplantation (LT) is a common finding^[2-4]. Normal blood glucose levels could even be maintained during LT procedure in humans^[2] and dogs^[5] with blood as the only source of dextrose. The mechanism is attributed to blood transfusion, which contains dextrose as part of the storage solution^[2,3]. Blood transfusion in LT was once a common feature in the past, however, with increasing LT experience, the blood loss and subsequent blood and blood product replacement during operations have decreased gradually^[6,7]. LT can now be performed without pack blood cell^[8] and fresh frozen plasma^[9]. In our living donor LT setting, some patients do not receive any banked blood products^[10] but hyperglycemia is still noted. Therefore, we hypothesize that hyperglycemia seen in LT might not be related to blood transfusion. In this study, blood glucose levels of pediatric patients with biliary atresia undergoing LT with or without blood transfusion and four massively-transfused adult patients vs 14 non-transfused adult patients were compared and analyzed.

MATERIALS AND METHODS

The approval of the Ethics Committee of the Department of Health, Taiwan, was obtained and written informed consent for anesthesia and surgery were also obtained from the adult patients or the parents of the pediatric patients. Sixty biliary pediatric patients who underwent living donor liver transplantation (LDLT) and 16 adult LT patients at Chang Gung Memorial Hospital, Kaohsiung Medical Center from January 1999 to April 2002 were included. The anesthesia records were analyzed retrospectively. The pediatric patients were grouped as transfused (GI) and non-transfused (GII) group. Since the transfusion of GI was minimal, an additional four adults in our cadaveric series, who were massively transfused over 10 L of blood products (GIV) were compared with 12 of our 28 adult LDLT patients (G III) who did not receive blood transfusion. Anesthesia for all patients was maintained with isoflurane in an oxygen-air mixture. Fentanyl was given whenever necessary and atracurium was used as muscle relaxant. Continuous monitoring with ECG, pulse oximetry, arterial blood pressure, central venous pressure, end tidal CO₂, urine output and naso-pharyngeal temperature was performed. At least four intravenous lines were set for

fluids and blood replacements. Crystalloids such as normal saline, half saline, lactated Ringer, dextrose 5% in 1/4 saline were used. Five percent albumin and crystalloids instead of blood products were used to replace blood, ascites and transudate losses to maintain a state of normovolemia. Transfusion threshold was set at hemoglobin of 6-7 g/dL for as long as the patients remained hemodynamically stable. The amount of packed RBC (preserved in CPDA-1) transfused was estimated to reach a Hb of no greater than 8-9 g/dL after transfusion. LDLT without veno-venous bypass was performed as reported previously^[11,12]. Continuous intravenous dopamine infusion was given at a rate of 2 µg/(kg·h) throughout the operation. Arterial blood gases and blood sugar were recorded after induction of the anesthesia, every 2 h during liver dissection, at anhepatic phase, 10 min after reperfusion and at the end of the operation. Blood glucose levels, total amount of fluids and blood components (including crystalloids with or without 5% dextrose, 5% albumin, packed red cell and fresh frozen plasma) were recorded. Blood glucose levels were compared between the transfused and non-transfused pediatric patients; likewise, comparison between the non-transfused adult patients and massively-transfused adult patients was made. Measurements between groups were compared using Mann-Whitney *U* test. All the data were given in mean±SD. Statistical calculations were performed using the SPSS advanced statistics module (SPSS Inc, Chicago, IL, USA). *P*<0.05 was regarded as significant.

RESULTS

Thirty-eight patients were included in the transfused group (GI), while twenty-two patients were included in the non-transfused group (GII). Table 1 shows the characteristics of the patients of GI and GII. The age, weight, height of the patients and anesthesia duration were not significantly different between groups. Higher hemoglobin levels were observed in GII. More intraoperative blood loss requiring packed blood cells and fresh frozen plasma transfusion was noted among GI patients. There was no difference between the two pediatric groups with regard to the mean amount of crystalloids with or without 5% dextrose and 5% albumin given. Mean urine output measured in pediatric groups showed no statistical difference. Blood glucose levels in both groups

were almost the same with a tendency towards hyperglycemia at 170-270 mg/dL (Figure 1). The changes in blood glucose levels in the four massively-transfused adult patients (with 8 020±6 544 mL red blood cells and 4 085±2 888 mL fresh frozen plasma) were similar to the changes seen in the GIII patients (Figure 2).

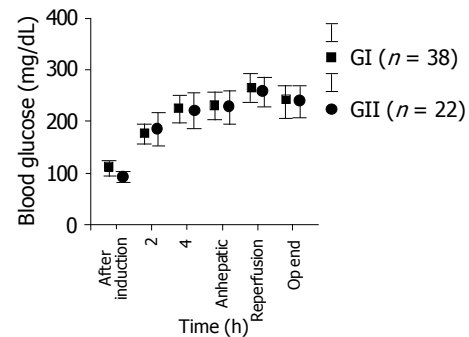


Figure 1 No significant changes of blood glucose level between groups during LT in biliary atresia patients with and without blood transfusion.

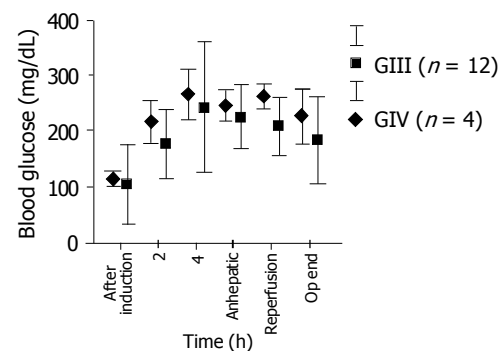


Figure 2 No significant changes of blood glucose level between groups during LT in adult patients with and without blood transfusion.

Table 1 Patients' characteristics between pediatric groups

	GI (n = 38)	GII (n = 22)	P
Age (yr)	2.18±0.4	3±1.9	0.35
Weight (kg)	10.6±4.9	12.6±4.3	0.46
Height (cm)	82.1±23.6	86.8±15.4	0.43
Anesthesia time (h)	14.2±2.6	12.8±1.4	0.56
Blood loss (mL)	172.1±134	66.1±53.6	0.000
RBC (mL)	171±110	0	0.000
FFP (mL)	101.5±169	0	0.002
5% Albumin (mL)	419±392	489±2 670	0.253
Platelet transfusion	0	0	
Crystalloids without dextrose (mL)	1 246±662	1 154±550	0.613
Crystalloid with 5% dextrose (mL)	499±279	367±209	0.096
Urine (mL/(kg·h))	3.0±1.4	3.3±1.1	0.137
Preoperative Hb (mg%)	8.2±1.8	10.7±1.1	0.000
Postoperative Hb (mg%)	7.6±1.1	7.7±2.0	0.638

DISCUSSION

The liver plays an important role in carbohydrate metabolism and glucose homeostasis. Impaired gluconeogenesis and glucogenolysis, glucose intolerance or insulin resistance may occur in patients with end stage liver disease, predisposing these patients to fasting hypoglycemia or postprandial hyperglycemia^[13-17]. In view of the complexity of glucose regulation and disease-specific anesthetic management^[18], our study primarily focuses on pediatric biliary atresia patients, who have had Kasai operation. This specific group of patients represents children of the same age group in a similar physiological state with the same disease entity. Figure 1 shows that the blood glucose increased significantly 2 h after induction of anesthesia from 108±46 to 174±60 and 92±22 to 185±73 mg/dL for GI and GII, respectively. Likewise, Figure 2 shows the similar changes of the blood glucose for GIII and GIV patients. The levels of the blood glucose for all groups remained lightly hyperglycemic until the end of the operation regardless of transfusion.

The volume of blood transfused in GI was statistically significant but the amount of the dextrose contained in the 272 mL of transfused blood products may be clinically

irrelevant when compared to GII. We find that the volume of blood transfused is not related to blood glucose level in the four massively-transfused adult patients given over 10 L blood components during LT (Table 2). The fresh blood stored in CPDA-1 contained dextrose of 432 mg/dL. This decreases to 282 mg/dL after 35 storage^[19]. Blood that is massively lost also contain dextrose. Blood glucose levels will not change despite exogenous dextrose loading if the amount of blood loss is greater than the volume transfused. This may explain why over 10 L of blood products given to GIV patients did not cause significant increase in blood glucose compared to GIII patients. Although Hb in blood product is usually higher than anemic patients requiring blood transfusion, the above mechanism may also explain why there is little increase in Hb level in patients receiving blood transfusion during massive bleeding in which losses are greater than that replaced. The preoperative and postoperative Hb showed that none of our patients was over-transfused (Table 1). It indicates that hyperglycemia encountered during LT in all groups is not related to blood transfusion per se. This observation is shared by other authors^[2]. Hyperglycemia is probably caused by stress during LT surgery and from two of methyl-prednisolone given during the operation. Both noxious stimuli and methyl-prednisolone are known to increase blood glucose levels^[20,21]. Furthermore, extrahepatic gluconogenesis^[22] and sudden release of glucose from the grafted liver in the reperfusion phase may also cause hyperglycemia^[5]. Recent discoveries reveal that extrahepatic gluconogenesis during anhepatic phase, most notably by the kidneys, contributes to endogenous glucose production in humans^[22]. This endogenous glucose production is sufficient to maintain normal blood glucose levels in the anhepatic phase of orthotopic LT. Corollary, reperfusion is usually associated with a sudden increase of blood glucose as a result of massive glucose release from the graft liver as seen in very high glucose levels in hepatic venous blood compared to arterial blood^[5].

Table 2 Patients' characteristics between adult groups

	GIII (n = 12)	GIV (n = 4)	P
Age (yr)	43±9.6	57±11.1	0.70
Weight (kg)	66±9.2	59.7±11.5	0.316
Height (cm)	168±7.7	167±4.9	1.0
Anesthesia time (h)	14. ±0.95	18.5±1.4	0.88
Blood loss (mL)	532±364	15 345±182 029	0.001
RBC (mL)	0	8 020±6 544	0.001
FFP (mL)	0	4 085±2 887	0.001
5% Albumin (mL)	1 337±505	2 966±550	0.253
Platelet transfusion (units)	0	24±24	0.003
Crystalloids (mL)	6 851±1 524	17 468±12 171	0.009

Hypo- as well as severe hyperglycemia should be avoided during surgery especially in LT. Our results showed that blood glucose levels of 170-270 mg/dL could be maintained with minimal administration of 499±279 or 367±209 mL 5% dextrose solution or 2.7 and 2 mg/(kg min) for transfused and non-transfused pediatric patients, respectively. The doses are only one-third to half of the recommended doses (4-6 mg/(kg min)) in routine non-liver transplant pediatric surgery. We conclude that no significant changes occur in the blood glucose levels among minimally-transfused and non-transfused, as well

as in non-transfused and massively-transfused, patients undergoing LT.

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• BRIEF REPORTS •

Survival outcome of lobar or segmental transcatheter arterial embolization with ethanol-lipiodol mixture in treating hepatocellular carcinoma

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Key words: Liver; Hepatoma; Transcatheter arterial embolization; Treatment; Survival outcome

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Abstract

AIM: To evaluate the clinical outcome and cost-effectiveness of transcatheter arterial ethanol-lipiodol embolotherapy on hepatocellular carcinoma (HCC).

METHODS: One hundred patients with HCC who were treated only by lobar or segmental transarterial embolization (TAE) with ethanol-lipiodol mixture were enrolled in this study. The 1st- and 2nd-year survival rates were analyzed to evaluate the feasibility of its method. These outcomes of our patients were individually correlated to the Child-Pugh classification and the computed tomographic features of HCC.

RESULTS: The overall 1st- and 2nd-year survival rates were 72% and 46%, respectively. The patients were classified into three groups according to their liver function status: 68 patients as Child-Pugh class A, 26 as Child B, and 6 as Child C. Child A had better survival rate than the Child B and/or C. The 1st-year survival rates of patients with Child A-C were 84%, 50%, and 33.3% respectively and the 2nd-year survival rates were 55.5%, 28.5%, and 33.3%, respectively. According to the computed tomographic features, solitary HCC with maximum diameter less than 5 cm had the best outcome with the 1st-year survival rate of 100% and the 2nd-year survival rate of 71.4%, while solitary HCC with maximum diameter over 5 cm and multiple HCC had the 1st-year survival rates of 75% and 63.7%, respectively, and the 2nd-year survival rates of 33.3% and 44.4%, respectively. Only one patient was complicated with abscess formation and was cured with antibiotic therapy. No mortality resulted from the procedures performed.

CONCLUSION: TAE with ethanol-lipiodol mixture is an economic, safe and feasible method for treating HCC, especially for the patients with smaller solitary HCC or with liver function status of Child-Pugh class A.

INTRODUCTION

Hepatocellular carcinoma (HCC) had been documented as the leading cause of cancer death in our country for years^[1]. Various methods of treatment including surgical and non-surgical therapies have been attempted. The non-surgical ones include transarterial embolization (TAE), transarterial chemoembolization (TACE), percutaneous ethanol injection, radiofrequency ablation, systemic chemotherapy, hormone therapy, immunotherapy, and radiotherapy. Among them, surgery is traditionally believed to be the best treatment, offering the chance of complete cure by tumor removal^[2]. Unfortunately, most of the HCC are unresectable at discovery, with poor survival rate of less than 6 mo^[2]. On the other hand, the recurrence of HCC is high in a cirrhotic liver secondary to infectious hepatitis.

In those cases with cirrhotic liver, deteriorating liver function is a common risk factor for undertaking a large area resection. An optimal therapy with safe procedure and minimal interruption on liver function is essential. Under such circumstances, minimal invasive nonsurgical management rather than invasive surgical approach may be the choice of therapy, in which TACE is one of the most commonly used methods. However, chemotherapeutic drugs are expensive and hepatic toxic. In this study, we report the survival outcomes of TAE of HCC using the less toxic ethanol-lipiodol liquid mixture, in order to evaluate the feasibility of this embolization method. To our knowledge, this is the first report to document the therapeutic results of TAE with ethanol-lipiodol liquid mixture in the patients with inoperable HCC.

MATERIALS AND METHODS

Reviewing the records of transcatheter arterial embolization (TAE) performed in our department from January 2000

to December 2002, we enrolled 100 HCC patients who received lobar or segmental TAE with the mixture of absolute ethanol and lipiodol. There were 70 males and 30 females, ranging from 39 to 82 years (mean: 63.62 ± 9.65 years). The technique of TAE was standardized, following the set up protocol since January 2000 in our department. In order to obtain an absolute therapeutic outcome of ethanol-lipiodol TAE, the patients that underwent other treatments such as percutaneous ethanol injection, radiofrequency therapy or TACE were excluded from our study. Those HCCs with the radiographic evidence of vascular shunts or with portal vein thrombosis were also eliminated.

All patients were requested to sign consents after full explanation. With the coaxial angiographic technique, a French-3 microcatheter was selectively catheterized beyond the lobar or segmental branch of the hepatic artery supplying the HCC. Absolute ethanol (99.5%) and lipiodol were mixed in emulsion as the embolizer for TAE. The composition of absolute alcohol and lipiodol was kept at 3 to 1 ratio. However, the amount of lipiodol used was proportional to the maximum diameter of targeted HCC measured on computed tomogram (CT). If the maximum diameter was 5 cm, the amount of lipiodol used would be 5 mL. In cases of multiple HCC, the amount of lipiodol (mL) used was adjusted according to the sum of maximum diameters of all targeted HCC in centimeters. However, the maximum amount of lipiodol was always limited to 10 mL.

Before starting the infusion of embolizing agents, each 25 mg Demoral was given intravenously and intramuscularly, respectively. To avoid the vasospasm, intra-arterial slow injection of 3 mL of 2% lidocaine diluted with 10 mL saline were routinely administered. With the stable vital signs continuously being monitored by pulse oximeter, the ethanol-lipiodol mixture was connected to an adjustable pumping machine and was slowly infused with a set rate at 0.5-1 mL per minute. The TAE would be finished when the infusion of embolizing mixture had been completed. In another situation, the embolization would also be terminated when the retrograde filling of the portal vein was observed (Figure 1). Finally, absorbable gelfoam particles were routinely administered until sluggish inflow of the contrast medium in the supplied arteries of the embolized HCC, in order to delay the washout of the embolizers.

According to the findings on triphasic CT, the HCCs of

our patients were classified into three categories. They included: (1) solitary HCC with maximum diameter less than 5 cm, (2) solitary HCC with maximum diameter greater than 5 cm, and (3) multiple HCCs of multinodular, infiltrating or mixed type. The patients were also classified into three other groups as Child A-C, according to their liver function status. We individually correlated the CT presentations of HCC and the Child-Pugh classification to the 1st- and 2nd-year survival outcomes.

RESULTS

Totally, 245 TAE procedures were successfully performed. Some cases of mild abdominal distension and mild fever were observed after the procedure, but all subsided within 24 h after the procedures. No major complications were immediately noticed during the procedures except one liver abscess (1%) was found 5 d after the TAE. The abscess was subsequently cured by antibiotic therapy. Based on the finding of follow-up CT, no bilioma was complicated. There was no procedure-related mortality.

According to the serum data, all our patients were hepatitis-infected, including 56 patients with viral hepatitis-B, 34 with viral hepatitis-C, six with both viral hepatitis B and C, and four with non-B and non-C viral hepatitis. At the time of TAE, 68 of our 100 patients were classified as Child-Pugh class A, 26 as Child B and six as Child C. According to the morphologic presentations of HCC, multiple HCCs were found in 69 patients, solitary HCC with maximum diameter less than 5 cm was found in 19 patients, and that greater than 5 cm was found in the remaining 12 patients.

Post-TAE follow up

Post-TAE serum levels of aspartate aminotransferase and alanine aminotransferase on the 3rd day increased in 195 procedures, of which an increment ranging from 50 to 300 IU/L was found in 123 procedures, ranging from 301 to 500 IU/L in 50 procedures, and >500 IU/L in the other 22 procedures. Slight elevation of the total bilirubin and a decrement in albumin levels were also noted in about 20% of our 245 TAE procedures. All these abnormal parameters returned to the initial levels within 2 wk. At that time, considerable decrement of alpha fetoprotein was noted in 172 of our TAE procedures. The alpha fetoprotein of our patients with HCCs with maximum diameter less than 5 cm was invariably improved. On the follow up triphasic CT after the first TAE, a complete package of embolized HCC without evidence of viable cancer was found in 73 patients (12 patients of HCC with maximum diameter less than 5 cm, 13 patients of HCC with maximum diameter greater than 5 cm, and 48 patients of multiple HCC). Residual viable tumors were noted in the other 27 patients (23 patients with multiple HCC and four with HCC larger than 5 cm of maximum diameter). In this series, in order to treat residual viable tumor ($n = 27$), local recurrence at the embolized areas ($n = 8$), or newly developed HCC in the otherwise liver areas ($n = 46$), 54 patients needed repeated TAE. Seventeen patients underwent second TAE and the other 37 patients underwent second and third TAEs.



Figure 1 Post-embolization radiographic image of upper abdomen shows homogeneously packed HCC and retrograde filling of multiple parenchymal portal veins.

Cost-effectiveness assessment

The benefits of ethanol-lipiodol TAE compared with TACE was assessed in the cost of embolizers and the duration of hospitalization. In our country, the cost of Doxorubicin in a single TACE procedure was \$US 60 for 20 mg. However, the cost of the 99.5% ethanol that we used in a single TAE procedure was \$US 0.05. On the other hand, all patients were discharged from hospital about 3 d after TAE procedures. Only one patient was admitted again due to the complication of liver abscess 5 d after TAE and required hospitalization for antibiotic treatment for 8 d. No additional systemic complication prolonging the hospitalization was noticed.

Survival outcome analysis

The overall 1st- and 2nd-year survival rates were 72% and 46%, respectively. The 1st-year survival rate distinctively varied to Child-Pugh classification with 84% for Child A, 50% for Child B and 33.3% for Child C. For the 2-year survival rate, Child A was still the best but the rate decreased to 55.56%. The 2-year survival rate of Child B and C were close to the survival rate of 28.57% and 33.3%, respectively (Figure 2). The cause of the same 1st- and 2nd-year survival rate (33.3%) of our Child C patients was that two of our six Child C patients had solitary HCC less than 5 cm and were well controlled by TAE in the period of 2 years.

The survival rates of patients with solitary HCC less than 5 cm, and that greater than 5 cm and multiple HCCs were 100%, 75%, and 63.77%, respectively for the 1st year and 71.43%, 33.33%, and 44.44%, respectively for the second year (Figure 3). The patients died of the uncontrollable progression of the disease including the decompensation of liver function in 18 patients, widespread dissemination of HCCs in 25, and the development of portal vein thrombosis in 9.

DISCUSSION

In Taiwan, most patients with HCC were cirrhotic related, accompanied by liver dysfunction. The liver function impairment or the HCC involvement often limit the surgery even though the survival rate of surgical cases is believed to be significantly higher than non-surgical cases^[3]. However, the survival rate had been improved by the progressive advances of angiographic techniques. With the development

of microcatheter system, we are able to superselectively catheterize a small angiocatheter into the tumor-feeding artery in order to achieve the lobar or segmental targeting embolization while avoiding arterial injury. With such a high technical performance, the embolizers can be effectively injected into HCC tumor beds.

Since the application of TAE on unresectable HCC, several reports have documented the effectiveness of this treatment on unresectable HCC^[4-7]. Various embolizers had been introduced. TACE was currently used in most centers due to the combined therapeutic effects of tumor chemotoxicity and ischemia. In a report of a Chinese population sample of inoperable HCC with Child A and B, the 1st- and 2nd-year survival rate after TACE were 86.3% and 78.8% respectively^[8]. In our result, the survival rates of the patients with Child A and B were 84% and 50%, respectively for the 1st year, and 55.5% and 28.5% respectively for the 2nd year. The survival rate of our patients with Child C maintained at 33.3% for the 1st- and 2nd-year survival rates, however, due to small populations in this group, such survival rate necessitated further investigation with a larger series.

In 1983, sonographically guided percutaneous ethanol injection therapy (PEIT) was introduced as a new alternative approach in treating small HCC^[9]. This method was subsequently agreed upon by many clinicians on the point of effectiveness in treating a small HCC^[10-13]. For a large HCC, PEIT has difficulties to fill the entire tumor tissue because of the complicated architecture of septa and inhomogeneous infiltration of ethanol within the tumor. The combined treatment with PEIT and TACE has been reported as effective in causing complete tumor necrosis^[12-14]. The capsular invasion or small daughter nodules can also be treated^[12-14]. The reported 1st- and 2nd-cumulative survival rates of combined therapy with PEIT and TACE were 78% and 54%, respectively^[15]. Such a result was similar to ours with ethanol-lipiodol TAE.

Currently, TAE or TACE are the mainstream in the treatment of unresectable HCC. Since chemotherapeutic drugs are hepatotoxic, we use a less toxic ethanol as an embolizer in order to preserve the liver function as much as possible. Ethanol is a traditionally cheap and safe liquid embolizer that has been used to treat vascular lesions or tumors. In 1997, the embolization effect of ethanol on HCC was established on the basis of animal models^[16,17].

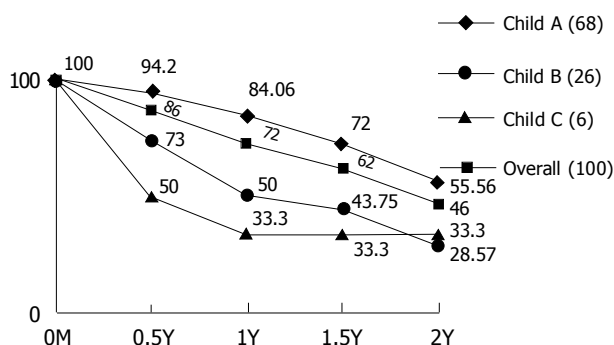


Figure 2 Survival rates of HCC in Child-Pugh classification.

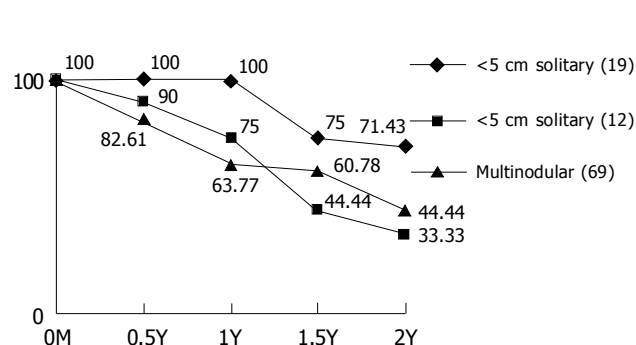


Figure 3 Survival rates of HCC in different morphology.

Kan *et al.*^[18], reported the pathways of iodized oil and silicon rubber solution shunting from the hepatic artery to the portal vein, and the mechanism of sinusoid embolization by iodized oil^[19]. On the other hand, two reports have documented the clinical efficacy of this ethanol-lipiodol TAE on HCC^[20,21]. The results showed successful liver lobar ablation with only minimal side effects.

From the fluoroscopic observation on an animal model, dual embolization will be induced by slow infusion of insoluble substance, such as a mixture of ethiodol and ethanol, which appears as small droplets passing through the hepatic sinusoids (the bridges between the hepatic artery and the portal vein) to the portal vein. This achieves complete thrombotic effect with embolizers package in both the arteries supplying the tumor and its adjacent parenchymal portal veins^[16,21]. A slow infusion of embolizers is the key point for the success of dual embolization. The slow infusion technique not only prevents the spasmodic change of the feeding artery, but also allows the embolizers to flow primarily to the fast stream tumor vessels. Once the tumor vessels have been filled up, the liquid, insoluble embolizers will then start to deposit along the feeding hepatic artery and the parenchymal portal veins.

In our results, tumor morphologic features and patient liver function status affected the survival rates of HCC patients after TAE with ethanol-lipiodol mixture. Better survival rates were observed in patients with solitary smaller HCC or with liver function status of Child A. The outcome of our study was similar to that of traditional TACE. Nevertheless, ethanol-lipiodol TAE is a cheap and less toxic alternative treatment feasible for HCC.

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• BRIEF REPORTS •

Association of the *myeloperoxidase*^{-468G→A} polymorphism with gastric inflammation and duodenal ulcer risk

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Abstract

AIM: To elucidate the relations between the *myeloperoxidase*^{-468G→A} polymorphism and the development of duodenal ulcer (DU), and to investigate the impacts of this host genetic polymorphism on the histopathological features of *Helicobacter pylori* (*H. pylori*)-related gastritis.

METHODS: In a case-control study of 115 consecutive DU patients and 182 controls, the *myeloperoxidase*^{-468G→A} polymorphism was genotyped. Additionally, gastric mucosal changes were examined according to the updated Sydney System.

RESULTS: The two study groups differed in the distributions of *myeloperoxidase* genotypes ($P = 0.008$). All six individuals carrying *myeloperoxidase* A/A genotypes were in the DU group. The carriage of *myeloperoxidase* allele A and *H. pylori* infection were associated with an increased risk of DU with odds ratios (OR) of 2.3 and 5.8, respectively. The combined risk of the carriage of *myeloperoxidase* allele A and *H. pylori* infection for DU was 8.7 (95% CI, 3.5-21.8). In the *H. pylori*-infected individuals, allele A carriers displayed higher bacterial density scores ($P = 0.04$) in the antrum than did non-carriers.

CONCLUSION: This work verifies for the first time the association of *myeloperoxidase*^{-468G→A} polymorphism with antral *H. pylori* density and DU disease. The mechanisms underlying this genetic polymorphism in developing DU disease merit further investigations.

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Key words: Duodenal ulcer; *Helicobacter pylori*; *Myeloperoxidase*; Polymorphism

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<http://www.wjgnet.com/1007-9327/11/2796.asp>

INTRODUCTION

The discovery of *Helicobacter pylori* (*H. pylori*) heralded a breakthrough in the field of gastroenterology. It is a well-recognized pathogen that chronically infects more than 50% of the world's population. Infection with the bacterium regularly leads to chronic gastritis. A subset of infected patients develops duodenal ulcer (DU), gastric ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (MAL-Toma)^[1-4]. The course of disease is affected by bacterial virulence factors, as well as genetic predisposition and environmental factors of the hosts.

It has been suggested that phenotypic or genotypic differences of the *cag* pathogenicity island, *vacA*, *iceA* and *babA* among bacterial strains may account for the development of severe diseases^[5-8]. Early studies indicated that the *cagA* gene/*CagA* protein was a marker for more severe diseases because it was more frequently associated with strains isolated from patients with ulcer diseases or gastric adenocarcinoma^[5,9]. However, the predominant strain of *H. pylori* that circulates in Asian countries is a *cagA*-positive, *vacA* s1 and *babA*2 genotype, unrelated to clinical outcomes^[9,10]. Bacterial virulent factors have so far failed to explain why the ulcer or gastric cancer phenotype develops^[11,12]. Recently, attention has been focused on the involvement of host factors that determine susceptibility to *H. pylori*-associated diseases, such as gender, blood groups, gastric acid physiology, human leukocyte antigen and genetic polymorphisms^[13-15].

It is well known that *H. pylori* infection is characterized by extensive infiltration of neutrophils. Neutrophils may

generate superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$) and nonradical oxidants, such as hypochlorous acid ($HOCl$), to kill invading micro-organisms^[16-18]. Myeloperoxidase is an important enzyme of neutrophils. It is the key enzyme for the formation of $HOCl$ from H_2O_2 in the presence of chloride ions. $HOCl$ is a potent oxidant known to have several cytotoxic effects on bacterial cells. The integrity of bacterial cell membrane may be violated by the oxidation of membrane proteins^[17]. Additionally, activated neutrophils and monocytes can also generate cytotoxic chloramines, tyroxyl radicals, and $\cdot OH$ via an myeloperoxidase-dependent pathway^[16,18].

The inter-individual variations in myeloperoxidase activity of neutrophils are genetically determined^[19]. A diallelic polymorphism at the promoter region of *myeloperoxidase* gene (-463 bp) was observed, and is related to the transcription activity of this gene^[20]. The G allele is the wild type with normal expression, while the A allele is a low-expression allele^[20]. Recently, Roe *et al.*^[21], disclosed that *myeloperoxidase* genotype critically determines the pathogenesis of atrophic gastritis subsequent to *H. pylori* infection. However, no data exist regarding the relationships between *myeloperoxidase* genotype and peptic ulcer disease. The purposes of this study were to elucidate the relations between the *myeloperoxidase* -468 G $\rightarrow$ A polymorphism and the development of DU, and to investigate the impacts of this host genetic polymorphism on the histopathological features of *H. pylori*-related gastritis.

MATERIALS AND METHODS

Subjects

One hundred and fifteen consecutive unrelated Taiwanese with DU, who attended the Kaohsiung Veterans General Hospital, were included in this study. The diagnosis of DU was confirmed by endoscopic examination. An ulcer was defined as a circumscribed mucosal break 5 mm or more in diameter, with a well-defined ulcer crater. The size of ulceration was measured by opening a pair of biopsy forceps of known span in front of the ulcer. One hundred and eighty-five consecutive ethnically matched asymptomatic healthy volunteers without active or past DU history served as healthy controls (HCs), and their endoscopic findings were all normal or showed gastritis only. The asymptomatic controls were enrolled from our health examination clinics. For them, panendoscopy was a routine examination of the general health checkup because the gastric cancer incidence was high in our country. To minimize ethnic bias, all patients and controls were Han Chinese; aboriginal and alien populations were excluded. Exclusion criteria for both groups included (1) history of esophageal or gastric ulcer, (2) previous history of anti-*H. pylori* therapy, (3) use of non-steroidal anti-inflammatory drug or proton pump inhibitors within one month of endoscopy, (4) associated gastrointestinal malignancy, and (5) serious medical illness. To adjust clinical characteristics, the following data were recorded for each subject: age, sex, blood type, smoking history and alcohol consumption. The study was approved by the Medical Research Committee of the Kaohsiung Veterans General Hospital. All patients and

controls gave informed consent.

Study design

Endoscopies were performed with the Olympus GIF XV10 and GIF XQ200 (Olympus Corp., Tokyo, Japan). During endoscopy, biopsies over antrum were performed for rapid urease test and histological examination. Prior to endoscopy, venous blood was drawn for serological test and *myeloperoxidase* genotyping. Serology was studied using a commercial IgG EIA kit (Premier *H. pylori*; Meridian Diagnostics Inc., Cincinnati, OH). The diagnosis of *H. pylori* infection was based on at least two positive results of histological findings, rapid urease test and serological assay.

To assess the significance of clinical characteristics, the following data were recorded for each patient: age, sex, blood type, smoking and alcohol consumption.

Histology

A histological examination of stomach was carried out during endoscopy for the subjects who provided informed consent for topographic histopathological study. Two specimens were taken from the antrum (pyloric gland area) and corpus (fundic gland area) at standard topographic sites. The biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. The sections were stained with a hematoxylin and eosin stain and a modified Giemsa stain as previously described^[22,23]. Sections were examined blinded to the patient's clinical diagnosis. Scores of acute inflammation (neutrophil infiltration), chronic inflammation (mononuclear cell infiltration), glandular atrophy, intestinal metaplasia and *H. pylori* density were graded from 0 to 3 as described by the updated Sydney system^[24].

Rapid urease test

The rapid urease test was performed according to our previous studies^[25]. Each biopsy specimen was placed immediately in 1 mL of a 10% solution of urea in deionized water (pH 6.8) to which two drops of 1% phenol red solution had been added and incubated at 37 °C for up to 24 h. If the yellowish color around the area of inserted specimen was changed to bright pink within the 24-h limit, the urease test was considered positive. In our laboratory, the sensitivity and specificity of the rapid urease test were 96% and 91%, respectively^[26].

Myeloperoxidase genotyping

Genomic DNA was extracted from 3 mL of whole blood by the use of a QIAamp DNA Extraction Mini Kit (QIAGEN Inc., Valencia, CA). The *myeloperoxidase* polymorphism analysis was performed using a PCR-restriction fragment length polymorphism method^[19]. The primers set to detect the polymorphic site at position -463 were forward primer 5'-CCGTATAGGCAGAGAATGGTGAG-3' and reverse primer 5'-GCAATGGTTCAAGCGATTCTTC-3'. The PCR product was then digested with *Acl*I and separated on a 2% agarose gel. Individuals homozygous for the G allele had three bands at 169, 120 and 61 bp, whereas those heterozygous alleles, *myeloperoxidase* (G/A), had four bands at 289, 169, 120 and 61 bp. Individuals homozygous for the A allele had two bands at 61 and 289 bp.

Statistical analysis

Statistical evaluations were performed using the SPSS/Windows computer software package (Chicago, IL). Two-sample *t*-tests were used to compare the mean values of the variables considered continuous in the DU patients and HCs. The χ^2 test with or without Yate's correction for continuity and Fisher's exact test when appropriate were applied to analyze the categorized variables. Differences were considered to be significant at $P < 0.05$. A multivariate analysis with logistic regression method was carried out to assess the odds ratios (ORs) of the risk factors of DU. The studied variables included the following: age (< 60 or ≥ 60 years), sex, blood type (O type or non-O type), history of smoking (< 1 or ≥ 1 pack/wk), history of alcohol consumption (< 80 or ≥ 80 g/d), *H. pylori* status (presence or absence) and the carriage of myeloperoxidase allele A (yes or no).

We estimated that a 20% difference in the susceptible factor could be present in DU patients and HCs. Based on this assumption, 95 subjects had to be studied in each group to yield a statistical power of 0.80 and an α value of 0.05.

RESULTS

Characteristics of the patients

Table 1 shows the demographic characteristics of DU patients and controls. Patients with DU were more likely to be males and to smoke than the HCs ($P = 0.012$ and 0.014 , respectively). The infection rate was significantly higher in the DU group than in the control group ($P < 0.001$). The two groups were similar with respect to age, blood type and history of alcohol consumption.

Table 1 Characteristics of DU patients and HCs

	HCs (<i>n</i> = 182, %)	DU (<i>n</i> = 115, %)	<i>P</i>
Age (yr)	53.4±14.1	52.9±14.10	0.755
Sex			0.012
Male	95 (52.2)	77 (67.0)	
Female	87 (47.8)	38 (33.0)	
Blood group			0.128
A	49 (26.9)	22 (19.1)	
B	55 (30.2)	28 (24.3)	
O	66 (36.3)	57 (49.6)	
AB	12 (6.6)	8 (7.0)	
Cigarette smokers	37 (20.3)	38 (33.0)	0.014
Heavy drinkers	7 (3.8)	8 (7.0)	0.233
<i>H. pylori</i> infection	87 (47.8)	93 (80.9)	<0.001

Myeloperoxidase genotypes in DU patients and HCs

Table 2 displays the distribution of myeloperoxidase genotypes in study groups. The distributions of this myeloperoxidase polymorphism were distinctively different between groups ($P = 0.008$). The G/G, G/A and A/A genotypes were 79%, 21% and 0% respectively in HCs, and 73%, 22% and 5% respectively in DU patients. All six individuals carrying myeloperoxidase A/A genotypes were in the DU group (myeloperoxidase A/A genotype: DU, 5%; HCs, 0%; $P = 0.003$).

Table 2 Genotypes and allele frequencies of myeloperoxidase gene in DU patients and HCs (*n*, %)

	HCs (<i>n</i> = 182)	DU (<i>n</i> = 115)	<i>P</i>
Genotypes			0.008
G/G	143 (78.6)	84 (73.0)	
G/A	39 (21.4)	25 (21.7)	
A/A	0 (0.0)	6 (5.2)	

Combined risk of myeloperoxidase polymorphism and *H. pylori* infection for the development of DU

Table 3 presents the carriage rate of myeloperoxidase allele A and the *H. pylori* status in the two studied groups. The carriage of myeloperoxidase allele A and *H. pylori* infection were associated with an increased risk of DU with OR of 2.3 [95%CI, 0.8-8.4] and 5.8 (95%CI, 2.9-11.8), respectively. The combined risk of the carriage of myeloperoxidase allele A and *H. pylori* infection for DU was 8.7 (95%CI, 3.5-21.8).

Comparison of histological gastritis between DU patients and HCs

Table 4 lists the histological gastritis scores in the antrum and the corpus. The scores of bacterial density, activity, inflammation, glandular atrophy and numbers of lymphoid follicles in the antrum were significantly higher in DU patients than in HCs ($P = 0.010$, 0.002 , 0.002 , 0.001 and 0.040 , respectively). The DU patients also had higher *H. pylori* densities, activity and inflammation scores in the corpus compared with HCs ($P = 0.025$, 0.021 and 0.003 , respectively).

Impact of the host myeloperoxidase genotypes on *H. pylori*-related gastritis

The relationships between *H. pylori* infection and the severity of gastritis were examined in this study. In the antrum, the activity, inflammation and atrophy scores and the number of lymphoid follicles were markedly higher in the *H. pylori*-infected individuals than in the non-infected individuals (1.83 ± 0.11 vs 0.18 ± 0.10 , 2.88 ± 0.06 vs 1.24 ± 0.20 , 1.21 ± 0.11 vs 0.29 ± 0.17 , and 0.57 ± 0.12 vs 0.00 ± 0.00 , respectively; $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.004$, respectively). The *H. pylori*-infected individuals also had higher activity, inflammation and atrophy scores in the corpus than the *H. pylori*-negative individuals (0.83 ± 0.13 vs 0.06 ± 0.06 , 2.05 ± 0.11 vs 1.25 ± 0.14 and 0.26 ± 0.08 vs 0.00 ± 0.00 ; $P < 0.001$, $P < 0.001$ and $P = 0.041$).

Figure 1 shows how the host myeloperoxidase genotypes impact *H. pylori*-related gastritis. Amongst the *H. pylori*-infected individuals, the myeloperoxidase allele A carriers had higher scores of *H. pylori* densities in the antrum than the non-carriers (2.00 ± 0.17 vs 1.52 ± 0.14 , $P = 0.044$). Additionally, the myeloperoxidase allele A carriers also showed a trend towards greater numbers of lymphoid follicles in the antrum and corpus than non-carriers (antrum: 0.87 ± 0.22 vs 0.41 ± 0.14 , $P = 0.074$; corpus: 0.20 ± 0.11 vs 0.04 ± 0.04 , $P = 0.089$).

DISCUSSION

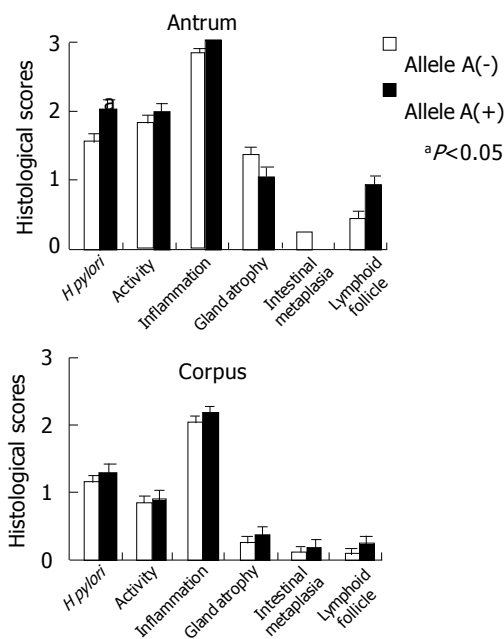
The current study found that the myeloperoxidase $^{-468}\text{G} \rightarrow \text{A}$ polymorphism was significantly associated with DU disease.

Table 3 The myeloperoxidase polymorphism and *H pylori* infection in the development of DU

Myeloperoxidase allele A carrier	<i>H pylori</i> infection	HC (n = 182, %)	DU (n = 115, %)	Odds ratio ¹ (95% CI)	P
(-)	(-)	72 (39.6)	13 (11.3)	-	
(+)	(-)	23 (12.6)	9 (7.8)	2.3 (0.8-8.4)	0.1257
(-)	(+)	71 (39.0)	71 (61.7)	5.8 (2.9-11.8)	<0.001
(+)	(+)	16 (8.8)	22 (19.1)	8.7 (3.5-21.8)	<0.001

¹Variables including age, sex and blood group have been adjusted.**Table 4** Comparison of gastric histological findings between DU patients and HCs

Histological parameters	HCs (n = 11)	DU (n = 49)	P
Antrum			
<i>H pylori</i>	0.55±0.25	1.37±0.14	0.010 ¹
Activity	0.50±0.27	1.53±0.13	0.002 ¹
Inflammation	1.60±1.07	2.57±0.12	0.002 ¹
Atrophy	0.20±0.13	1.10±0.11	0.001 ¹
Intestinal metaplasia	0.00±0.00	0.10±0.05	0.387
Lymphoid follicle	0.00±0.00	0.49±0.11	0.040 ¹
Corpus			
<i>H pylori</i>	0.27±0.14	0.96±0.14	0.025 ¹
Activity	0.10±0.10	0.73±0.12	0.021 ¹
Inflammation	0.12±0.20	1.96±0.10	0.003 ¹
Atrophy	0.00±0.00	0.23±0.07	0.133
Intestinal metaplasia	0.00±0.00	0.08±0.05	0.454
Lymphoid follicle	0.00±0.00	0.08±0.04	0.330

¹Significant difference.**Figure 1** Impact of host myeloperoxidase genotypes on *H pylori*-related gastritis.

The G/G, G/A and A/A genotypes were 79%, 21% and 0%, respectively in HCs and 73%, 22% and 5%, respectively in DU patients. The two study groups differed in myeloperoxidase genotype distributions. Interestingly, the six individuals carrying myeloperoxidase A/A genotype were in the DU group. None of the 185 HCs had this special genotype. Another

study by Roe *et al.*^[21], also showed no myeloperoxidase A/A genotype present in 127 Korean gastritis patients. Recently, we have examined the myeloperoxidase genotypes of 269 gastric cancer patients, and none of them had the A/A genotypes (unpublished data). These results, taken together, suggest that the individuals carrying myeloperoxidase A/A genotype are prone to develop DU.

Myeloperoxidase is an important enzyme of neutrophils, related to oxidant burst for bacterial killing. Neutrophils are one of the professional phagocytes in humans. They manufacture O_2^- by the one-electron reduction of oxygen at the expense of NADPH^[8,16,27]. Most of the O_2^- reacts with itself to form H_2O_2 . From these agents a large number of highly reactive microbicidal oxidants are formed, including HOCl, $\cdot OH$, peroxyne and many others^[28]. Uniquely, myeloperoxidase readily oxidizes chloride ions to the strong nonradical oxidant, HOCl, which have several cytotoxic effects on bacterial cells^[27-30]. Recent reports demonstrated that myeloperoxidase activity of neutrophils is genetically determined^[19,20]. A G-to-A substitution polymorphism in the promoter region of myeloperoxidase gene has been suggested to decrease gene transcription due to the disrupted SP1 binding site^[20], meaning less enzyme would be available to form HOCl. In our histological study, *H pylori*-infected allele A carriers had higher scores of bacterial density. This phenomenon may be caused by low myeloperoxidase activity in the allele A carriers, whose neutrophils had decreased ability to generate HOCl and other reactive oxygen species for bacterial killing^[16,30].

Recent studies suggested that the bacterial load is one of the determinants related to the outcomes of *H pylori*-infected individuals. Bacterial densities of DU patients were significantly higher than those of gastritis patients^[31,32]. In addition, the higher the *H pylori* load, the worse the associated gastritis^[33]. Recently, Richter-Dahlfors *et al.*^[33], demonstrated that co-culture of antral epithelial cells with *H pylori* increased basal gastrin secretion of epithelial cells. Furthermore, Talamini *et al.*^[34], disclosed that high *H pylori* density was an independent risk factor of DU. We therefore propose that the *H pylori*-infected individuals with high bacterial loads may stimulate more antral gastrin release, which can lead to excessive acid secretion from the corpus and result in DU diathesis.

H pylori infection is widely accepted as the most important factor in the pathogenesis of DU and MALToma. In our histological studies, the *H pylori*-infected individuals displayed higher scores of activity, inflammation and gland atrophy in both antrum and body than the non-infected individuals.

The *H. pylori*-infected individuals who carried the *myeloperoxidase* allele A had higher bacterial scores in the antrum and a trend towards increased lymphoid follicles in the antrum and corpus than infected non-carriers ($P = 0.074$ and 0.089 respectively). Currently, the host factors affecting the growth of mucosa-associated lymphoid tissues and MALToma remain unclear. Whether the low-expression *myeloperoxidase* genotype is related to the pathogenesis of MALToma deserves further study.

The major paradox in *H. pylori* research is the apparent association of the infection with divergent and mutually exclusive clinical outcomes^[11,13]. The infection increases the risk of DU, a condition characterized by antral-predominant gastritis and high acid secretion while also heightening the risk of gastric cancer, a condition characterized by corpus-predominant gastritis and hypochlorhydria. Roe *et al*^[21], revealed that *myeloperoxidase* genotype is a critical determinant in the pathogenesis of atrophic gastritis subsequent to *H. pylori* infection. A strong positive correlation between the levels of gastric atrophy was found in wild *myeloperoxidase* (G/G) genotype but not in low expression (G/A) genotype. This implies that wild *myeloperoxidase* genotype is linked with gastric carcinogenesis. Interestingly, we observed that the carriage of *myeloperoxidase* allele A is related to the development of DU disease. Aforementioned studies suggest that *myeloperoxidase* genotype may be a critical turning factor for the outcomes of *H. pylori*-infected individuals.

To our knowledge, this study is the first to verify the association of *myeloperoxidase* -468G→A polymorphism with DU disease. The *H. pylori*-infected allele A carriers had higher bacterial load in the antrum than did infected non-carriers. More work is mandatory to clarify the relationship between low-expression *myeloperoxidase* genotype, the reactive oxygen species of neutrophils and the fates of *H. pylori*-infected individuals.

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• BRIEF REPORTS •

Effect of water-soluble contrast in colorectal surgery: A prospective randomized trial

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Abstract

AIM: Postoperative gastrointestinal-tract motility is normally delayed. Early feeding after colorectal surgery has been reported recently, but late feeding is common. Gastrografin not only enhances bowel peristalsis, but also decreases bowel-wall edema. Whether contrast medium allows early oral feeding and reduces the duration of hospitalization requires clarification.

METHODS: Fifty patients underwent elective colorectal surgery in a regional medical center. Patients were prospectively randomized into a Gastrografin group or control group ($n = 25$ each). Patients in the Gastrografin group began their feeding schedule with 100 mL of 5% dextrose water with 100 mL of Gastrografin on postoperative d 3 and were advanced to a full liquid diet when the contrast reached the colon in 4 h. Patients in the control group began their feeding schedule with 200 mL of 5% dextrose water on postoperative d 3 and were advanced to a full liquid diet after the passage of flatus and stool. Nasogastric tubes were inserted for persistent postoperative vomiting. Fullness, nausea, vomiting, complications, time of anesthesia, time of operation, time of mobilization, time of oral feeding, and duration of hospital stay were recorded and analyzed with Student's *t*-test.

RESULTS: In the Gastrografin group, one patient had aspiration pneumonia and one patient had anastomotic leakage resulting in sepsis and eventual death. This mortality was excluded from the subsequent statistical analysis. In the control group, two patients had wound infections. There was no significant difference between the two groups at the time of anesthesia, time of operation, or time of mobilization. There were significant differences between the two groups in the time of oral feeding (3.3 ± 0.3 d in

the Gastrografin group vs 4.8 ± 0.4 d in the control group; $P =$ odds ratio--, 95%CI [-0.5 to +0.7 d]) and in the length of hospital stay (7.6 ± 1.1 d in the Gastrografin group vs 10.2 ± 1.3 d in the control group; $P =$ odds ratio--, 95% CI [-1.2 to +1.4 d]).

CONCLUSION: Gastrografin not only allowed early oral feeding but also reduced the duration of hospitalization after elective colorectal surgery.

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Key words: Colorectal surgery; Contrast; Gastrografin

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INTRODUCTION

Postoperative intestinal motility is normally delayed and returns in a predictable manner^[1,2]. Recent investigations have shown that early oral feeding after abdominal surgery is safe and generally well tolerated^[3]. This has potential cost-saving implications in this age of rising medical expenses and diminishing reimbursement. Patients who are fed early should be able to be discharged from hospital earlier, and this shorter hospital stay should decrease the overall cost.

The role of water-soluble contrast medium in acute small-bowel obstruction has recently been evaluated. Studies have documented the diagnostic value of this contrast medium in assessing the need for surgical treatment^[4-7]. A possible therapeutic effect of this agent has also been suggested^[8-11]. Gastrografin (Schering AG, Berlin, Germany) is the water-soluble contrast medium that has been most widely studied. This contrast medium may shorten the period of postoperative ileus^[12] and have other therapeutic effects after elective colorectal surgery. Therefore, we designed this prospective randomized trial to determine whether Gastrografin facilitates early oral feeding and shorter hospitalization in patients undergoing elective colorectal surgery.

MATERIALS AND METHODS

Patients

Between June 1 2003 and March 1 2004, 50 patients underwent elective colorectal surgery in a regional medical

center, where about 1 500 colorectal surgical procedures are performed yearly. Our patients were prospectively randomized into two different postoperative treatment arms via their case numbers: the even numbers were assigned to the Gastrografin group ($n = 25$) and the odd numbers to the control group ($n = 25$) after informed written consent was given by all patients.

Patients younger than 16 years or who had emergency operations were excluded. Patients with obstruction, perforation, or active intra-abdominal infection were also excluded. No laparoscopic procedures were performed.

Patient characteristics were as follows (Table 1). The mean age was 63.7 ± 11.5 years (range, 20–83 years) in the Gastrografin group and 64.2 ± 10.3 years (range, 25–81 years) in the control group. The procedures were evenly matched in both groups, with the following results: segmental resection with ileocolostomy, five in the Gastrografin group and six in the control group; resection with colocolostomy, 10 in the Gastrografin group and 11 in the control group; abdominoperineal resection, three in the Gastrografin group and two in the control group; total proctocolectomy with ileoanal pouch, one in the Gastrografin group and two in the control group; and ostomy closure, six in the Gastrografin group and four in the control group. Diagnoses were also distributed evenly across groups: carcinoma was most common, with 18 in the Gastrografin group and 16 in the control group; followed by diverticular disease, with six in the Gastrografin group and seven in the control group; then inflammatory bowel disease, with one in the Gastrografin and two in the control group. Both groups had similar numbers of patients who had undergone prior abdominal surgery (Gastrografin, five; control, seven).

Table 1 Patient characteristics

	Gastrografin group (25)	Control group (25)
Mean age (yr)	63.7 $\pm$ 11.5	64.2 $\pm$ 10.3
Operation		
Ileocolostomy	5	6
Colocolostomy	10	11
Abdominoperineal resection	3	2
Proctocolectomy and ileoanal pouch	1	2
Ostomy closure	6	4
Diagnosis		
Carcinoma	18	16
Diverticular disease	6	7
Inflammatory bowel disease	1	2
Prior abdominal surgery	5	7

Methods

Patient management: Patients were maintained on a clear liquid diet from noon on the day prior to surgery. Bowel preparation, consisting of phosphosoda catharsis and oral neomycin and metronidazole, was administered the day before surgery. All patients had orogastric tubes placed intraoperatively for gastric decompression, which were removed immediately upon arrival in the postanesthetic care unit.

Patients in the Gastrografin group began their feeding schedule with 100 mL of 5% dextrose water with 100 mL of Gastrografin on postoperative d 3 and were advanced to a full liquid diet when the contrast medium reached the colon after 4 h, confirmed by plain abdominal radiography. If the patients consumed 1 000 mL or more of a full liquid diet in a 24-h period, they were advanced to a regular diet the next day. Patients were discharged when they could tolerate more than two-thirds of a regular diet.

Patients in the control group began their feeding schedule with 200 mL of 5% dextrose water on postoperative d 3 and were advanced to a full liquid diet after evidence of return to normal bowel function with the passage of flatus or stool, and no nausea or vomiting. These patients were advanced to a regular diet when they consumed 1 000 mL or more of a full liquid diet in a 24-h period. They were discharged when they could consume more than two-thirds of a regular diet.

All patients received general anesthesia and meperidine was administered during the initial postoperative phase. In both groups, nasogastric tubes were inserted for vomiting unresponsive to antiemetics. All patients were monitored for fullness, nausea, vomiting, and complications such as wound infection, aspiration pneumonia, and anastomotic leakage; and time of operation, time of anesthesia, time of early mobilization, time of oral feeding, and length of hospital stay were recorded.

Statistical analysis

The χ^2 test was used for qualitative variables and Student's *t*-test for continuous variables. Results of the two groups were compared using Student's *t*-test. A two-day difference in the duration of hospitalization was deemed to be a clinically significant difference, and the sample size was based on the detection of this difference with 90% power. A 95% CI for the difference in duration of hospitalization was computed. $P < 0.05$ indicated statistical significance. Analyses were performed using S-Plus®2000 for Windows statistical software (CANdiensten, Amsterdam, The Netherlands).

RESULTS

The feeding schedule in the Gastrografin group was well tolerated, starting with 100 mL of 5% dextrose water and 100 mL of Gastrografin on postoperative d 3 in all patients. Two patients (8%) in the control group experienced fullness postoperatively, but no patient in the Gastrografin group did. Three patients (12.5%) in the Gastrografin group and six patients (24%) in the control group experienced nausea (Table 2). Three patients (12%) in the control group experienced vomiting postoperatively, but none in the Gastrografin group. All patients in both groups were treated initially with antiemetics. However, three patients (12%) in the control group required nasogastric decompression and intravenous fluids for persistent vomiting, whereas no patient in the Gastrografin group required this treatment. None of these differences was statistically significant.

Similar complication rates were seen in both groups; one case of aspiration pneumonia and one of anastomotic leakage in the Gastrografin group; two cases of wound

infection in the control group.

There were no significant differences between the two groups at the time of anesthesia, time of operation, or time of mobilization.

There were significant differences between these two groups in the time of oral feeding (Gastrografin, 3.3 ± 0.3 d *vs* control, 4.8 ± 0.4 d; $P < 0.05$, 95%CI, -0.5 to +0.7 d) and duration of hospital stay (Gastrografin, 7.6 ± 1.1 d *vs* control, 10.2 ± 1.3 d; $P < 0.001$, 95%CI, -1.2 to +1.4 d).

Table 2 Clinical features and complications of Gastrografin and control groups

	Gastrografin group (24)	Control group (25)
Fullness	0	2
Nausea	3	6
Vomiting	0	3
Nasogastric decompression	0	3
Complications		
Wound infection	0	2
Aspiration pneumonia	1	0
Anastomotic leak	1	0
Time of operation (h)	4.3 ± 1.2	4.2 ± 1.1
Time of anesthesia (min)	34.3 ± 5.4	32.7 ± 6.3
Time of mobilization (d)	5.1 ± 1.0	5.3 ± 1.2
Time of oral feeding (d)	3.3 ± 0.3^1	4.8 ± 0.4
Hospital stay (d)	7.6 ± 1.1^1	10.2 ± 1.3

¹Significantly different.

DISCUSSION

A dynamic ileus after abdominal surgery is characterized by a lack of mobility caused by neuromuscular inhibition with sympathetic overactivity. It occurs after all abdominal procedures and motility typically returns to the small bowel within 24 h, to the stomach within 48 h, and to the colon within 3-5 d^[1,2]. Because of this phenomenon, we designed our study so that our patients began their feeding schedule with 5% dextrose water on postoperative d 3, after which a liquid diet was initiated and advanced to a regular diet over 1-2 d.

Reissman *et al*^[3], reported a prospective, randomized trial of 161 patients who underwent elective colon resection, and compared early postoperative feeding with traditional postoperative feeding. This study showed that early oral feeding was safe and also demonstrated no difference in the duration of ileus, the incidence of nausea and vomiting, or major postoperative morbidity. Hartsell *et al*^[13], also showed that early oral feeding after elective colorectal surgery was safe. Most patients tolerated early feeding. However, there was no significant difference in the duration of hospitalization in these patients.

Given this information, it is reasonable to suppose that patients who tolerate early oral feeding after treatment with Gastrografin can be discharged from hospital sooner, thereby reducing the duration of hospitalization and medical costs.

The role of water-soluble contrast medium in predicting the need for surgery for adhesive small-bowel obstruction has recently been evaluated. Other studies have evaluated its possible therapeutic effects^[8,9]. Gastrografin is the contrast

medium most commonly cited. It is an ionic bitter-flavored mixture of sodium diatrizoate, meglumine diatrizoate, and a wetting agent (polysorbate 80), with an osmolality of 1 900 mOsm/L, approximately six times that of extracellular fluid^[8]. It promotes the shifting of fluid into the bowel lumen and increases the pressure gradient across the anastomotic site^[8]. The bowel contents are diluted, and in the presence of the wetting agent, the passage of the bowel contents through the anastomotic site is facilitated. Gastrografin also decreases edema of the bowel wall and enhances bowel motility^[6,9,14]. Gastrografin is water-soluble and relatively safe, even if anastomotic leakage occurs. Complications from the use of Gastrografin are rare, although anaphylactoid reactions and lethal aspiration have been reported^[15-17]. Gastrografin may also shorten postoperative ileus^[12], thus providing another therapeutic effect in elective colorectal surgery. We designed this prospective randomized trial to determine whether treatment with Gastrografin facilitates early oral feeding and shorter hospitalization in patients undergoing elective colorectal surgery.

In our study, there were significant differences between the two groups in the duration of hospitalization and time of oral feeding. The primary hypothesis that treatment with Gastrografin leads to early oral feeding and shorter hospitalization in patients undergoing elective colorectal surgery was confirmed in this series.

No difference in the incidence of complications was noted between the two groups. In the Gastrografin group, an anastomotic complication occurred that was related to surgical technique. However, Gastrografin allowed early detection of the site of anastomotic leakage. Another patient suffered aspiration pneumonia that may have been due to Gastrografin, but there was no subsequent lethal effect. In the control group, two patients suffered wound infections that were unrelated to feeding. Furthermore, the fact that the anastomotic leak and aspiration pneumonia noted in two patients receiving Gastrografin might have been caused by the fact that they got Gastrografin in the first place. Certainly, the fluid loaded in the gut could have led to the anastomotic leak and the aspiration pneumonia might have been made worse if the patient aspirated Gastrografin. Thus, Gastrografin was not only safe, but also provided a further diagnostic advantage in patients undergoing gastrointestinal-tract surgery. However, we could not forget the lethal complications of Gastrografin when using it.

No patient suffered from fullness in the Gastrografin group, whereas 8% suffered from fullness in the control group, leading to a delay in oral feeding. Although no significant difference between the two groups was observed, Gastrografin may have reduced the incidence of fullness, contributing to the shorter hospitalization in the Gastrografin group.

There were some defects in our study because the study was single blinded. When taking care of the patients and making decisions regarding whether or not to give them food and whether or not to send them home might have been influenced by knowing which group they were in. Therefore, another double blinded study should be designed to overcome the disadvantage in the future.

In recent years, several studies have demonstrated that routine nasogastric decompression is unnecessary in colorectal

surgery^[13,18]. In the Gastrografin group, only 12% of patients suffered from nausea and none required a nasogastric tube. In the control group, 24% patients suffered from nausea and 12% of patients required nasogastric tubes because of vomiting. Although none of these differences was statistically significant, perhaps the lower incidence of nausea and vomiting in the Gastrografin group was related to the effects of the contrast medium.

The time of anesthesia and the time of operation did not differ significantly between the two groups, so there was no effect on postoperative care and consequently no significant difference in the time of mobilization between the two groups. Therefore, in our study, early oral feeding and shorter hospitalization might be attributable to the effects of Gastrografin rather than to other causes.

The type of anesthesia and appropriate analgesia also have important effects on postoperative care in colorectal surgery. Bradshaw *et al*^[9], reported that the return of bowel function and the duration of hospitalization of patients undergoing colon surgery were improved if perioperative epidural anesthesia and analgesia were provided. Carli *et al*^[20], showed that thoracic epidural analgesia had distinct advantages in providing superior quality analgesia and shortening the duration of postoperative ileus.

Our patients received general anesthesia and analgesia agents during the initial postoperative phase. Gastrografin not only allowed early oral feeding but also reduced the hospital stays of our patients. Further randomized studies, including larger doses of Gastrografin and perioperative epidural anesthesia, may enhance this effect and allow even shorter hospitalization.

We conclude that Gastrografin not only facilitates early oral feeding but also reduces hospitalization after elective colorectal surgery.

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• BRIEF REPORTS •

How can colorectal neoplasms be treated during colonoscopy?

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Abstract

AIM: For many physicians who ordinarily treat patients with colonic diseases, colonoscopy is considered a prime study interest. Developments in colonoscopic equipment and methods have led to a large number of endoscopic diagnoses and treatment for colorectal neoplasms. The purpose of this investigation is to evaluate the efficacy and outcomes of endoscopic treatment for colorectal neoplastic lesions.

METHODS: From September 1999 to May 2003, 11 447 consecutive colonoscopic examinations in 9 864 patients were gathered; totaling 5 502 endoscopic treatments for colorectal neoplasms. Macroscopic characteristics of the neoplasms were classified into protruded ($n = 3\,953$), sessile ($n = 1\,402$), lateral spreading tumor ($n = 139$) and depressed lesions ($n = 8$). Snare polypectomy was conducted in 3 984 lesions, hot forcep removal in 1 368 lesions, and endoscopic mucosal resection in 150 lesions.

RESULTS: Histological diagnoses were 4 596 neoplastic lesions (4 376 adenomas and 220 adenocarcinomas) and 906 non-neoplastic lesions (891 hyperplastic and 15 inflammatory polyps). For the adenocarcinoma group, 31 instances involved submucosal invasion or resection margin, who received further operations, while 13 surgical specimens discovered no residual tumors. Three perforations and 96 bleedings were found following endoscopic treatment. No procedure-related mortality was found and no recurrent malignancy was found after 14-56 mo follow-up.

CONCLUSION: To lower the incidence and mortality of colorectal cancer, endoscopic treatment for colorectal neoplasms is a simple and safe procedure.

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Key words: Colonoscopy; Snare; Hot forcep; Endoscopic mucosal resection

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INTRODUCTION

For many physicians who normally treat patients with colonic diseases, colonoscopy is considered a prime study interest. The advantage of colonoscopy is its capacity to visualize lesions in situ and also to biopsy or resect both benign and malignant mucosal lesions. Developments in colonoscopic equipment and methods have led to larger numbers of endoscopic resections for colorectal neoplasms including any macroscopic morphology. Macroscopically, colorectal neoplasms can be categorized into protruded, sessile, depressed lesions and lateral spreading tumors. Protruded lesions were protruding lesions with or without a stalk; sessile lesions are superficial, slightly raised lesions with a diameter of no more than 5 mm, and while lateral spreading tumors are bigger, flat lesions with a diameter above 5 mm, and depressed colonic lesions are superficial flat lesions with depressed centers. Several variables, including size, shape, and depth of invasion, have been investigated in attempts to optimize therapy^[1-4]. While the protruded lesions can be easily excised by snare polypectomy; other lesion types should be resected by hot forcep polypectomy or endoscopic mucosal resection.

Colorectal neoplasms can be histologically categorized into neoplastic (include adenoma and adenocarcinoma) and non-neoplastic lesions. Ethically, all the neoplastic lesions should be completely resected to prevent colorectal malignancy. This study presents experience with endoscopic treatments for different types of colorectal neoplasms.

MATERIALS AND METHODS

From September 1999 to May 2003, 11 447 consecutive colonoscopic examinations in 9 864 patients at the digestive therapeutic endoscopic center of Chang Gung Memorial Hospital (Taipei, Taiwan) were gathered. There were 6 138 male and 3 726 female patients, with a mean age of 53 years (ranging from 16 to 97 years). After the patients were readied with ingestion of an electrolyte lavage solution (2 000 mL of polyethylene glycol, PEG), colonoscopic examination was performed with a CF200Z or CF240ZI electronic videoendoscope (Olympus, Tokyo). The colonoscope was inserted up to the cecum in all the included patients. Lesions were gauged by putting an opened standard biopsy forceps beside the lesions, and then the lesions were resected endoscopically if morphologically neoplastic lesions were



Figure 1 Protruded polyp with a stalk.

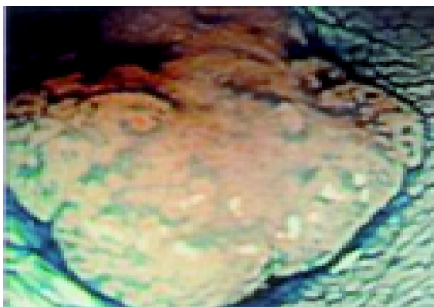


Figure 2 Sessile lesion.



Figure 3 Lateral spreading tumor.



Figure 4 Depressed lesion.

suspected.

Macroscopic characteristics of the lesions were classified into protruded, sessile, depressed lesions and lateral spreading tumors. The resection methods included snare polypectomy, hot forcep polypectomy and endoscopic mucosal resection (EMR). An electrocautery device performed the polypectomy,

while EMR was conducted using techniques introduced by Kudo *et al*^[5]. First the lesion was elevated by injecting 5-20 mL normal saline into the submucosal layer using a standard 25-G injection needle (Olympus) inserted beside the lesion base. For lesions draped over a fold, the distant aspect was injected first to sustain or increase lesion visualization. The volume of normal saline injected varied but was sufficient to attain the desired goal of a large submucosal swelling beneath the lesion and nearby parts of the mucosa. Next the lesion was completely and securely ensnared with a spiked snare and finally, the lesion was resected with a 40-W pure cutting current with an electrosurgical unit (UES-20, Olympus). The wound was then clamped with hemoclips.

Endoscopic resection specimens were embedded in paraffin and then stained with hematoxylin and eosin. Microscopic examinations of specimens were performed by a pathologist specializing in gastrointestinal pathology. If the pathologic finding indicated malignancy, a follow up colonoscopy would be performed 1 mo later, or for the adenomatous group, otherwise followed up 6 mo later.

RESULTS

Cumulatively 5 502 endoscopic treatments were conducted for colorectal neoplasms in this period. Macroscopic features of the neoplasms were classified into protruded ($n = 3\,953$) (Figure 1), sessile ($n = 1\,402$) (Figure 2), lateral spreading tumor ($n = 139$) (Figure 3) and depressed lesions ($n = 8$) (Figure 4). Snare polypectomy was performed in 3 984 lesions, hot forcep removal in 1 368 lesions, and endoscopic mucosal resection in 150 lesions. The position of colorectal neoplasms was mostly in the left side colon and rectum (Table 1). The mean sizes of lesions were 19.3 mm for pedunculated (4-60 mm), 4.6 mm for sessile (3-5 mm), 7.1 mm for depressed lesions (4-10 mm) and 20.3 mm for lateral spreading tumors (6-60 mm).

For protruded lesions, all were excised by snare polypectomy, which pathologically showed 3 473 (87.86%) neoplastic and 480 non-neoplastic lesions. For neoplastic lesions, there were 2 465 adenomas without dysplasia, 842 with moderate to severe dysplasia, 148 mucosal cancer and 18 adenocarcinomas with submucosal involvement (Table 2). Twenty-two patients with malignant lesions in this group underwent further operation due to resection margin or submucosal involvement, and eight of them showed no residual tumors from surgical specimens. Lymph nodes metastases were observed in two cases with submucosal cancers in this group.

Hot forcep polypectomy was used in 1 368 sessile lesions, while 31 lesions were excised by snare polypectomy, and three were resected by EMR. Pathologic findings were 983 (70.11%) neoplastic and 419 non-neoplastic lesions. Neoplastic lesions included 743 adenomas, 232 adenomas with moderate to severe dysplasia, six mucosal cancer and two adenocarcinomas with submucosal involvement. Two submucosal cancerous lesions received further operation and one case without residual tumor was noted by surgical specimen.

The lateral spreading tumors were all resected by EMR methods pathologically revealing 133 (95.68%) neoplastic and six non-neoplastic lesions while 38 adenomas, 53

Table 1 Characteristics of 5 502 resected colorectal lesions

Morphology	Size (mm)	Location					Total
		R	S	D	T	A & C	
Protruded	≤5	161	135	209	63	161	729
	6-10	1 105	717	478	72	254	2 626
	11-20	118	121	173	46	75	533
	21-30	13	22	14	0	1	50
	>30	7	8	0	0	0	15
Sessile	≤5	353	495	271	143	140	1 402
LST	6-10	25	11	5	5	11	57
	11-20	12	8	3	2	12	37
	21-30	15	21	1	0	2	39
	>30	4	2	0	0	0	6
Depressed	≤5	0	1	0	0	2	3
	6-10	2	0	1	0	2	5
Total		1 815	1 541	1 155	331	660	5 502

R, rectum; S, sigmoid colon; D, descending colon; T, transverse colon; A, ascending colon; C, cecum; LST, lateral spreading tumor.

Table 2 Histological finding of 5 502 resected lesions

Morphology	Size (mm)	Adenoma		Adenocarcinoma		Non-neoplastic	Total
		(-) Dysplasia	(+)	M (%)	SM (%)		
Protruded	≤5	293	19	1 (0.14)	0	416	729
	6-10	2 053	505	7 (0.27)	0	61	2 626
	11-20	118	312	99 (18.6)	1 (0.19)	3	533
	21-30	1	5	39 (78)	5 (10)	0	50
	>30	0	1	2 (13.3)	12 (80)	0	15
Sessile	≤5	743	232	6 (0.43)	2 (0.14)	419	1 402
LST	6-10	18	27	8 (14)	0	4	57
	11-20	15	10	10 (27)	0	2	37
	21-30	5	15	18 (46.2)	1 (2.6)	0	39
	>30	0	1	3 (50)	2 (33.3)	0	6
Depressed	≤5	1	0	1 (33.3)	0	1	3
	6-10	0	2	2 (40)	1 (20)	0	5
Total		3 248	1 128	196 (3.56)	24 (0.44)	906 (891 HP, 15 IP)	5 502

LST, lateral spreading tumor; M, mucosal cancer; SM, cancer with submucosal involvement; dysplasia (+), with moderate to severe dysplasia; HP, hyperplastic polyp; IP, inflammatory polyp.

adenomas with moderate to severe dysplasia, 39 mucosal cancers and 3 submucosal cancers were discovered. Six patients received further operations, but in three of them residual tumors could not be found in the surgical specimens. Lymph node metastases were noted in two cases with submucosal cancers in this group.

The eight depressed lesions were resected successfully by EMR. Seven (87.5%) were neoplastic lesions and one was non-neoplastic lesion. Two adenomas and one adenoma with moderate to severe dysplasia were found in the neoplastic lesion; the other three were mucosal cancers and one was adenocarcinoma with submucosal invasion; the last patient underwent further operation but no residual tumor was discovered.

Across all the endoscopic treatments, only two colonic perforations occurred after snare polypectomy and one in hot forcep polypectomy, and all three patients received surgical procedures without mortality. Post treatment bleeding was identified in 89 patients following snare polypectomy and seven after hot forcep polypectomy; while endoscopic hemostasis was accomplished by heat probe coagulations or hemoclips. For the malignant lesions, no recurrent tumors occurred 14-56 mo following endoscopic resections.

DISCUSSION

Polyps are significant owing to their well-recognized relationship to colorectal cancer^[6]. Researchers generally recognize that colorectal cancer extends from benign adenomas -the "adenoma-adenocarcinoma sequence"^[7], and thus that excising adenomatous polyps lower the risk of colorectal cancer^[8]. Histologically, polyps are categorized into neoplastic and non-neoplastic polyps. Non-neoplastic polyps encompass hyperplastic polyps, hamartomas, lymphoid aggregates, and inflammatory polyps, which are not potentially malignant.

Colonoscopic treatments, including hot forcep polypectomy, snare polypectomy, and endoscopic mucosal resection^[9,10], carry risks of bleeding and perforation. Beside these risks, colonoscopic treatments are also financially expensive. However, this expensive and risky method has been normally employed, because colorectal polyps are frequently discovered during screening colonoscopy^[11]. Ideally, the criteria for colonoscopic treatments should be restricted to an adenomatous polyp with the potential to transform into invasive colorectal carcinoma. To avoid needless polypectomy following screening colonoscopy, distinguishing neoplastic from non-neoplastic polyps may help lower this risk and thus the attendant costs. Conventional colonoscopic examination thus far has provided

no morphological criterion^[12-15]. Although a small polyp of less than 5 mm is histologically hyperplastic in 80-90% of cases^[16,17], recent data has implied that up to 40-60% of diminutive colorectal polyps can be neoplastic^[18,19]. Furthermore, small, flat adenomas have been shown to carry elevated risk of dysplasia^[20,21]. In this study, the predictive value of macroscopic diagnosis for neoplastic polyps was 83.26%. However, a previous study of magnifying colonoscopy with the 0.1% indigo carmine dye spreading method puts the diagnostic accuracy at higher than 90%^[22]. If magnifying colonoscopy and chromoendoscopy were applied to all the colonic polyps, this would raise diagnostic accuracy and avoid some needless colonic treatments. A major problem with these procedures is the risk of bleeding and perforation following treatment. In fact, complication rates following treatment have ranged from 0.4% to 1.7%^[23-25]. In this study, the perforation rate was 0.05% and bleeding rate was 1.7% without any mortality. The perforation rate is connected to coagulation current energy and would be diminished by the use of cutting current. However, pure cutting current increases the bleeding rate, although it is easily controlled by the hemoclips method. It is considered as a simple and safe procedure.

The locations of colon lesions were mostly in rectum (32.99%) and sigmoid colon (28.00%), a result comparable with previous reports. As widely recognized, most colorectal carcinomas develop via malignant transformation in benign adenomatous polyps^[26]. Large studies of colonoscopic polypectomies indicate at least two factors connected with malignant degeneration of polyp size and tissue type. Carcinoma is histologically detected in 0.1% of polyps measuring less than 5 mm in diameter. This increases to 1.0% in cases with a 10 mm diameter polyp and may attain 40% in cases with polyps exceeding 20 mm^[27]. Colonoscopic treatment, which includes snare polypectomy, hot forcep polypectomy and endoscopic mucosal resection, is acknowledged as an established method of removing adenomatous polyps and for diminishing the incidence of and mortality from colorectal cancer. This study indicated that while colorectal lesions less than 5 mm had a 0.45% incidence of malignancy; lesions between 6 and 10 mm had a malignancy rate of 0.67%; for lesions between 11 and 20 mm was 19.3%; for lesions between 21 and 30 mm was 70.8%; and those larger than 30 mm had a 90.5% incidence of carcinoma. The overall incidence of malignancy (4.00%) was higher than in previous reports, which may be related to the fact that, because the Chang-Gung Memorial Hospital is a referral center, some bigger colonic lesions found by local hospitals or clinics will be transferred there. Colorectal lesions larger than 10 mm should be completely resected and subjected to carefully pathologic review for the rising malignancy incidence. The pedunculated lesions could be resected by snare polypectomy, while LST larger than 10 mm should be resected by EMR method. Although previous studies show a low level of malignant transformation in small adenomas, this investigation indicated that it also had a 0.45% incidence of carcinoma. Therefore if neoplastic lesions were suspected macroscopically, even at less than 5 mm, they should be excised completely by hot forcep polypectomy or EMR method. According to the morphology of lesions, there was a 4.2% incidence of malignancy in pedunculated lesions, 0.57% in sessile lesions,

30.21% in LST, and 50% in depressed lesions. This result resembles the previous report by Dr. Kudo that depressed colorectal lesions display a significantly higher rate of malignancy.

There were 31 (14.09%) malignant cases which received further operation following endoscopic treatment, 24 of which were submucosal invasion, and seven were unclear resection line. Of these cases, 13 identified no residual tumors from the surgical specimens. Four cases (16.67%) with cancer of submucosal invasion, revealed lymph node metastases in surgical specimens. This implies that, if the submucosal tissues were involved with carcinoma, a further operation is needed. In all malignant cases treated endoscopically or surgically, no recurrent tumors were discovered after 14-56 mo. Cristie published a study establishing five criteria for determining colonoscopic cure by polypectomy: (1) the stalk was uninvolved with carcinoma; (2) no lymphatic or vascular invasion was apparent; (3) the adenomas included a well-differentiated or moderately-differentiated malignancy; (4) early follow-up colonoscopic examination (3 mo) showed no recurrence of cancer at the polypectomy site; and (5) the carcinoma was restricted to the head of the adenoma. The authors contend that all mucosal cancers could be cured endoscopically.

In conclusion, to lower the incidence and mortality of colorectal cancer, colonoscopic treatment is a simple and secure technique of removing colorectal neoplastic lesions. Complete resection of neoplastic lesions is essential, particularly for LST and depressed lesions. Snare polypectomy for protruded lesions, hot forcep polypectomy or EMR for sessile lesions, and EMR for LST and depressed lesions could resect the lesions completely. Detailed histological examinations are essential for deciding surgery indication. If unclear resected margins or submucosal invasions are discovered, further operation is indicated. Early follow-up (1-3 mo) colonoscopy after mucosal cancer resection is essential for deciding colonoscopic cure.

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• BRIEF REPORTS •

Monitoring microarray-based gene expression profile changes in hepatocellular carcinoma

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Abstract

AIM: To find out key genes responsible for hepatocarcinogenesis and to further understand the underlying molecular mechanism through investigating the differential gene expression between human normal liver tissue and hepatocellular carcinoma (HCC).

METHODS: DNA microarray was prepared by spotting PCR products of 1 000 human genes including 445 novel genes, 540 known genes as well as 12 positive (housekeeping) and 3 negative controls (plant gene) onto treated glass slides. cDNA probes were prepared by labeling normal liver tissue mRNA and cancer liver tissue mRNA with Cy3-dUTP and Cy5-dUTP separately through reverse transcription. The arrays were hybridized against the cDNA probe and the fluorescent signals were scanned. The data obtained from repeated experiments were analyzed.

RESULTS: Among the 20 couple samples investigated (from cancerous liver tissue and normal liver tissue), 38 genes including 21 novel genes and 17 known genes exhibited different expressions.

CONCLUSION: cDNA microarray technique is powerful to identify candidate target genes that may play important roles in human carcinogenesis. Further analysis of the obtained genes is helpful to understand the molecular changes in HCC progression and ultimately may lead to the identification of new targets for HCC diagnosis and intervention.

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Key words: cDNA microarray; Gene expression profile; Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent human malignancies in the world. It is the fifth most common cancer with an estimated 437 000 new cases diagnosed annually (5.4% of all new cancer cases). It ranks fourth in mortality rate, following lung, stomach and colon cancers. HCC is a leading cause of cancer-related deaths in adults in Asia and sub-Saharan Africa. Initiation and progression of HCC are thought to be due to an accumulation of genetic changes involving numerous genes^[1]. Genes potentially involved in HCC include the p53 gene on chromosome 17p13.1^[2-4]. However, it is likely that many of the genes involved in the initiation and progression of HCC are currently unknown.

Identification and evaluation of new molecular parameters are of utmost importance in cancer research and treatment. Genetic information of new genomic technologies and approaches is accumulated at a rapid pace. It is now possible to use cDNA array to identify candidate target genes that play important roles in human carcinogenesis^[5]. To identify and monitor gene expression profile changes in HCC specimens may explore not only the cause of these pathological changes, but also provide the opportunity to identify novel targets for disease detection and intervention. In this paper, we constructed a cDNA microarray for liver tissue by selecting genes that were nonredundant and preferentially expressed in hepatocellular tissue. We described the assembly and utilization of liver cDNA microarray designed to assess the differences in gene expression between normal liver and tumor tissues.

MATERIALS AND METHODS

Construction of microarrays

The microarray consisted of 445 novel genes, 540 known genes as well as 12 positive (housekeeping) and 3 negative controls (plant gene) provided by Shanghai Cancer Institute. PCR products were used as universal primers. After completion of PCR, 5 µL of each reaction product was checked by agarose gel electrophoresis, purified and dissolved in 50% DMSO solution. These target genes were arrayed from 384-well microtiter plates onto silylated

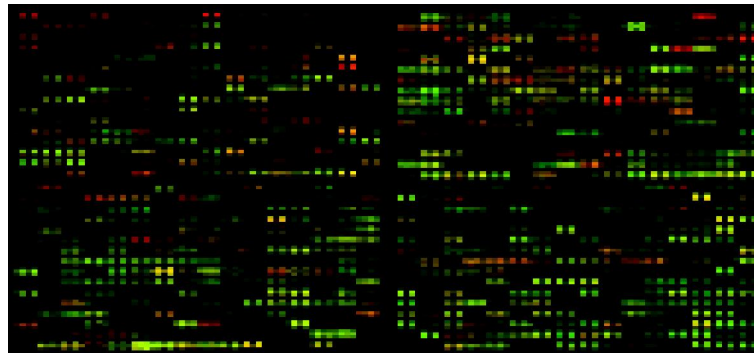


Figure 1 Two-color fluorescent scans of human cDNA microarray. The array

was hybridized with cDNA probe mixture of HCC (Cy5) and normal liver (Cy3).

microscope slides (Cel Associate, Inc.) using PixSys 7500 spotting Robotics (Cartesian Technologies Inc.). Printed arrays were UV crosslinked (65 mJ/cm) and ready for use.

Fluorescent probe preparation

Total RNA was extracted from 20 couple cancerous liver tissues and normal liver tissues using TRIzol reagent. The amount of total RNA from each sample was measured by a spectrophotometer, and its quality was checked by agarose-gel electrophoresis. mRNA was purified using Oligo dT. The fluorescent cDNA probes were made through reverse transcription.

The probes from normal tissues were labeled with Cy3-dUTP, those from cancerous tissues with Cy5-dUTP. Reverse transcriptase was Superscript II RNase H- (GIBCO). Cy3-dUTP and Cy5-dUTP were purchased from Amersham. Each reverse transcription reaction contained 2.5 µg of mRNA. Following the reverse transcription reaction, samples were treated with 2.5 µL of 1 mol/L sodium hydroxide for 10 min at 37 °C, then neutralized by adding 2.5 µL of 1 mol/L Tris-HCl (pH 6.8) and 2.0 µL of 1 mol/L HCl. Probe mixtures were precipitated by ethanol and suspended in 20 µL of hybridization buffer (5× SSC+0.2% SDS).

Microarray hybridization and washes

The slides were boiled in water for 30 s to denature the cDNA, the probe mixtures were heated to 99 °C for 4 min, immediately chilled on ice for 3 min and applied to slides. The slides were covered with glass coverslips, hybridized overnight, incubated at 60 °C in a humid chamber. The slides were washed in 2× SSC+0.1% SDS for 5 min, 0.1× SSC+0.1% SDS for 5 min at room temperature. After a quick rinse in 0.1× SSC, the slides were air dried for scanning.

Detection and analysis

Microscope slides were scanned for Cy3 and Cy5 fluorescences using the Gene Pix4000B (Axon Instruments) at two wavelengths, and the obtained data (16-bit tiff image) were analyzed by GenPix Pro4.0 software, converting the signal intensity of each spot into a text format. The intensities of each spot at the two wavelengths represented the quantity of Cy3-dUTP and Cy5-dUTP respectively, and the overall intensities were normalized according to the ratios of the located 12 housekeeping genes. In each sample, the

Cy3/Cy5 ratio values were log transformed, and global equalization to remove a deviation of the signal intensity between whole Cy3- and whole Cy5-fluorescences was performed by subtracting a median of all log(Cy3/Cy5) values from each log(Cy3/Cy5) value. Accurate differential expression measurement was obtained by taking the average of the ratios of three independent hybridizations. The processed overlay image of a hybridized slide is shown in Figure 1.

RESULTS

Pretreatment

To investigate the gene expression profile changes in the development of HCC, a cDNA microarray was assembled with selected clones. The array consisted of a total of 1 000 genes, including 115 novel genes, 120 unknown function genes with homology to cDNA sequences, 210 unknown function genes with homology to genomic sequences, 540 known genes as well as 12 positive (housekeeping) and 3 negative controls (plant gene). Each gene was spotted twice. Thus, there were 2 000 spots on the array. The probes made from purified mRNA from HCC samples were labeled with the fluorescent dye Cy5 and mixed with the probes made from purified mRNA from normal liver tissues with the fluorescent dye Cy3.

In order to reduce the error occurrence in statistics, normal liver tissue mRNA was labeled separately with Cy3-dUTP and Cy5-dUTP as mixed probes. The array was hybridized independently thrice with the above mixed probes. The mean value from ratios of Cy5/Cy3 was applied to a normalization factor in further experiments. In addition, we noticed that these negative control spots showed a low intensity of signals after hybridization, which proved the reliability of statistics.

Expression profile of genes

To investigate and monitor the gene expression profile changes in liver cancers, replicates of the fabricated cDNA array were hybridized independently with cDNA probes that were generated from 20 couples of different specimens of normal liver and cancer liver tissues (Figure 1). To examine the reliability of hybridization signals and experimental variables unrelated to the differences in hybridization probes, each couple specimens from normal liver and cancer liver tissues were hybridized twice

independently. Forty groups of data were obtained. With each gene, variance among the cancer and normal samples was calculated.

Scatter plots of the values of Cy3 and Cy5 fluorescent signals (Figure 2) also revealed a pattern of distribution and were clustered in a diagonal line as expected.

After the hybridization results were analyzed, 38 genes whose ratios between Cy5 signals and Cy3 signals were greater than 2 or less than 0.5 were selected for further analysis, including 21 novel genes (Table 2). Of these, 20 genes with a ratio between Cy5 and Cy3 signals greater than 2 showed a higher expression in cancerous tissues, 18 genes with a ratio between Cy5 and Cy3 signals less than 0.5 showed a lower expression in cancerous tissues. Seventeen of the 38 genes were known genes registered in GenBank (Table 1).

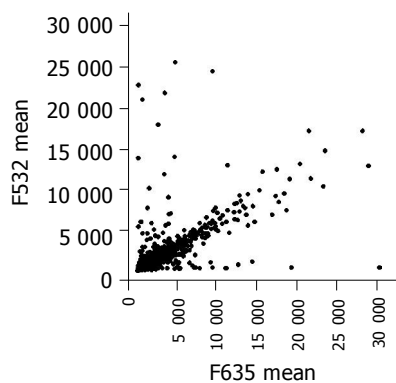


Figure 2 Scatter plots for HCC (Cy5) and normal liver (Cy3) control hybridization.

The green spots represent the genes that were more abundant in HCC than in normal tissues. The red spots represent the genes that were less abundant in HCC than in normal tissues. Yellow spots represent the genes that were equally expressed. Black spots represent the genes that were not expressed.

Our results indicated that the micro-array approach described here could identify differentially expressed novel genes. It might be the result of these novel genes obtained by screening the human normal liver cDNA library.

DISCUSSION

Among 20 couple samples investigated, mRNA profile analysis indicated that 38 genes including 21 novel genes and 17 known genes exhibited different expressions.

The 17 known genes cover a broad range of functional activities: transcription (TA), signaling transduction (ST), cell-cycle regulation (CC), protease/inhibitor (extracellular and cytoplasmic) metastasis related (P), redox regulator (energy related) (Redox), cancer (tumor) suppressor (TS), proto-oncogene (O), immune response related (I), DNA replication and repair (DR), matrix/stroma (M/S).

HCC is one of the most malignant tumors with a high mortality, and is characterized by aggressive growth behaviors and a high recurrence rate. During proliferation of a primary tumor or the establishment of metastatic foci, there was continuous remodeling of the extracellular matrix including various degrees of biosynthesis, reformation and degradation.

Some genes were overexpressed in HCC samples in our study. Metastatic lymph node 51 (MLN51) cDNA was

Table 1 Differentially expressed known genes in HCC and normal liver

CloneID	GB-ACC	Type	Code	Identity	Remarks
192	Z11793	Known-related	Redox	Selenoprotein	Related to redox
273	AF132609	Known-related	DR	Histone deacetylase 6	Related to DNA replication and transcription
568	Y07846	Known-related	TS	GAR-22 meningioma deleted gene, 22q12	Deleted in meningioma
671	X80199	Known-related	O	MLN51	Possibly related to breast cancer progression (17q11-q21.3)
1483	U50330	Known-related	P	PCP-2	Procollagen C-proteinase
2648	AJ223951	Known-related	CC	CDK2	CDK2
2803	E12644	Known-related	CC	Q6 (quiescin)	Cell proliferation inhibitor
2820	U89867	Known-related	ST	Nuclear matrix protein 55 (nmt55), related to breast cancer metastasis	Possibly related to ER negative phenotype and tumor progression metastases in breast cancer
2939	M25915	Known-related	P	Complement cytolysis inhibitor, CLI.	Complement cytolysis inhibitor
3120	X86691	Known-related	TA	Helicase (presumed), Mi-2 protein (218 ku), involved in transcriptional activation	
3373	U60068	Known-unrelated	M/S	Fibronectin	Matrix protein fibronectin
3423	AF027740	Known-related	Redox	Transferase, microsomal glutathione S-transferase 1-like 1 (MGST1L1)	Related to redox
4367	AF064770	Known-related	ST	Kinase, DAG (diacylglycerol) kinase alpha	Kinase for diacylglycerol
6655	AF069987	Known-related	TS	Nitrilase 1 (NIT1)	A putative tumor suppressor, with diadenosine triphosphate (A-ppp-A) hydrolase activity
7072	X57352	Known-related	I	Interferon-inducible gene family (1-8 U gene)	
8343	X13905	Known-related	ST	Ras-related rab1B protein	
9039	U41767	Known-related	P	Metargidin	A membrane-anchored metalloprotease-disintegrin protein with an RGD integrin binding sequence

Table 2 Differentially expressed novel genes in HCC and normal liver

Clone ID	GB-ACC	Type	Identity
6	-	Novel	-
50	AC007078	Novel-only DNA	Clone NH0340A14
432	-	Novel	-
775	G42524	Novel-only DNA	STS genomic (sequence tagged site)
894	-	Novel	-
1008	G27158	Novel-only DNA	STS SHGC-31700
1485	Z68162	Novel-only DNA	Cosmid L145E5
1511	AB002307	Novel-unknown-function	mRNA, KIAA0309
1863	AL110272	Novel-unknown-function	DKFZp564C0716
2202	AF086469	Novel-unknown-function	Full-length insert cDNA clone ZD87A06
3227	-	Novel	-
3429	AB020685	Novel-unknown-function	mRNA, KIAA0878
4189	-	Novel	-
6068	-	Novel	-
7478	AF052114	Novel-only DNA	Clone 23887
7518	-	Novel	-
9427	AB002339	Novel-unknown-function	mRNA, KIAA0341
12723	-	Novel	-
13734	AF030453	Novel-only DNA	Chromosome 7q22, 7q22-q31.1
14214	-	Novel	-
14733	-	Novel	-

PS: Novel: Genes without any homology in GenBank; Novel-only DNA: Unknown function genes with homology with genomic sequences in GenBank; Novel-unknown-function: Unknown function genes with homology with cDNA sequences in GenBank; Known-related: Known function genes related with cellular growth; Known-unrelated: Known function genes unrelated with cellular growth.

isolated by screening a human breast cancer metastasis cDNA library. MLN51 cDNA encodes a novel human protein of 703 residues that shares no significant homology to any known protein. However, MLN51 is well conserved between vertebrate and invertebrate species suggesting an important biological function. The amino terminal half of the protein contains a coiled-coil domain and two potential nuclear localization signals. MLN51 is ubiquitously expressed in normal tissues. Human breast carcinomas show MLN51 overexpression in malignant epithelial cells. The uncommon association of protein-protein interaction domains often found either in nuclear or in cytoplasmic signaling proteins has raised a possible nucleo-cytoplasmic function for MLN51^[6]. PCP-2 is a human receptor protein tyrosine phosphatase of the MAM domain family in human pancreatic adenocarcinoma cells. It is co-localized with beta-catenin and E-cadherin at cell junctions. Its intracellular part consists of two tandem phosphatase domains and a relatively large juxtamembrane region that is homologous to the conserved intracellular domain of cadherins, suggesting a role in the regulation of cell adhesion. PCP-2 is endogenously expressed at the cell surface and unregulated by increased cell density. PCP-2 is a negative regulator for cell migration. Interaction of PCP-2 with its substrate beta-catenin might be involved in the process of cell-cell contact^[7]. Fibronectin (FN) is a glycoprotein component of connective tissue. It is involved in cancer progression. FN connects with cancer cells via its receptors, including integrins $\alpha 5 \beta 1$ and $\alpha v \beta 3$. The ability in adhesion to FN and migration increased markedly, after HCC cells were transfected with H-ras oncogenes. In addition, FN has been reported to be the primary protein involved in the displacement of MMP-2 produced by adjacent normal cells

to cancer tissues^[8]. MMP-2 is associated with cell surface through its COOH-terminal and hemopexin-like domain via a number of mechanisms, including binding to cell-associated collagens IV and I. This may enhance the FN-induced displacement of MMP-2, and facilitate invasion of cancerous cells. IFN-inducible genes 1-8U that were overexpressed in cancer tissues may be cancer-related genes. IFN-inducible genes mediate several kinds of functional roles. IFN-inducible genes such as double-stranded RNA-dependent protein kinase could exert an antiviral and antiproliferative effects^[9]. 1-8U expression in severely inflamed mucosae and colitis-associated cancer tissues in ulcerative colitis (UC) may also be important in protection against the proliferation of inflammation-mediated cells and tumor cells. The study indicated that expression of 1-8 U gene in colonic mucosa might be a useful marker in the identification of high-risk groups of UC-associated colon cancer^[10].

In our study, we also found that some genes were down-regulated in cancer tissues. Selenium (Se) has been shown to prevent cancer in animal model systems when fed at levels exceeding the nutritional requirements^[11,12]. A landmark study by Clark *et al*^[13] showed cancer chemopreventive efficacy using a Se supplement in humans. The term "selenoprotein" is restricted to the proteins containing selenocysteine^[14]. Selenoproteins such as mammalian thioredoxin reductase might play a role in Se-mediated cancer prevention^[15]. A human 15 ku selenoprotein (Sep15) has been identified to contain selenium in the form of Sec in human T-cells and is present in various human tissues, such as liver, kidney, testis, brain, but the level of this selenoprotein was reduced substantially in a malignant prostate cell line and in HCC^[16]. Quiescent Q6 (QSCN6) expression was induced just as

fibroblasts began to leave the proliferating cycle and entered quiescence. QSCN6 is located on human chromosome 1q24, near the putative hereditary prostate cancer locus (HPC1). Expression of QSCN6 mRNA was suppressed in transformed fibroblasts. Quiescin QSCN6 potentially involves cell growth and the redox state of key proteins. The identification of QSCN6 as a gene that was highly expressed in quiescent cells compared to actively growing cells suggested that QSCN6 might play a role in the process by which normal cells enter a reversible quiescence. Inhibition of QSCN6 activity could thus play a role in cancer. The molecular characterization of the promoters of quiescence-induced genes and their molecular structures would shed new lights upon the mechanisms, which regulate the onset of quiescence and how these are deranged in cancer. nmt55/p54nrb may be critical to cell growth and function; decreased nmt55/p54nrb expression in ER- human breast tumors or the expression of nmt55/p54nrb variants in ER+ tumors may indicate loss of normal growth. nmt55/p54nrb has been shown in several studies to bind to RNA and interact with PSF and topoisomerase^[17-21]. These results associated with several splicing factors essential for spliceosome formation (unpublished) and tumor hormonal status^[22] suggest that nmt55/p54nrb may be involved in pre-mRNA processing. There is evidence for the role of RNA processing in oncogenesis^[23,24]. The protein MGST1-L1, involved in redox regulation, has been reported to be under the control of p53^[25]. The protein was also independently identified as an EST clone, and the full-length sequence was deposited in the GenBank database. The finding that MGST1-L1 was upregulated after p53 expression in a colorectal cancer cell line^[25] suggests an important biological function possibly associated with cancer or apoptosis. These genes down-expressed in HCC in our study may play the same role.

These data indicated that we obtained the same results by using cDNA microarray by other methods and confirmed the feasibility, accuracy and effectiveness of microarray as a method to investigate the expression profiles of liver cancer.

The genes discussed here are those with a better-known function. However, the recognition of altered expression of genes with unknown function in Table 2 can help assign it a role, so these new genes will be subjected to further experiments to study their function.

In this paper, we demonstrated the utility of cDNA microarray in analyzing the molecular changes in HCC (Cy5) and normal liver (Cy3). Most importantly, the hybridization results might provide valuable information for further understanding the primary function of these new genes and molecular pathways or areas that may be involved in hepatic carcinogenesis. Monitoring and understanding the molecular changes in HCC progression will lead to the identification of new targets for HCC diagnosis and intervention in the future.

DNA microarrays have become an indispensable tool for the study of gene expression on a genomic scale^[26,27]. At present, human cDNA collection remains the most completely representative and best characterized of all mammalian species. Human cDNA microarray is therefore the most powerful and convenient tool among all kinds of microarrays. We presented the methodology and results of using human cDNA microarray to study oncogenes and

related genes. In addition, this method can contribute to analyzing multiple tumor samples in a rapid fashion. Using cDNA microarrays to define alterations in gene expression associated with a specific cancer may be an efficient way to uncover the clues to specific molecular derangements that account for its pathogenesis and thus identifying potential targets for therapeutic intervention. Moreover, recognition of pathognomonic alterations in gene expression might provide a basis for improved diagnosis and therefore allowing selection of the most appropriate therapeutic strategies.

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• BRIEF REPORTS •

Variations of very low-density lipoprotein receptor subtype expression in gastrointestinal adenocarcinoma cells with various differentiations

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Abstract

AIM: This study is aimed at investigating the expression and possible significances of very low-density lipoprotein receptor (VLDLR) subtypes in gastroenteric adenocarcinoma tissues and cells with various differentiations.

METHODS: Thirty-one cases of gastroenteric carcinoma/adjacent normal tissues were enrolled in the study, which were diagnosed and classified by the clinicopathological diagnosis. The expression of VLDLR subtypes was detected in gastroenteric carcinoma/adjacent normal tissues and three various differentiated human gastric adenocarcinoma cell lines (MKN28, SGC7901 and MKN45) by reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis.

RESULTS: Two VLDLR subtypes, namely, type II VLDLR and type I VLDLR, were found to express changes in gastroenteric carcinoma tissues, their adjacent normal tissue, and gastric adenocarcinoma cell lines as well. Type II VLDLR is predominantly expressed in poorly- or moderately-differentiated gastroenteric carcinoma tissues and gastric adenocarcinoma cell lines, whereas type I VLDLR is mainly detected in well-differentiated intestinal carcinoma tissues and gastric adenocarcinoma cells compared with the adjacent normal tissues.

CONCLUSION: The results suggested that the variations of the VLDLR subtype expression might be correlated with the progress and differentiation of gastroenteric carcinoma.

INTRODUCTION

Very low density lipoprotein receptor (VLDLR) is a member of the low-density lipoprotein receptor (LDLR) superfamily. VLDLR closely resembles LDLR in structure, but differs from LDLR in function^[1,2]. According to the presence of O-linked sugar region located outside the VLDLR membrane, VLDLR is divided into two subtypes: full-length VLDLR (type I) and O-linked sugar region lacked VLDLR (type II)^[3]. The studies about the distribution of these two VLDLR subtypes suggested that their distribution presents obvious tissue- and cell-specificity. For example, type I VLDLR is mainly distributed in tissues of heart and skeletal muscles with high lipid metabolism, while type II VLDLR predominates in the non-muscle tissues, such as kidney, spleen, adrenal gland, lung, brain, testis, uterus and ovary^[4,5]. A recent study showed that VLDLR subtypes are relevant with the differentiation and development of tissues and cells. For instance, during embryonic development in mice, mRNA level of VLDLR is significantly fluctuated in the heart, kidney and brain tissues; however, it is relatively constant in skeletal muscles^[6]. The mice with VLDLR deficiency only demonstrate slower development^[7]. In addition, type II VLDLR has been found to be the major expressed receptor in embryonic tissues and germ cells in chicken, necessary for reproduction^[8,9]. Other reports indicated that some cancer tissues and cells also express two VLDLR subtypes that possess inhomogeneity^[10-12].

Abnormal differentiation of cells leads to cancer formation. However, the reports so far are hardly ever concerned about the identification of VLDLR subtype expression in various differentiated carcinoma tissues and cells. Therefore, we sought to determine whether the expression of VLDLR subtypes could show a degree of difference in gastroenteric carcinoma tissues, their adjacent normal tissue, and three various differentiated human gastric adenocarcinoma cell lines (MKN28, SGC7901 and MKN45) as well, in order to understand the possible

implication of VLDLR subtype expression during the occurrence, differentiation and progress of cancer.

MATERIALS AND METHODS

Tissues and cells

Thirty-one cases of gastroenteric carcinoma/adjacent normal tissue specimens were collected during 2001-2002 from patients in Tongji hospital (Wuhan, PR China), and were diagnosed and classified by the clinicopathological diagnosis. Of these, 10 cases of gastric carcinomas were poorly- or moderately-differentiated adenocarcinomas, and 21 cases were large intestine carcinomas, of which there were 6 cases of well-differentiated adenocarcinomas and 15 cases of poorly- or moderately-differentiated adenocarcinomas. The collected specimens were immediately placed in RNA TRIzol and stored in liquid nitrogen. Well-, moderately- and poorly-differentiated human gastric adenocarcinoma cell lines (MKN28, SGC7901 and MKN45) were donated from the department of gastroenterology, Union Hospital. Anti-VLDLR polyclonal antibodies for Western blot analysis were prepared at our laboratory^[13].

Human gastric adenocarcinoma cell culture

Three of the variedly differentiated human gastric adenocarcinoma cell lines (MKN28, SGC7901 and MKN45) were cultured in RPMI1640 supplemented with 10% FBS, 0.22 sodium bicarbonate and 0.25% HEPES at 37 °C and 50 mL/L CO₂. After 48 h, all cultured cells were digested with 0.25% pancreatin, and harvested for RT-PCR and Western blot.

RT-PCR analysis

Total RNA was isolated from 2 g tissue specimens or 5×10⁸ cells in reference to the manual of RNA Trizol. Four micrograms total RNA was reverse-transcribed to cDNA for PCR experiment. The sequences of PCR primers referring to Nakamura^[14] were 2162F: CAACCTGAA-TGATGCCCAAGA, and 2496R: CTTTGGGGGAA-CACTGACCT, which just span the O-linked sugar region of human VLDLR, thereby distinguishing two VLDLR subtypes. Cycling conditions were: denaturing at 94 °C for 4 min, then at 94 °C for 1 min, at 56 °C for 1 min, at 72 °C for 2 min for 35 cycles. Finally the mixture was extended at 72 °C for 10 min. The PCR products were electrophorized on 2% agarose gels, stained by ethidium bromide, photographed under ultraviolet lamp, and then analyzed with GIS-2000 scanning and analyzing system.

Western blot analysis

For Western blot, the cultures were rinsed twice in ice-cold PBS, lysed in buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.02% sodium azide, 100 µg/mL phenylmethanesulfonyl fluoride, 1 µg/mL aprotinin, followed by spinning at 13 000 g at 4 °C for 20 min. The supernatants were removed and denatured immediately by adding the same volume of 2× laemmli buffer. The protein concentration was determined with Lowry Protein Assay Reagent. The equal amounts of protein (80 µg) were isolated in 7.5%

SDS-polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked with 5% BSA in TBS for 2 h at room temperature, then immunostained with the polyclonal antibodies against human VLDLR. The Western blots were developed by using enhanced chemiluminescence detection reagents, as described by the manufacturer.

Statistical analysis

The apex area ratio of VLDLR types II/I in all specimens was analyzed by *t* test with Microsoft Excel 2002. A *P* value less than 0.05 was considered statistically significant.

RESULTS

mRNA level of VLDLR subtypes in intestinal carcinomas compared to the adjacent tissues

Type I VLDLR (335 bp) and type II VLDLR (252 bp) were detected in both intestinal carcinoma and adjacent tissues. In comparison of carcinoma with normal tissues, however, expression of VLDLR subtypes showed obvious difference. Two hundred and fifty-two base pair bands were predominantly expressed in poorly- or moderately-differentiated intestinal carcinoma, whereas well-differentiated intestinal carcinoma mainly presented 335 bp bands (Figure 1). Statistical analysis also showed a significant difference of *P*<0.05 (Table 1).

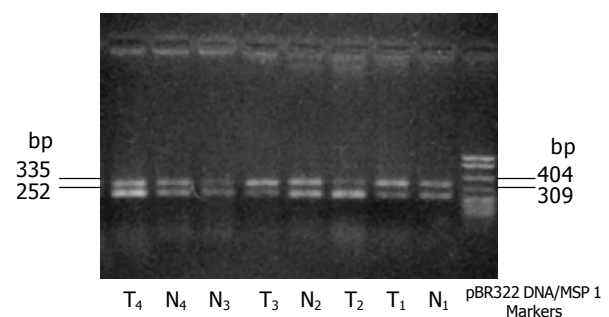


Figure 1 Detection of mRNA of VLDLR subtypes: type I (335 bp) and type II (252 bp) in intestinal carcinoma vs. adjacent tissues, T₁ and T₃: well-differentiated intestinal cancer, T₂ and T₄: moderately- or poorly-differentiated intestinal cancer, N₁, N₂, N₃ and N₄: adjacent tissues.

Table 1 VLDLR subtype expression ratio in gastroenteric cancer

	The apex area ratio of VLDLR type II/I	
	Tumor tissue	Adjacent tissue
Intestinal cancer		
Well-differentiated (<i>n</i> = 6)	0.518±0.021 ^a	0.834±0.011
Moderately- or poorly-differentiated (<i>n</i> = 15)	2.091±0.495 ^c	1.072±0.107
Gastric cancer		
Moderately- or poorly-differentiated (<i>n</i> = 10)	2.214±0.215 ^e	1.403±0.189

^a*P*<0.05; ^c*P*<0.05; ^e*P*<0.05 vs adjacent tissue.

mRNA level of VLDLR subtypes in gastric carcinomas compared to the adjacent tissue

Both type I VLDLR (335 bp) and type II VLDLR (252 bp) appeared in both gastric carcinoma and the adjacent tissues. However, compared to normal tissues, the mRNA level of type II VLDLR in poorly- and moderately-differentiated gastric carcinoma were significantly higher than type I VLDLR, with a significant difference of $P < 0.05$ (Figure 2, Table 1). Additionally, the collected gastric carcinoma specimens with lower expression of type I VLDLR were from the patients who had highly malignant degree of gastric carcinoma. These results implied that the expression of VLDLR subtypes might be related to the malignancy of gastric carcinoma.

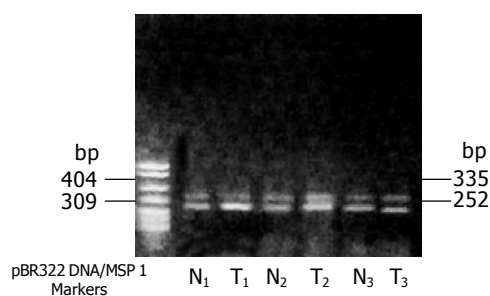


Figure 2 Detection of mRNA of VLDLR subtypes: type I (335 bp) and type II (252 bp) in gastric carcinoma vs adjacent tissues, T₁, T₂ and T₃: moderately- or poorly-differentiated gastric cancer, N₁, N₂ and N₃: adjacent tissues.

mRNA level of VLDLR subtypes in three various differentiated gastric adenocarcinoma cell lines

Both type I VLDLR (335 bp) and type II VLDLR (252 bp) presented in three various differentiated gastric adenocarcinoma cell lines (MKN28, SGC7901 and MKN45). However, poorly- or moderately-differentiated SGC7901 and MKN45 mainly expressed type II VLDLR, while well-differentiated MKN28 showed significantly higher level of type I VLDLR (Figure 3). These results coincided with that of gastric carcinoma tissues.

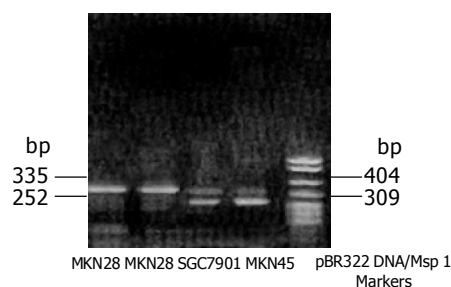


Figure 3 Detection of mRNA of VLDLR subtypes (335 and 252 bp) in MKN45, SGC7901 and MKN28 cell lines.

Protein level of VLDLR subtypes in various differentiated gastric adenocarcinoma cell lines

Protein of VLDLR subtypes was detected by Western blot. The results showed the protein expression of both type I

VLDLR (155 ku) and type II VLDLR (130 ku) in well-differentiated MKN28, but the 155 ku band was the major one. Significantly, in poorly- or moderately-differentiated SGC7901 and MKN45 cell lines, only type II VLDLR (130 ku) was detected (Figure 4). These results were consistent with RT-PCR results of human gastric adenocarcinoma cell lines.

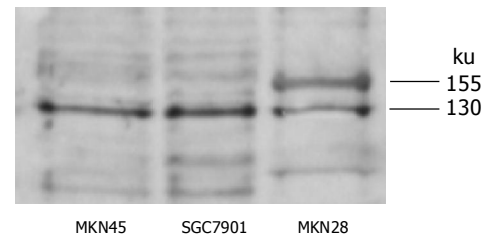


Figure 4 Detection of protein of VLDLR subtypes (160 and 110 ku) in MKN28, SGC7901, and MKN45.

DISCUSSION

Since VLDLR was cloned in 1992 and found to have its subtypes, there have been more and more researches about the distribution and function of VLDLR subtypes^[2-5]. The cause of VLDLR subtype formation is the result of the alternative splicing for 16 exon. Yet, tissues and cells per se do not contain VLDLR genes that express two VLDLR subtypes respectively. O-linked sugar region is the only distinction between two VLDLR subtypes, and plays an important role in enhancing VLDLR stability and binding capacity with ligand^[4,15]. It is well known that, compared to type II VLDLR, type I VLDLR has relatively stronger affinity to VLDL and β -VLDL and is more stable, while both subtypes have no significant differences in internalization, dissociation, and degradation of ligand^[4,16]. It has been believed that the function of VLDLR is mainly to bind lipoproteins including apoE and participate in triglyceride metabolism. Now some studies have found that this receptor functions as a signal receptor on the membrane, binding many ligands, inducing extracellular transport and signal transduction of signal molecule, and then producing many biological effects^[17-19]. However, the functional difference of VLDLR subtypes, especially the distinct function and signification of type II VLDLR has still not been defined yet.

The previous researches showed that the expression level of VLDLR subtypes changes during the development of embryonic tissues in mouse or chicken, indicating that it is correlated with tissue growth and development^[6-9]. Particularly, Nakamura *et al.*^[20,21] found that type II VLDLR is the major receptor in the early phase of fetal brain development, and that type I VLDLR mainly presented in adult brain, both of which are related to brain development. Furthermore, the recent research suggested that in the embryogenesis of brain, VLDLR and apoER₂ may induce the signal transduction between extracellular Reelin and intracellular Disabled-1 (Dab1), which phosphorylates Dab1 and activates intracellular kinase to affect the migration and accurate localization of

developed neuron^[22,23]; type II VLDLR is possibly the important receptor binding Reelin in developed brain and inducing Reelin signal transduction pathway^[20]. However, after adipocytic 3T3-L1 cells were induced to differentiate into adipocyte-like cells, the expressions of both VLDLR subtypes have a concomitant increase, especially for type I VLDLR^[24]. This is significantly beneficial for adipocyte to absorb and store triglyceride. Therefore, the variations of VLDLR subtype expression fairly reflect the requirement of special function during various phases of tissue growth and development. In addition, we observed previously that the expression level of VLDLR subtypes also changes in some pathologic tissues^[25]. For example, type II VLDLR in spleen and adipose tissues disappeared from the patients with liver cirrhosis and in adipose tissues of patients with diabetes did not have obvious changes, yet the mRNA level of type II VLDLR significantly decreased or disappeared^[25], implying that there might be some linkages between special pathological changes. Thereby, according to the tissue-specific distribution of VLDLR subtypes and the changes mentioned above, VLDLR subtypes, especially type II VLDLR, are likely to have other important functions besides lipid metabolism.

Our research firstly found that type I and II VLDLR are differentially expressed in various differentiated gastroenteric carcinoma tissues and cells. In particular, type II VLDLR is mainly expressed in poorly- or moderately-differentiated gastroenteric carcinoma. The mechanism and/or cause of these variations remains unknown. In addition, type II VLDLR also predominates in fetal normal intestinal epithelial, gastric adenocarcinoma cell (AGS) and many adenocarcinomas such as adenocarcinoma of the breast, gallbladder, lung, colon, ovary and parotid gland^[14]. It has recently been found that VLDLR's ligands such as tissue factor pathway inhibitor (TFPI), urokinase-type plasminogen activator-type-1 and plasminogen activator inhibitor complex (uPA-PAI-1) play some roles in tumor proliferation, differentiation, infiltration and metastasis by different signal pathways, respectively^[26-32]. The phenomenon strongly showed that type II VLDLR is closely related to the occurrence, differentiation and progress of carcinoma. The VLDLR subtype expressing changes in variedly differentiated carcinoma are different in their respective functions in tissue growth and development. The variations of VLDLR subtypes in differentiated cancer cell may be caused by several aspects, namely, the different ability to bind the same or different ligands, the relevant signal pathways, and the subsequent biological effects. VLDLR subtypes can adapt or regulate the differentiation of cancer cells, and simultaneously produce different effects on the differentiation and proliferation of cancer cells. In addition, the construction of cytoskeleton needs lots of phospholipids during cell differentiation and proliferation, where type II VLDLR may play an important role in myelin intake^[21]. These might be the causes of the different expression of VLDLR subtype at various differentiations of cancer cells. However, whether the variation of VLDLR subtype expression might be a mark of cancer differentiation and whether it could be used as a clinical evidence of early diagnosis and treatment for cancer, needs to be further investigated.

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Sulindac induces apoptosis and protects against colon carcinoma in mice

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Abstract

AIM: To study the effect of sulindac on colon cancer induction in mice.

METHODS: The chemo-preventive action of 80 ppm sulindac fed during initiation and post-initiation and 100 ppm sulindac fed during progressive stages of induction of colon carcinogenesis in mice was investigated using 1,2-dimethylhydrazine (DMH). Using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique and PCNA immunohistochemical staining, we observed the apoptotic and proliferative cell density changes at different carcinogenic stages and the effect of sulindac on these two phenomena.

RESULTS: Dietary sulindac significantly inhibited the incidence of colonic neoplasmas in mice. Compared with the control group, feeding sulindac during initiation and post-initiation stages inhibited the incidence by 46.7-50.4%, and feeding sulindac during progressive stages inhibited the incidence by 41.1%. Animals that were fed sulindac showed less serious pathological changes than those that were fed the control diet ($P < 0.01$, $H = 33.35$). There was no difference in the density of proliferating cells among those groups which were or were not fed sulindac. In the same period, feeding sulindac resulted in a higher density of apoptotic cells than feeding control diet.

CONCLUSION: Sulindac has an anti-carcinogenic function in mice. Its effect on preventing colon carcinogenesis is better than its effect on treating established tumors. By inducing apoptosis, sulindac inhibited the development of colon cancer and delayed canceration. Sulindac has no effect on proliferation. The anti-carcinogenic properties of sulindac are most effective in the moderate and severe stages of dysplasia and canceration.

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Key words: Large intestine carcinoma; Apoptosis; Proliferation; Sulindac

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INTRODUCTION

Large intestinal carcinoma is a common malignant tumor of the digestive system and its incidence is in an ascending trend. More and more evidence shows that dietary factors play an important role in the progression of large intestinal carcinoma. Sulindac, a type of non-steroidal anti-inflammatory drug (NSAID), gets metabolized into a sulindac sulfone^[1]. In recent years, more and more studies concerning sulindac in preventing familial adenomatous polyposis and influencing large intestinal cancer cellular proliferation, differentiation and apoptosis have been published^[2]. However, the anti-tumor mechanism of the effects of sulindac during different stages of large intestinal canceration remains to be elucidated. In our study, 1,2-dimethylhydrazine (DMH) was used as a model agent experimentally to induce large intestinal carcinoma in mice in order to observe the dynamic effects of sulindac's influence on the apoptotic and proliferative cell density; whether the anti-tumor effects of sulindac are due to induction of the tumor cell apoptosis or not was examined. Furthermore, prevention by sulindac in the early stage of tumor formation and its curative effect in an advanced stage will be assessed. In summary, experimental data relating to the ability of sulindac to prevent and cure large intestinal cancer will be offered in this study.

MATERIALS AND METHODS

Experimental animals

One hundred and sixty KUNMING strain mice, male, 5 wk of age, 24-30 g, were randomly divided into the following groups: model-agent (48), sulindac preventative (48), sulindac curative (40) and control (24) and used in our study after feeding 1 wk.

Diet preparation

Normal diet (AIN-76A) and sulindac diet were produced by the Animal Department of Tianjin Chinese Traditional Medical University. The animals were given food containing sulindac at 80 mg/kg in the sulindac-preventative group and at 100 mg/kg in sulindac-curative group. All food was prepared once every 3-4 wk and maintained at room temperature.

Reagents

Reagents used in this study were DNH and sulindac (Sigma Inc., USA), TUNEL assay (BM Inc., Germany) and ABC immunohistochemical compounds (Vector Inc., USA).

Experimental procedure

Model-agent group: DMH dissolved in normal saline was injected subcutaneously into the model-agent group mice at a dosage of 20 mg/kg. The mice received the normal diet and were killed at the 12th (10), 18th (10), 24th (10) and 32nd (18) wk after injection.

Sulindac preventative group: the mice were fed a Sulindac diet with the dosage of 80 mg of sulindac/kg for 26 wk at the time of DNH injection and were killed at the 12th, 18th, 24th and 32nd wk.

Sulindac curative group: the mice were fed a Sulindac diet with the dosage of 100 mg of sulindac/kg starting at 8 wk after the DNH injection and for 24 more wk. The mice were killed at the end of the 32nd wk.

Control group: the mice were fed normal diet and the same quantity of normal saline was injected. The mice were killed at the end of the 18th and 32nd wk.

Histological indexes and procedures

The mice were killed by cervical dislocation and the whole large intestine and important organs were removed and examined visually. The tissues were fixed in 40 g/L formaldehyde, dehydrated, paraffin-embedded and sectioned at 5 μ m thickness. The investigation included three parameters.

Pathological observation The large intestine was opened lengthways and the number, size and infiltrative depth of the tumors were observed. The grade of the tissue lesions under microscopic examination included mild, moderate and severe dysplasia and cancer (Figures 1A-C).

Distribution and density of apoptosis The distribution and density of apoptosis were demonstrated with TUNEL.

Distribution and density of proliferation The distribution and density of proliferation were recorded by counting cells in aG₁/S phase determined by PCNA immunohistochemistry.

Staining and counting methods for apoptotic and proliferative cells

TUNEL staining The sections were deparaffinized and

put into 3% hydrogen peroxide for 20 min at room temperature. After nonspecific binding sites were blocked, the sections were incubated for 60 min at 37 °C with TUNEL and stained with 3,3'-diaminobenzidine(DAB) chromogen for 10 min at room temperature. Finally, all of the sections were counterstained with hematoxylin. The sections were rinsed with PBS after every step.

PCNA immunohistochemical staining Five-micrometer formalin-fixed paraffin- embedded tissue sections were mounted on poly-L-lysine slides. The slides were air-dried and the tissue deparaffinized. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. The sections were rehydrated and washed with PBS and then pretreated with citrate buffer (0.01 mol/L citric acid, pH 6.0) for 20 min at 100 °C in a microwave oven. Then the sections were incubated overnight at 4 °C with mouse monoclonal anti-PCNA. The sections then were rinsed with PBS, incubated with biotinylated goat anti-mouse IgG for 20 min at 37 °C, incubated with the third antibody and then stained with DAB. Finally, all of the sections were counterstained with hematoxylin.

Method of counting The number of positive cells in the sections with TUNEL and PCNA were counted under microscope magnified 400 $\times$ with a 16D gridding. All positive cells in the 16D gridding were counted except those cells that lay to the right and on the line at the bottom. More than five 16D-gridding microscopic fields were counted in 1 section. The average was considered as the positive-cell density. The area of a 16D-gridding is about 0.1 mm². The density of proliferation and apoptosis was calculated according to the positive cell number in 1 mm².

Statistical analysis

Rank sum test and analysis of variance were performed using SPSS statistical analytic software. $P < 0.05$ was considered significant.

RESULTS

Pathological examination

Macroscopic examination Single or multiple nodes whose diameters ranged from 0.1 to 0.3 cm situated on the surface of the large intestinal mucosa were found in 22.2% of the model-agent group at the 24th wk. Multi nodes with the maximum diameter of 1.8 cm were demonstrated in

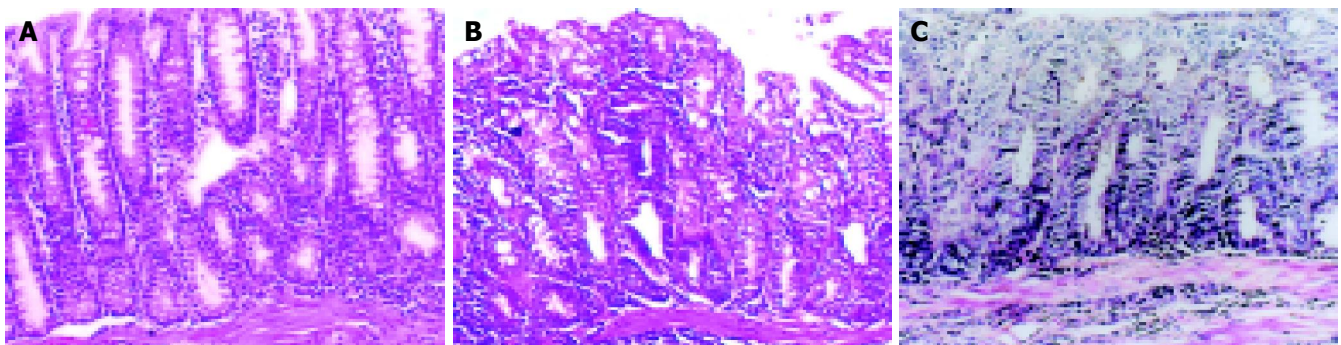


Figure 1 Grade of the tissue lesions under microscopic examination. **A:** mild dysplasia ($\times 100$); **B:** moderate dysplasia ($\times 100$); **C:** severe dysplasia in model group ($\times 200$).



Figure 2 Macroscopic examination. **A:** Model-agent group (32nd wk). Multiple nodes of tumors located in the large intestinal mucosa with the intestinal wall becoming thicker and rigid; **B:** Sulindac-preventative group (32nd wk). Only two

nodes of tumors of diameter about 2 mm are located at the large intestinal mucosa; **C:** Curative group (32nd wk). Several nodes of tumor are located in the large intestinal mucosa with disappeared plica.

86.7% of the mice at the 32nd wk with necrosis and ulcer formation in some nodes. The nodes usually were seen at the distal end of the large intestine. Thickening of the large intestinal wall, rigidity, and adhesion were observed in some mice. The large intestinal mucosa of the mice showed mild, moderate and severe dysplasia in turn in model-agent group, preventive group and curative group during the progression of carcinogenesis and large intestinal carcinomas developed in some mice (Figures 2A-C). One mouse in the model-agent group died of lung infection and three mice of the sulindac curative group died of intestinal obstruction from a tumor. Tables 1 and 2 show the number and size of the tumors at the 24th and 32nd wk.

Table 1 Number of mice with tumor and incidence of the tumors at the 24th and 32nd wk in model-agent and control group

Wk	<i>n</i>	≤5 mm	≤10 mm	≤15 mm	>15 mm	Incidence
24 th	9	1	1	0	0	22.2
32 nd	15	2	6	3	2	86.7
Control	12	0	0	0	0	0

n = number of mice.

Table 2 The average diameter (mm) of tumors at the 24th and 32nd wk in the tumor-induced groups

Wk	<i>n</i>	0	≤1	≤2	≤3	≤4	≤5	>5
24 th	9	7	0	1	1	0	0	0
32 nd	15	2	0	5	2	1	3	2

n = number of mice.

Microscopic examination Mild, moderate and severe dysplasia were observed in the large intestinal mucosa of the mice during the process of tumor induction. Cancer development in the large intestine appeared by the 24th wk. The histological type of these tumors was mainly adenocarcinoma with only few adeno-squamous cell carcinomas. The infiltrative depth of tumors reached the muscle and outer layer. Three mice had liver metastatic foci. The pathological changes in the large intestinal mucosa at various times are shown in Table 3. The incidence of

large intestinal carcinoma was found in 66.7% of the model-agent group and 20% in the sulindac preventative group at the 24th wk, at 32nd wk in 93.3% in the model-agent group, 42.9% in the sulindac preventative group and 52.2% in the sulindac curative group.

Table 3 The pathological changes of large intestinal mucosa at different tumor-inducing stages

Week	<i>n</i>	Normal	Dysplasia			Adenocarcinoma	
			Mild	Moderate	Severe	Intramucous carcinoma	Infiltrative carcinoma
12 th	10	2	3	4	1	0	0
18 th	10	0	2	4	4	0	0
24 th	9	0	0	1	2	5	1
32 nd	15	0	0	0	1	2	12

Outcome of rank test: *H* = 33.35; *n* = number of mice.

Apoptotic and proliferative cell density in the large intestinal mucosa in multistage carcinogenesis

Apoptotic and proliferative cell density developed an increasing trend as the degree of dysplasia increased. In the cancerous stage, the density of apoptosis was lower than it was in a dysplasia stage. On the contrary, the proliferative cells density increased and the ratio of apoptosis and proliferation decreased.

The density of apoptosis in the sulindac-preventative group was higher than in the other groups and there was a statistically significant difference during moderate dysplasia and the cancerous stage. The density of apoptosis in the sulindac-curative group was also higher than it was in the other groups, but there was only a statistically significant difference during the cancerous stage. The density of apoptosis in the sulindac-preventative group was higher than that in the sulindac-curative group and there was a significant difference during the moderate dysplasia and canceration stages.

The difference in the density of proliferation between sulindac-preventative group and sulindac-curative group was not statistically significant during the whole carcinogenic stage but the proliferative cell density in the sulindac-preventative group and sulindac-curative group was higher than in the control group. The proliferative cell density in

the model-agent group was higher than in sulindac-preventative group, sulindac-curative group and control groups (Tables 4-7).

The distribution and density of the proliferation and apoptosis in the large intestinal mucosa in multistage carcinogenesis

The brown-yellow positive material with the TUNEL and

Table 4 Comparison of the density of proliferation and apoptosis with mild dysplasia in the model-agent group, sulindac-preventative group and sulindac-curative group (number/mm²)

Group	<i>n</i>	Proliferative cell density (mean±SD)	Apoptotic cell density (mean±SD)	Ratio (apoptosis/proliferation)
Model-agent	5	88.8±25.7	46.6±19.1 ^a	0.52
Preventive	13	88.2±20.7	65.9±16.3	0.78
Curative	3	92.3±9.02	54.7±11.6 ^a	0.78

n = number of mice. Results with analysis of variance: the difference between groups of proliferative and apoptotic cells density had no statistically significant difference. ^a*P*<0.05 *vs* preventive group.

Table 5 The density comparison of proliferation and apoptosis with moderate dysplasia in model-agent group, sulindac-preventative group and sulindac-curative group (number/mm²)

Group	<i>n</i>	Proliferative cell density (mean±SD)	Apoptotic cell density (mean±SD)	Ratio (apoptosis/Proliferation)
Model-agent	9	114.7±31.7	57.1±17.3 ^a	0.50
Preventive	7	118.9±21.4	107.8±35.0	0.91
Curative	3	133.0±27.5	62.7±20.3 ^a	0.47

n = number of mice. ^a*P*<0.05 *vs* preventive group the cell density of proliferation and apoptosis in the model-agent group and sulindac-curative group were higher than in the sulindac-preventative group resulting in a statistically significant difference.

Table 6 Comparison of the density of proliferation and apoptosis with severe dysplasia in the model-agent group, sulindac-preventative group and sulindac-curative group (number/mm²)

Group	<i>n</i>	Proliferative cell density (mean±SD)	Apoptotic cell density (mean±SD)	Ratio (apoptosis/proliferation)
Model	8	159.0±45.9	61.0±24.6	0.38
Preventive	3	155.8±31.9	88.5±14.6	0.57
Curative	5	159.8±22.7	64.2±19.1	0.40

n = number of mice. ^a*P*<0.05 *vs* preventive group results with analysis of variance: the difference of proliferation and apoptotic cells density between groups was not statistically significant.

Table 7 Comparison of the density of proliferation and apoptosis in a cancerous stage in the model-agent group, sulindac-preventative group and sulindac-curative group (number/mm²)

Group	<i>n</i>	Proliferative cell density (mean±SD)	Apoptotic cell density (mean±SD)	Ratio (apoptosis/proliferation)
Model	20	214.7±24.1	30.7±17.0 ^a	0.14
Preventive	3	211.3±33.7	110.0±16.5	0.52
Curative	12	190.8±35.5	72.1±38.5 ^a	0.38

n = number of mice. ^a*P*<0.05 *vs* preventive the cell density of proliferation and apoptosis in the model-agent group and sulindac-curative group was higher than in the sulindac-preventative group result in a statistically significant difference.

PCNA assays was located in the nucleolus. In normal mucosa, TUNEL-positive cells were on the epithelial layer and few PCNA-positive cells were on the bottom layer. In dysplasia, the number of TUNEL positive cells increased and distributed from the epithelium to the bottom layer. The density of distribution increased with the dysplasia grade. The number of PCNA-positive cells also increased with the dysplasia grade and the distribution of PCNA-positive cells was the same as the apoptotic cells. In the cancerous stage, the number of TUNEL-positive cells obviously decreased and distributed randomly. The number of PCNA positive cells was distinctly increased and distributed evenly (Figures 3A-D).

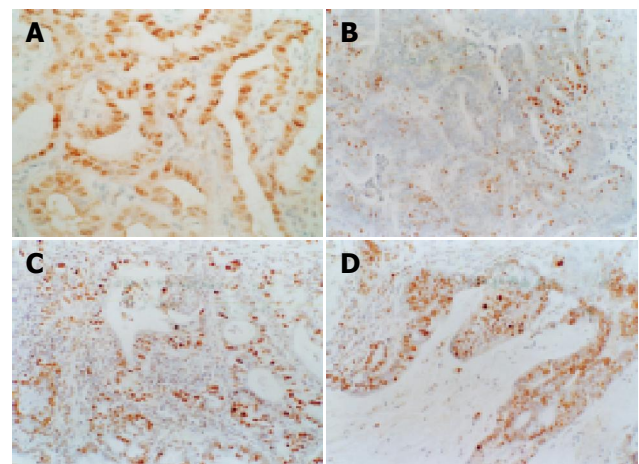


Figure 3 The distribution of proliferation and apoptotic cells. **A:** Model-agent group: there are a large number of positive cells with PCNA staining in large intestinal cancer and evenly distributed, ×200; **B:** Model-agent group: there are a small number of apoptotic cells in the large intestinal cancer. TUNEL, ×200; **C:** Curative group: there are a large number of apoptotic cells in the large intestinal cancer. TUNEL, ×200; **D:** Preventive group: there are a large number of apoptotic cells in the large intestinal cancer. TUNEL, ×200.

DISCUSSION

Chemoprevention of malignant tumors is a field of intense interest. In China, the incidence of large intestinal cancer has been increased year after year and the patients with this cancer are tending to be younger. Therefore, safe and effective medicine needs to be identified for prevention and cure of colon carcinoma. It has been reported that non-steroidal anti-inflammatory drugs (NSAID), such as sulindac and aspirin, have some ability to prevent and cure large intestinal carcinoma^[3], esophageal carcinoma and cancer of the pancreas, bladder, liver, breast and lung^[3,4]. Results from our study showed that not only the incidence of large intestinal cancer in the sulindac-preventative and curative groups was lower than in model-agent group but that the grade of pathologic lesions in the sulindac-preventative and curative groups were less than in the model-agent group. The incidence of large intestinal cancer in the model-agent group was reduced 46.7-50.4% in the sulindac-preventative group and 41.1% in the sulindac-curative group. All of these results showed that sulindac had beneficial effect on colon cancer prevention and treatment. However, the exact mechanism by which sulindac

prevents large intestinal cancer is unknown. Because there is more prostaglandin production from tumor tissue than normal tissue, the anti-tumor mechanism of NSAIDs has been suggested to be due to a reduction in prostaglandin formation^[5-7].

In recent years, the degree of apoptotic activity has been considered to play a role in tumorigenesis^[8]. The results from our study have shown that the dynamic balance between proliferation and apoptosis sustained a steady number in normal tissue which demonstrated that apoptotic cells were on the surface layer of the mucosa and formed all apoptotic cell zone with the proliferating cells located at the bottom of the mucosa forming a proliferative cells zone. The dynamic balance between cellular proliferation and apoptosis in normal tissue was destroyed in cancer tissue with an abnormal number and distribution of cellular apoptosis and proliferation. The distributive relationship between proliferation and apoptosis disappeared^[9]. The results of our new study demonstrated that the density of proliferative and apoptotic cells increased gradually with the enhancing dysplasia. However, in cancer tissue the density of proliferative cells increased and the density of the apoptotic cells decreased. All the results indicate that cell proliferation and apoptosis were active in the precancerous stage but with tissue canceration the high-apoptosis cells were removed. At the same time, cells with a high-proliferative rate were saved. The ratio between apoptosis and proliferation decreased continuously. Based on these data, we proposed the concept of Selective Cell Proliferation relating to an imbalance between proliferation and apoptosis during the process of large intestinal epithelium canceration^[10,11]. That is to say, there are a large number of proliferative and apoptotic cells in precancerous lesions but only those cells with a high-proliferation and a low-apoptosis are maintained so those cells with a high-apoptotic potential are diminished^[12]. From this point of view, the Selective Cell Proliferation concept suggests that tumor tissue progression is a process of cellular adaptation to the environment.

In studying sulindac inhibition of large intestinal carcinogenesis in mice we have drawn the conclusion that sulindac can suppress the emergence of large intestinal cancer. In different stages of canceration, the density of apoptotic cells in the sulindac-preventative group and curative group was higher than in the model-agent group. The extent of apoptosis increased with degrees of the pathologic change and this change influenced the growth rate of the tumors, which delayed the process of tumorigenesis.

In a word, sulindac can induce cellular apoptosis causing a delay in the development of tumors. However, cancerous progression is regulated by multigenes and the exact mechanism by which sulindac induces the tumor cell apoptosis is unknown^[13-15]. It is clear that the relationship between sulindac inhibition of large intestinal carcinogenesis and Selective Cell Proliferation needs further study.

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• BRIEF REPORTS •

Detection of T lymphocyte subsets of children with *Helicobacter pylori* infection

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is one of the causes of many upper digestive diseases, which relates with chronic gastritis and peptic ulcer, in addition it is also closely related to adenocarcinoma of stomach, mucosa-associated lymphoid tissue and lymphoma. The pathogenicity of *H pylori* may differ based on bacteria types, the age of patients and body response. The influence of *H pylori* infection on the immunity in children is unclear and there are few researches on it. We applied ELISA and Western blot (immunoblot) technique in 98 children and T lymphocyte subsets in peripheral blood were determined by flow cytometrical analysis to study the influence of *H pylori* infection on the immunological situation in children that will provide the new information for preventing *H pylori* infection.

MATERIALS AND METHODS

Study population

Ninety-eight Chinese children in Guangxi Luocheng county primary school (9 Han nationality, 26 Zhuang nationality, 31 Mulao nationality and 32 other nationalities) were studied. The age of them is from 6 to 14 years (average 9.40 ± 1.88 years), 56 boys and 42 girls.

Samples collection

As their parents had agreed upon, all of them had no breakfast. Four milliliters of venous blood were taken for the next analysis (2 mL for collecting serum for *H pylori* examination and 2 mL with EDTA for T leukocytes subsets examination).

H pylori antibodies examination

H pylori antibodies were determined by serodiagnosis with ELISA (kit from China Shenzhen Jingmei Biotechnologies Co. Ltd.). The ELISA procedure assay was performed according to the manufacturer's instructions and the results were determined in A value with 450 nm wave on BIO-RAD apparatus Model 450 Microplate Reader. All positive serum samples were assayed in duplicate. Because borderline samples tested positive, they were retested to minimize the chance

Abstract

AIM: To study the transformation of T lymphocyte subsets in children with *Helicobacter pylori* (*H pylori*) infection.

METHODS: The *H pylori* infection status were determined by a combination of ELISA and Western blot (immunoblot) technique in 98 children and T lymphocyte subsets in peripheral blood were determined by flow cytometrical analysis.

RESULTS: There were 75 children positive with *H pylori* infection and 23 negative in 98 children. Comparing the proportion of peripheral blood T lymphocytic subsets in children with *H pylori* infection and without *H pylori* infection, it was found that a higher proportion of CD4 T-cells in infected children (39.02 ± 7.71 vs 34.25 ± 10.73 , $t = 2.246$, $P < 0.05$) and higher value of CD4 to CD8 T-cells ratio (1.51 ± 0.52 vs 1.25 , $t = 2.104$, $P < 0.05$) were present, but there were not significant differences in CD3 T-cells and CD8 T-cells (73.11 ± 10.02 vs 69.49 ± 17.08 , 27.22 ± 6.07 vs 28.27 ± 8.67 , $P > 0.05$).

CONCLUSION: Th1 cell-mediated immune responses may be induced by *H pylori* infection in children.

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Key words: Children; *H pylori*; T lymphocyte subsets

of false positivity. Negative samples were not retested because they all showed clear negative A value.

Determination of VacA and CagA of *H pylori*

VacA and CagA of *H pylori* were determined by Immunoblotting technique (kit from China Huizhou Allic Biotechnologies, Inc.) and the Immunoblot testing was performed according to manufacturer's instructions. A non-reactive control and reactive control were run with each assay. Reactions that showed a band at CagA(128 116 ku) or any three bands from among VacA(959 187 ku), or the urease subunits (66 ku) were considered positive.

T leukocytes subsets examination

The lymphocyte subset was determined by three color flow cytometry for CD4-FITC/CD8-PE/CD3-Cy5 and the control group with IgG1-FITC/IgG1-PE/Ig1-PE-Cy5. Analyses were performed using a Coulter EPICSXL flow cytometer with 5×10^3 cells per count apparatus from Beckmancoulter. Fluorochrome-tagged (FITC or PE), CyTOROL, and other agents were purchased from Beckmancoulter. The experiments were carried out as follows: (1) standard examination samples: 20 μ L of three color monoclonal antibody was added into 100 μ L whole peripheral blood (with EDTA antiagglutination) in a 12 mm $\times$ 75 mm tube, mixed and after room temperature for 20 min, treatment in samples apparatus, added agent for splitting decomposition of red blood cells. Twenty microliters IgG1-FITC/IgG1-PE/Ig1-PE-Cy5 of mice were added into 100 μ L whole peripheral blood (with EDTA antiagglutination) for negative control test. (2) T leukocytes subsets were examined by flow-check, all fluorochrome signs were <2 . Fluorochrome I is FITC, Fluorochrome II is PE, Fluorochrome IV is PE-Cy5, Chromewave filter were 525, 575 and 675 nm. At the same time, we carried out the negative control tests. The results were obtained and analyzed on flow cytometry apparatus and SYSTMIL.

Standard diagnosis of *H pylori* infection

From the results of ELISA and Immunoblotting, if both the results of experiments were positive, they were recognized as *H pylori* infection.

Statistical analysis

Statistical analysis was performed using the SPSS package (Version 10.0 for Windows). t -test and variant analysis were conducted for statistical analyses of all results.

RESULTS

The results of T lymphocyte subsets

T lymphocyte subsets are not significantly different among

98 children from different nationalities (by analysis of variance, $P>0.05$, Table 1).

T lymphocyte subsets in different situations of *H pylori* infection

Seventy-five children tested positive for *H pylori* infection and 23 tested negative. There is no significant difference in proportion of CD3⁺T and CD8⁺T between children with *H pylori* infection (+) group and *H pylori* infection (-) group (by t -test, $P>0.05$). But there is a significant difference in proportion of CD4⁺T between two groups ($t = 2.246$, $P < 0.05$). The value of proportion of CD4⁺T is comparatively high in *H pylori* infection (+) group; and the ratio of CD4⁺T / CD8⁺T is also different ($t = 2.104$, $P < 0.05$, Table 2).

Table 1 T lymphocyte subsets

Nationalities	Samples	mean $\pm$ SD		
		CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)
Zhuang nationality	26	75.542 $\pm$ 11.699	39.788 $\pm$ 8.668	28.500 $\pm$ 5.884
Mulao nationality	31	68.648 $\pm$ 12.579	36.816 $\pm$ 9.742	25.087 $\pm$ 6.559
Han nationality	9	73.867 $\pm$ 3.096	36.222 $\pm$ 4.118	29.844 $\pm$ 5.047
Other nationality	32	72.419 $\pm$ 9.193	37.753 $\pm$ 7.098	28.119 $\pm$ 6.365
Total	98	72.188 $\pm$ 10.923	37.856 $\pm$ 8.235	27.419 $\pm$ 6.327

DISCUSSION

T lymphocyte subsets included T cell population CD4⁺, antigen restricted by class II major histocompatibility complex and CD8⁺, which is restricted by class I major histocompatibility complex are called cytotoxic T lymphocytes. T cell population CD4⁺ are differentiated into three subsets Th0 cells, Th1 cells and Th2 cells that depend on their different cytokines. After accepting the stimulation from antigens, Th0 cells then split up into Th1 or Th2 cells. Th1 cells secrete IL-2, IL-12, IFN- γ , TNF- α , etc., which participate in cell-mediated immune response, that affects the intracellular pathogens (viruses, bacteria, parasites, etc.). And Th2 cells secrete IL-4, IL-5, IL-6, IL-10, IL-13, etc., which stimulate the production of antibodies IgA, IgG, IgE, etc. which induce humoral immune response. Th1 or Th2 cells are mutually inhibited, Th1 cells secrete IL-2, IFN- γ can restrain the proliferation of Th2 cells, but Th2 cells secrete IL-4, IL-10 which could control the cytokine-production of Th1 cell. CD8⁺ has an important effect on immunity due to the immunological inhibition of T cell, the activation of CD8⁺ T needs CD4⁺Th, IL-2 from Th1 secretion is the factor required for the proliferation of CD8⁺ T. Lymphocytes in the circulating blood come into a select lymphatic tissue, e.g. lymphocytes from lymph nodes

Table 2 T lymphocyte subsets in different situation of *H pylori* infection

<i>H pylori</i>	Samples	mean $\pm$ SD			
		CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	CD4 ⁺ /CD8 ⁺
+	26	73.107 $\pm$ 10.015	39.020 $\pm$ 7.714	27.219 $\pm$ 6.067	1.5134 $\pm$ 0.520 ^a
-	31	69.485 $\pm$ 17.078	34.250 $\pm$ 10.730	28.270 $\pm$ 8.670	1.2490 $\pm$ 0.409

Positive group compares with negative group: CD4⁺T $P < 0.05$ (39.020 $\pm$ 7.714 vs 34.250 $\pm$ 10.730). CD4⁺T / CD8⁺T $P < 0.05$ (1.5134 $\pm$ 0.520 vs 1.2490 $\pm$ 0.409).

come into the circulating blood, which usually go back to the lymph nodes, lymphocytes from gastrointestinal associated lymphoid tissue (GALT) come into the circulating blood that go back to GALT after circulating in the body. Besides T lymphocytes are major particles that circulate in the blood^[1], so we can examine T lymphocyte subsets in peripheral blood to evaluate the immune response of the body.

Some researches showed that there are high concentrations of IL-12, IFN- γ , TNF- α , *etc.* secreted from Th1 cells in children with *H pylori* infection, but IL-4, IL-10, *etc.* from Th2 cell secretions do not vary much^[2-5]. The results showed that *H pylori* infection in children principally stimulates Th1 but not Th2 cell response.

There are no reports that relate to the variation of T lymphocytes in peripheral circulation in children with *H pylori* infection in China. Our study shows that there are no significant differences of T lymphocyte subsets in children of different nationalities; there is a significant difference of proportion of CD4⁺T between *H pylori* infection (+) group and *H pylori* infection (-) group. The ratio of CD4⁺T/CD8⁺T also has a significant difference. Our results are the same as those of the reports of Krenska-Wiacek^[6], but there is no significant difference of proportion in CD3⁺T and CD8⁺T between the two groups, these results also ascertained that *H pylori* infection in children principally stimulates Th1 cell response.

The immunity function of Th1 cells is the effect of the intracellular pathogens, cell-mediated immunity has no effect on *H pylori*, which is negative in normal gastric mucosa. After infection with *H pylori*, the antigens are treated with class II MHC of gastric mucosa, and then transferred to Th0 cell. Antigens of *H pylori* stimulate Th0 cell, which become active immune cells and stimulate Th1 to split up and activate. Th1 cells secrete IL-12, IFN- γ which can stimulate the leukocytes and macrophages continuously and these cells secrete IL-12, IFN- γ , TNF- α furthermore, Th0 cells go a step further splitting into Th1, but IL-12, IFN- γ inhibit Th2 from secreting IL-4, IL-10, that cause the gastric local mucosa to decrease secreting IgA, which is not enough to kill *H pylori*, which may be the reason for the prolonged infection of *H pylori* during a person's lifetime. In the study

of Jiang^[7], the author discovered that the progress of acute response to *H pylori* infection from children and adults is the same but there are different changes between gastric mucosa of children and adults, which may suggest that the immune response between children and adults are different. Our study shows that the increase in CD3⁺T and CD8⁺T is not significant, the reason is unclear until now. Further studies are required to clarify the potential biological mechanism.

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• BRIEF REPORTS •

Gastric mucosal injury due to hemorrhagic reperfusion and efficacy of *Salvia miltiorrhizae* extract F and cimetidine

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Abstract

AIM: To observe the gastric mucosal injury caused by hemorrhagic shock and reperfusion and to compare the effect between *Salvia miltiorrhizae* extract F (SEF) and cimetidine (CI) on it.

METHODS: A model of hemorrhage/reperfusion injury was produced by Itoh method. Wistar rats were randomly divided into three groups: 0.9% sodium chloride treatment group (NS group), SEF treatment group (SEF group), and CI treatment group (CI group). Saline, SEF and CI were injected respectively. The index of gastric mucosal lesions (IGML) was expressed as the percentage of lesion area in the gastric mucosa. The degree of gastric mucosal lesions was categorized into grades 0, 1, 2, 3. Atom absorption method was used to measure the intracellular calcium content. Radioimmunoassay was used to measure the concentrations of prostaglandins.

RESULTS: IGML (%) and grade 3 (%) were 23.18 ± 6.82 , 58.44 ± 9.07 in NS group, 4.42 ± 1.39 , 20.32 ± 6.95 in SEF group and 3.74 ± 1.56 , 23.12 ± 5.09 in CI group, and the above parameters in SEF group and CI group decreased significantly (IGML: SEF vs NS, $t = 6.712$, $P = 0.000 < 0.01$; CI vs NS, $t = 6.943$, $P = 0.000 < 0.01$; grade 3: SEF vs NS, $t = 8.386$, $P = 0.000$; CI vs NS, $t = 8.411$, $P = 0.000$), but the grade 0 and grade 1 damage in SEF group (22.05 ± 5.96 , 34.12 ± 8.12) and CI group (18.54 ± 4.82 , 30.15 ± 7.12) were markedly higher than those in NS group (3.01 ± 1.01 , 8.35 ± 1.95 ; grade 0: SEF vs NS, $t = 8.434$, $P = 0.000 < 0.01$; CI vs NS, $t = 7.950$, $P = 0.000 < 0.01$; grade 1: SEF vs NS, $t = 8.422$, $P = 0.000 < 0.01$; CI vs NS, $t = 8.448$, $P = 0.000 < 0.01$). The intracellular calcium

content ($\mu\text{g}/\text{mg}$) in SEF group (0.104 ± 0.015) and CI group (0.102 ± 0.010) was markedly lower than that in NS group (0.131 ± 0.019 , SEF vs NS, $t = 2.463$, $P = 0.038 < 0.05$; CI vs NS, $t = 3.056$, $P = 0.017 < 0.05$). The levels (pg/mg) of PGE_2 , 6-keto- $\text{PGF}_{1\alpha}$ and 6-keto- $\text{PGF}_{1\alpha}/\text{TXB}_2$ were 540 ± 183 , 714 ± 124 , 17.38 ± 5.93 in NS group and 581 ± 168 , 737 ± 102 , 19.04 ± 8.03 in CI group, 760 ± 192 , $1\,248 \pm 158$, 33.42 ± 9.24 in SEF group, and the above parameters in SEF group markedly raised (PGE_2 : SEF vs NS, $t = 2.282$, $P = 0.046 < 0.05$; SEF vs CI, $t = 2.265$, $P = 0.047 < 0.05$; 6-keto- $\text{PGF}_{1\alpha}$: SEF vs NS, $t = 6.583$, $P = 0.000 < 0.000$; SEF vs CI, $t = 6.708$, $P = 0.000 < 0.01$; 6-keto- $\text{PGF}_{1\alpha}/\text{TXB}_2$: SEF vs NS, $t = 3.963$, $P = 0.003 < 0.001$; SEF vs CI, $t = 3.243$, $P = 0.009 < 0.01$), whereas TXB_2 level in SEF group (45.37 ± 7.54) was obviously lower than that in NS group (58.28 ± 6.74 , $t = 3.086$, $P = 0.014 < 0.05$) and CI group (54.32 ± 6.89 , $t = 2.265$, $P = 0.047 < 0.05$). No significant difference was shown between NS group and CI group (PGE_2 : $t = 0.414$, $P = 0.688 > 0.05$; 6-keto- $\text{PGF}_{1\alpha}$: $t = 0.310$, $P = 0.763 > 0.05$; TXB_2 : $t = 1.099$, $P = 0.298 > 0.05$; 6-keto- $\text{PGF}_{1\alpha}/\text{TXB}_2$: $t = 0.372$, $P = 0.718 > 0.05$).

CONCLUSION: Both SEF and CI could inhibit reperfusion-induced injury in gastric mucosa, but with different mechanisms. SEF could not only enhance the protective effect of gastric mucosa, but also abate the injury factors, while CI can only abate the injury factors.

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Key words: Hemorrhagic shock; Reperfusion injury; Gastric mucosa; Radix *Salvia miltiorrhizae*; Cimetidine

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INTRODUCTION

Under certain circumstances, reperfusion after hemorrhage can lead to multiple organ injury called reperfusion injury. Gastric mucosa mainly manifests as stress ulcer, hemorrhage, necrosis, and perforation^[1-3]. In the present study, we observed the resistance of *Salvia miltiorrhizae* extract F (SEF) and H_2 receptor antagonist cimetidine (CI) to mucosal injury in gastric corpus caused by hemorrhagic shock reperfusion and probed into their mechanisms to provide a theoretic basis for exploring effective drugs

against reperfusion injury of gastric mucosa.

MATERIALS AND METHODS

Drugs

SEF was extracted from *S. miltiorrhizae* provided by Chemical Assay Center of China Medical University. CI was produced in Guangdong Xiaolan Pharmaceutical Factory (batch no. 900603).

Animal models

A hemorrhagic shock/reperfusion model was duplicated using modified Itoh method^[4]. Healthy male Wistar rats, weighing 260–300 g, were fasted for 24 h before experiments. The rats were then anesthetized intraperitoneally with 5 mg/100 g of 20% urethane. Tracheostomy was performed and a PE-250 tube was inserted into the trachea to ensure an open airway. The blood pressure was monitored through a polyethylene tube placed in the right carotid artery. A femoral artery was cannulated to withdraw and reinfuse the shed blood, caudal vein was punched for injection of fluid or medication. The abdomen was opened and gastric lumen was washed gently with warm saline till pH 6.0. Normal saline, SEF or CI was then administered (0.03 mL/min), and 25 min later 0.1 mol/L HCl [1 mL/(min·100 g)] was instilled into the stomach via a gastric tube. Five minutes after intragastric HCl induction, blood was withdrawn from the femoral artery. The mean arterial blood pressure was reduced to 2.67–4.00 kPa and maintained for 20 min. The shed blood was then reinfused, and 20 min later the rats were killed and the model of hemorrhagic shock/reperfusion injury was established.

Grouping

Rats were randomly divided into three groups: NS group (treated with normal saline), SEF group, and CI group. NS, SEF (1 g/100 g), or CI (6.5 mg/100 g) was injected respectively.

Index of gastric mucosal lesion

Index of gastric mucosal lesion (IGML) was expressed as a percentage of lesion area in corpus^[5].

Depth of gastric mucosal lesion (DGML)

Mucosa taken from anterior gastric corpus was divided into the following grades under a light microscope^[6]: grade 0: normal gastric mucosa; grade 1: surface mucosa cells were damaged; grade 2: in addition to extensive luminal damage, cells lining the gastric pits were also disrupted and exfoliated; grade 3: cell destruction extended into the gastric gland. Samples analyzed by scanning electron microscopy (SEM) were evaluated as follows^[7]: grade 0: normal gastric mucosa; grade 1: surface cells were flattened with irregular shape, and gaps between individual cells were present; grade 2: the basal lamina was exposed and largely devoid of surface mucous cells, but still showed continuity; grade 3: most of the basal lamina were disrupted, and only parts of it were still intact, regular surface was no longer present. According to the ratio of damaged length to the whole slide, grading between light microscopy and SEM had a close correlation ($r = 0.846$, $P < 0.01$).

Intracellular calcium concentration

Atom absorption spectrometry was used to measure the intracellular calcium content.

Prostaglandin level

Prostaglandin E₂ (PGE₂), 6-keto-PGF_{1α} (6-keto, a metabolite of PGI₂), TXB₂ (a metabolite of TXA₂) kits were provided by Biochemistry Laboratory of General Hospital of the PLA and assayed with radioimmunoassay.

Statistical analysis

Data were represented as mean±SD and analyzed by *t* test in SPSS10.0. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of IGML among different groups

IGMLs (%) were 4.42 ± 1.39 and 3.74 ± 1.56 in SEF ($n = 7$) and CI ($n = 6$) groups respectively. They were significantly lower than 23.18 ± 6.82 in NS ($n = 9$) group (SEF *vs* NS, $t = 6.712$, $P = 0.000 < 0.01$; CI *vs* NS, $t = 6.943$, $P = 0.000 < 0.01$). There was no significant difference between SEF and CI groups ($t = 0.855$, $P = 0.413 > 0.05$).

Comparison of DGML among different groups

As shown in Table 1, DGMLs in normal gastric mucosa (grade 0) and mildly injured mucosa (grade 1) in SEF and CI groups were much higher than those in NS group ($P < 0.01$), and DGML in severely injured mucosa (grade 3) was much lower than that in NS group ($P < 0.01$). There was no obvious difference between SEF and CI groups (grade 0: $t = 1.326$, $P = 0.214 > 0.05$; grade 1: $t = 0.952$, $P = 0.354 > 0.05$; grade 3: $t = 0.799$, $P = 0.443 > 0.05$).

Table 1 Comparison of depth of gastric mucosal lesion among different groups (% , mean±SD)

Group	<i>n</i>	Damage grade			
		0	1	2	3
NS	7	3.01±1.01	8.35±1.95	31.32±4.49	58.44±9.07
SEF	6	22.05±5.96 ^b	34.12±8.12 ^b	25.96±10.04	20.32±6.95 ^b
		$t = 8.434$, $P = 0.000$	$t = 8.422$, $P = 0.000$		$t = 8.386$, $P = 0.000$
CI	6	18.54±4.82 ^b	30.15±7.12 ^b	26.59±8.32	23.12±5.09 ^b
		$t = 7.950$, $P = 0.000$	$t = 8.448$, $P = 0.000$		$t = 8.411$, $P = 0.000$

^b $P < 0.01$ *vs* NS group.

Comparison of intracellular calcium concentration among different groups

Intracellular calcium concentrations (μg/mg) were 0.1044 ± 0.0147 and 0.1020 ± 0.0103 in SEF ($n = 6$) and CI ($n = 6$) groups, respectively, much lower than 0.1308 ± 0.0194 in NS ($n = 7$) group (SEF *vs* NS, $t = 2.463$, $P = 0.038 < 0.05$; CI *vs* NS, $t = 3.056$, $P = 0.017 < 0.05$). There was no significant difference between SEF and CI groups ($t = 0.433$, $P = 0.674 > 0.05$).

Comparison of prostaglandin concentration among different groups

The levels of PGE₂, 6-keto-PGF_{1α} and 6-keto-PGF_{1α}/TXB₂ in SEF group were much higher than those in NS and CI groups ($P < 0.01$, $P < 0.05$) and TXB₂ was much lower than that in NS and CI groups ($P < 0.05$). In CI group, PGE₂ and the ratio of 6-keto-PGF_{1α}/TXB₂ were higher than those in NS group, while TXB₂ was lower than that in NS group. There was no significant difference between these two groups (PGE₂: $t = 0.414$, $P = 0.688 > 0.05$; 6-keto-PGF_{1α}: $t = 0.310$, $P = 0.763 > 0.05$; TXB₂: $t = 1.099$, $P = 0.298 > 0.05$; 6-keto-PGF_{1α}/TXB₂: $t = 0.372$, $P = 0.718 > 0.05$, Table 2).

Table 2 Comparison of PGE₂, 6-keto-PGF_{1α} and TXB₂ concentrations in gastric mucosa among different groups (mean±SD)

Group	n	PGE ₂ (pg/mg)	6-keto-PGF _{1α} (pg/mg)	TXB ₂ (pg/mg)	6-keto-PGF _{1α} /TXB ₂
NS	6	540±183	714±124	58.28±6.74	17.38±5.93
SEF	6	760±192 ^a $t = 2.282$, $P = 0.046$	1 248±158 ^b $t = 6.583$, $P = 0.000$	45.37±7.54 ^a $t = 3.086$, $P = 0.014$	33.42±9.24 ^b $t = 3.963$, $P = 0.003$
CI	6	581±168 ^c $t = 2.265$, $P = 0.047$	734±102 ^d $t = 6.708$, $P = 0.000$	54.32±6.89 ^c $t = 2.265$, $P = 0.047$	19.04±8.03 ^d $t = 3.243$, $P = 0.009$

^a $P < 0.05$, ^b $P < 0.01$ vs NS group; ^c $P < 0.05$, ^d $P < 0.01$ vs SEF group.

DISCUSSION

More and more researches have focused on gastric mucosa reperfusion injury. Though scholars both at home and abroad have done many studies on it, there is no satisfactory therapeutic method or drug yet. The present study showed that both SEF and CI could obviously reduce injury area and depth of gastric mucosa caused by hemorrhagic shock and reperfusion, and protect gastric mucosa. Although the inhibitory rate of these drugs on gastric mucosa injury was similar, SEF had advantages in preventing severe injury, making injured gastric mucosa easier to recover. Furthermore, clinical observations showed *Salvia* water decoction (used to produce SEF) had no rebounding after stopping medication, and fewer side-effects. Some patients had reddened faces, which showed spontaneous recovery. With highly purified drugs, we would get better therapeutic effects and fewer side-effects.

Reperfusion injury of gastric mucosa is a kind of acute gastric mucosa lesion. Gastric mucosa is affected by both injury factors (such as gastric acid and pepsin)^[8,9] and protective factors (such as prostaglandin and gastric mucus)^[10,11]. Normally, the protective factors surpass injury factors so that human gastric corpus could perform digestive function, prevent stomach and duodenum from injury. According to the equilibrium theory by Sun and Shen, the increase of injury factors and/or abating of protective factors could cause gastric mucosa injury. This study showed that SEF could increase the concentration of prostaglandin in gastric mucosa and reduce intracellular calcium content, while CI could lower intracellular calcium content besides being a H₂ receptor antagonist. The mechanisms of these two drugs are different. SEF could increase both protective factors

and weakened injury factors in gastric mucosa, while CI could merely weaken injury factors.

It is generally believed that under stress a large amount of adrenal glucocorticoid secretion can increase gastric acid secretion, decrease mucus and increase histamine release from mast cells. Histamine combines with H₂ receptors on parietal cells to stimulate the latter to secrete gastric acid. CI is a H₂ receptor antagonist, it competes with histamine to combine with H₂ receptors to diminish gastric acid secretion caused by histamine. SEF could increase the level of PGE₂ and 6-keto-PGF_{1α} in gastric mucosa and reduce the content of TXB₂. PGE₂ could obviously inhibit basal gastric acid secretion and stimulate gastric acid secretion caused by histamine, pentagastrin and food in dogs and humans. Prostaglandin has cell protective effects. PGE₂ could obviously increase gastric mucus content, thicken gastric mucosa gel layer. PGE₂, PGI₂ could dilate blood vessels, increase blood flow and carbohydrate secretion, enhance resistance of gastric mucosa against injury. In addition, prostaglandin could extend life span of epithelia and thicken mucosa layer^[12-16]. This study showed that both SEF and CI could inhibit intracellular calcium overload. Increase of intracellular calcium content could activate xanthine dehydrogenase and cause many xanthine oxidases accumulated in body. Thus, after reperfusion, gastric mucosa gained oxygen again and produced superoxide anion explosively, and caused gastric mucosa injury. Moreover, inhibition of intracellular calcium overload could enhance energy metabolism, reduce membrane phospholipid decomposition^[17-20]. So, the resistant mechanisms of these two drugs to reperfusion injury after gastric mucosa ischemia have similarities as well as differences. Whether combination of these two drugs could enhance therapeutic effects still needs further exploration.

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• BRIEF REPORTS •

Effects of bile reflux on gastric mucosal lesions in patients with dyspepsia or chronic gastritis

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Abstract

AIM: To investigate the influences of bile reflux on profiles of gastric mucosal lesions in patients with dyspepsia or chronic gastritis.

METHODS: A total of 49 patients diagnosed with dyspepsia and chronic gastritis underwent 24-h ambulatory and simultaneous monitoring of intragastric bilirubin absorbance and pH values, and then they were divided into bile reflux positive group and bile reflux negative group. Severity of pathological changes in gastric mucosa including active inflammation, chronic inflammation, intestinal metaplasia, atrophy and dysplasia as well as *Helicobacter pylori* (*H. pylori*) infection at the corpus, incisura and antrum were determined respectively according to update Sydney system criteria. The profiles of gastric mucosal lesions in the two groups were compared, and correlations between time-percentage of gastric bilirubin absorbance >0.14 and severity of gastric mucosal lesions as well as time-percentage of gastric pH >4 were analyzed respectively.

RESULTS: Thirty-eight patients (21 men and 17 women, mean age 44.2 years, range 25-61 years) were found existing with bile reflux (gastric bilirubin absorbance >0.14) and 11 patients (7 men and 4 women, mean age 46.2 years, range 29-54 years) were bile reflux negative. In dyspepsia patients with bile reflux, the mucosal lesions such as active inflammation, chronic inflammation, intestinal metaplasia, atrophy or *H. pylori* infection in the whole stomach, especially in the corpus and incisura, were significantly more severe than those in dyspepsia patients without bile reflux. Moreover, the bile reflux time was well correlated with the severity of pathological changes of gastric mucosa as well as *H. pylori* colonization in the near-end stomach, especially in the corpus region. No relevance was found between the time of bile reflux and pH >4 in gastric cavity.

CONCLUSION: Bile reflux contributes a lot to mucosal

lesions in the whole stomach, may facilitate *H. pylori* colonization in the corpus region, and has no influence on acid-exposing status of gastric mucosa in patients with dyspepsia or chronic gastritis.

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Key words: Bile reflux; Chronic gastritis; Dyspepsia; *H. pylori*; Gastric mucosa; Corpus

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INTRODUCTION

Reflux of bile and other contents of duodenum, along with gastric acid and *Helicobacter pylori* (*H. pylori*) infection, are the main etiological factors which play roles in the pathophysiological processes leading to gastric mucosal lesions in patients with chronic gastritis, and to some extent, these factors may act synergistically^[1,2]. However, the exact pathogenic features of bile reflux as well as its contributions to gastric mucosal lesions in this disease are still remaining unrevealed, *e.g.*, little is known about the relevance between bile reflux and profile of pathological changes of gastric mucosa, such as the locations and histological characteristics of these changes in chronic gastritis. In addition, the relationships between bile reflux and *H. pylori* infection as well as the acid-exposing status of gastric mucosa are also not clear.

In the present study, by means of monitoring dynamically intragastric bilirubin absorbance, which reflects the status of bile reflux in stomach, along with the pH values in gastric cavity, we assessed the influences of bile reflux on severity of mucosal pathological changes, status of *H. pylori* infection and the acid environment of mucosa in stomachs of patients with dyspepsia or chronic gastritis, with intention to shed new light on the exact role of bile reflux in the pathophysiological processes of chronic gastritis.

MATERIALS AND METHODS

Study subjects

We enrolled 49 consecutive patients who came to our out-patient department in the period of November 2000 to March 2002 (28 men and 21 women, mean age 44.5 years, range 25-61 years), all the patients had a diagnosis of chronic

gastritis or dyspepsia established by both gastroscopy and biopsy of gastric mucosa.

Assessment of profile of gastric mucosal lesions

In gastroscopy, five biopsies were taken from gastric mucosa in three different regions of the stomach according to the principles of update Sydney system^[3], two from the antrum, 2–3 cm to pylora; two from the corpus, 8 cm to cardia, one in lesser curvature and the other in greater curvature; the fifth from the incisura. All the serial sections (5- μ m thick) prepared from formalin-fixed, paraffin-embedded biopsy samples underwent hematoxylin and eosin staining (for conventional histological determination), AB-PAS (pH 2.5) staining (for evaluation of intestinal metaplasia) and modified Giemsa staining (for detection of *H pylori* infection). Each specimen was assessed for the grade of active inflammation, chronic inflammation, intestinal metaplasia, hyperplasia, atrophy, dysplasia and *H pylori* infection of gastric mucosa at corpus, incisura and antrum according to the criteria of update Sydney system (classified as +, ++ or +++, which were respectively given a score of 1, 2 or 3 in statistical processing; *H pylori* infection status was classified as negative or positive, which was given a score of 0 or 1 respectively in statistical processing).

Assessment of bile reflux and pH in gastric cavity

This work was performed in our clinic. Using a Bilitec 2000 bile reflux monitor and Synectics Digtapper MKIII dynamic pH monitor respectively, all the patients underwent a 24-h ambulatory and simultaneous monitoring of bilirubin absorbance and pH in gastric cavity. Any drug that may inhibit gastric acid secretion, promote gastrointestinal motility, protect gastric mucosa or neutralize bile and antibiotics were stopped 2 wk before monitoring. In order to determine the lengths of esophagus and locations of lower esophageal sphincter (LES), esophagus pressure examination was conducted in all subjects before monitoring. The fiberoptic bilirubin probe and pH electrode were located 5 cm below LES inframargin. Nothing restricted patients' behavior, except ingesting diets free from substances with a similar absorption wavelength to that of bilirubin. The single-channelled, combined analysis of bile reflux and pH was conducted using Synectics analysis program. Patients with bilirubin absorbance >0.14 were regarded as bile reflux positive^[4,5]. The time-percentages of bile reflux or pH >4 in gastric cavity were recorded respectively.

Statistics and ethical approval

The statistical differences in profiles of gastric mucosal lesions between bile reflux positive group and bile reflux negative group were determined using Student's *t* test. The prevalent rates of *H pylori* infection were compared using Fisher's exact test. The correlations between time-percentages of bile reflux and profiles of gastric mucosal lesions or time-percentages of pH >4 were analyzed using Pearson correlation analysis. All the statistical analyses were performed using the SAS statistical software package (SAS Institute Inc., Cary, NC, USA). *P* values <0.05 were considered statistically significant. Informed consent was obtained from all participants in concurrence with the Declaration of Helsinki.

RESULTS

Profiles of gastric mucosal lesions in patients with or without bile reflux

All the 49 patients fulfilled gastric bilirubin absorbance and pH monitoring. Among these patients, 38 (21 men and 17 women, average age 44.2 years, range 25–61 years) were found existing with intragastric bile reflux (bilirubin absorbance >0.14), whose mean time-percentage was $19.9 \pm 14.3\%$ (range 5.02–61.57%). No bile reflux was found in the other 11 patients (7 men and 4 women), whose average age was 46.2 years (range 29–54 years). The bile reflux positive patients were well matched in age, sex, history and course with those without bile reflux.

The profiles of gastric mucosal lesions in dyspepsia patients with or without bile reflux are summarized in Table 1. The pathological grades of gastric mucosa in patients with bile reflux, e.g. active inflammation, chronic inflammation, intestinal metaplasia and atrophy in the near-end stomach (the corpus and incisura, $P < 0.0001$ –0.005), active inflammation and atrophy in the antrum ($P < 0.05$), were significantly more severe than those in patients without bile reflux.

Profiles of *H pylori* infection in dyspepsia patients with or without bile reflux

The incidence of *H pylori* infection in the corpus of dyspepsia patients with bile reflux was significantly higher than that in the corpus of dyspepsia patients without bile reflux ($P < 0.01$), while no significant differences were found in prevalence of *H pylori* infection in the incisura and antrum in these two groups. The overall *H pylori* infection rate [36.9% (14/38)] of bile reflux positive patients was lower than that [54.5% (6/11)] of patients without bile reflux, but with no statistical significance.

Correlation between bile reflux time and profile of gastric mucosal lesions in dyspepsia patients with bile reflux

The time-percentages of bile reflux in gastric cavity were well correlated with active inflammation ($r = 0.3949$, $P < 0.05$), chronic inflammation ($r = 0.8938$, $P = 0.0001$), atrophy ($r = 0.4619$, $P < 0.005$) and *H pylori* colonization ($r = 0.8938$, $P = 0.0001$) in the corpus. Similarly, chronic inflammation ($r = 0.6234$, $P = 0.0001$) and *H pylori* colonization ($r = 0.4992$, $P < 0.005$) in the incisura were also found to be positively correlated with time-percentages of gastric bile reflux. No association was found between bile reflux time and gastric mucosal lesions in the antrum.

Correlation between time of bile reflux and pH >4 in gastric cavity in dyspepsia patients with bile reflux

In 38 dyspepsia patients with bile reflux, the time-percentages of bile reflux and pH >4 in gastric cavity were $19.9 \pm 14.3\%$ and $25.4 \pm 16.7\%$ respectively. No obvious correlation was found between the time of gastric bile reflux and pH >4.

DISCUSSION

Similar with the results of studies about remnant gastric mucosa after gastric operation^[6,7], our study demonstrated that bile reflux positive patients with chronic gastritis had

Table 1 Profile of gastric mucosal lesions in dyspepsia patients with or without bile reflux, % (n)

	Antrum		Incisura		Corpus	
	BR+	BR-	BR+	BR-	BR+	BR-
Active gastritis	P<0.05		P<0.001		P<0.001	
Negative	13.1 (5/38)	0 (0/11)	50.0 (19/38)	54.5 (6/11)	39.5 (15/38)	72.7 (8/11)
+	39.5 (15/38)	63.6 (7/11)	15.8 (6/38)	45.5 (5/11)	42.1 (16/38)	27.2 (3/11)
++	34.2 (13/38)	18.2 (2/11)	28.9 (11/38)	0 (0/11)	15.8 (6/38)	0 (0/11)
+++	13.1 (5/38)	18.2 (2/11)	52.6 (2/38)	0 (0/11)	2.6 (1/38)	0 (0/11)
Chronic gastritis	P<0.001		P<0.001		P<0.001	
Negative	0 (38/38)	0 (0/11)	0 (38/38)	18.2 (2/11)	0 (38/38)	100 (11/11)
+	94.7 (36/38)	81.8 (9/11)	84.2 (32/38)	81.8 (9/11)	52.6 (20/38)	0 (0/11)
++	5.2 (2/38)	18.2 (2/11)	10.5 (4/38)	0 (0/11)	42.1 (16/38)	0 (0/11)
++	0 (0/38)	0 (0/11)	5.2 (2/38)	0 (0/11)	7.9 (3/38)	0 (0/11)
Intestinal metaplasia	P<0.001		P<0.001		P<0.001	
Negative	0 (38/38)	0 (0/11)	0 (38/38)	63.6 (7/11)	23.7 (9/38)	100 (11/11)
+	84.2 (32/38)	72.7 (8/11)	100 (38/38)	36.4 (4/11)	55.3 (21/38)	0 (0/11)
++	15.8 (6/38)	27.3 (3/11)	0 (0/38)	0 (0/11)	15.8 (6/38)	0 (0/11)
++	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)	5.3 (2/38)	0 (0/11)
Atrophy	P<0.05		P<0.001		P<0.001	
Negative	0 (38/38)	0 (11/11)	18.4 (7/38)	63.6 (7/11)	21.1 (8/38)	100 (11/11)
+	78.9 (30/38)	72.7 (8/11)	73.6 (28/38)	27.3 (3/11)	71.1 (27/38)	0 (0/11)
++	21.0 (8/38)	18.2 (2/11)	7.9 (3/38)	0 (0/11)	7.9 (3/38)	0 (0/11)
++	0 (0/38)	9.0 (1/11)	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)
Dysplasia						
Negative	89.5 (34/38)	100 (11/11)	95.8 (36/38)	100 (11/11)	86.8 (33/38)	100 (11/11)
+	10.5 (4/38)	0 (0/11)	7.9 (3/38)	0 (0/11)	13.2 (5/38)	0 (0/11)
++	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)
++	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)	0 (0/38)	0 (0/11)
<i>H. pylori</i> infection					P<0.01	
Positive	28.9 (11/38)	54.5 (6/11)	31.5 (12/38)	18.2 (2/11)	36.9 (14/38)	9.0 (1/11)
BR+			36.9 (14/38)			
BR-			54.5 (6/11)			

more severe mucosal lesions, suggesting that bile reflux deteriorates mucosal lesions in the whole stomach. The exact mechanisms by which bile as well as other refluxing contents of duodenum cause gastric mucosal damage are still unclear. It has been indicated that interaction of bile acid, a component of bile, with M3 muscarinic receptor subtype expressed in chief cells may contribute to mucosal damage, manifested as active inflammation, intestinal metaplasia, glandular atrophy and focal hyperplasia, and other pathophysiological consequences of bile reflux^[1,7,8-10].

Regurgitating via pylorus to gastric or esophageal cavity and sticking to mucous surface, refluxed bile might result in direct injuries of mucous epithelial cells and the tight junctions between these cells. It could provoke inflammatory responses in the whole mucous layer and submucous layer. Furthermore, by changing the chemical environment of mucous surface, regurgitated bile might affect the pathogenic patterns of other damage-causing factors such as gastric acid and *H. pylori*, and potentiate the actions of these factors^[1,2]. Consequently, bile reflux exerts harmful effects on gastric mucosa in the whole stomach.

Previous studies have shown that bile reflux is associated with the near-end gastric mucosal damage (including cardia cancer and intestinal metaplasia of cardia mucosa) as well as the mucosal lesions in bile reflux esophagitis^[9,10]. Also, in the present study, the severity of mucosal pathological changes in the near-end stomach and *H. pylori* infection in the corpus in bile reflux positive patients was significantly

greater than that in patients without bile reflux. In addition, an obviously positive correlation was found between bile reflux time and gastric mucosal lesions including *H. pylori* colonization in the near-end stomach, especially in the corpus, suggesting that bile reflux deteriorates gastric mucosal damage in the corpus region to a more extent, and bile reflux may facilitate *H. pylori* colonization in gastric corpus. This phenomenon involves complex mechanisms. On the one hand, as described above, adhering to the surface of gastric corpus mucosa, refluxed bile might impair directly and strengthen the deleterious effects of gastric acid and pepsin on gastric corpus mucosa. On the other hand, as a result of adherence and destructive effects of regurgitated bile, the secretory and brushing capacity of gastric corpus mucosa was obviously decreased, which might facilitate *H. pylori* colonization from the antrum to the corpus region and hence exacerbating gastric mucosal lesions in the corpus. The overall *H. pylori* infection rate of bile reflux positive patients was lower than that of patients without bile reflux, but with no statistical significance. However, it was coincident with previous studies^[1,2].

Combined with the pH monitoring system, the ambulatory Bilitec 2000 fiberoptic system could provide good approaches for simultaneous investigation of the dynamic processes of bile reflux and pH in stomach. The study of Barrett *et al*^[5] showed that the method using Bilitec 2000 system for monitoring of bile reflux had a high sensitivity *in vivo*, while its sensitivity was much lower *in*

vitro, with a false-negative rate of 23%. In the acidic environment of the stomach, the values of bilirubin absorbance detected with this system were lower than their true values; the likelihood of false-positive results was very small. Consequently, it has been recognized that, although it may not be sufficiently reliable to be regarded as the gold standard for the evaluation of gastroduodenal reflux, this system is currently the most reliable method for dynamical monitoring of bile reflux, the results obtained can reflect the actual status of human body. The overall accuracy of this system should be sufficient for clinical use^[5,11-15].

Theoretically, the existence of bile reflux in stomachs may at least, to some extent, reflect alkaline regurgitation from duodenum, which may result in decreased gastric H⁺ concentration. However, in our study, no obvious correlation was found between time of bile reflux and pH >4 in stomachs of dyspepsia patients. This was further confirmed in the real-time overlapped charts of bile absorbance and pH values. These findings may be due to the complex feedback regulatory networks between gastric acid secretory, gastric motion and emptying functions. The exact reasons why bile reflux failed to influence the acidic environment of gastric mucosa in dyspepsia patients remain to be determined.

In conclusion, the results of our study indicate that bile reflux affects both the anatomical and histological features of gastric mucosal damage and *H. pylori* colonization in bile reflux positive patients. It may exacerbate near-end gastric mucosal damage, and facilitate *H. pylori* colonization in the corpus region. Besides, bile reflux has no obvious influences on acid-exposing status of gastric mucosa in dyspepsia patients.

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• BRIEF REPORTS •

Dissociation, culture and morphologic changes of interstitial cells of Cajal *in vitro*

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Abstract

AIM: To study the method of dissociation, culture and investigate its morphologic changes *in vitro* of interstitial cells of Cajal (ICC).

METHODS: Enzymatic digestion and Ficoll density centrifugation were used to dissociate ICC from the ileal segment of mice. Factors including contamination, Ca^{2+} , Mg^{2+} and collagenase, and stem cell factor, *etc.*, were investigated. ACK2, the antibody of c-kit, was used to identify the cultured ICC. Both light microscope and fluorescence microscope were used to observe the changes of ICC *in vitro*.

RESULTS: The method for dissociation and culture of ICC *in vitro* was successfully established. After 24 h, cultured ICC exhibited a few axis-cylinders, and longer axis-cylinders were observed to form synapse of each other after 3 d. More widespread connections formed within 7 d *in vitro*. The changes of its morphologic character were obvious within 7 d; however, there were no obvious morphologic changes after 30 d.

CONCLUSION: Many factors can influence the dissociation and culture of ICC.

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Key words: Interstitial cells of Cajal; Culture; Morphologic change

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INTRODUCTION

Interstitial cells of Cajal (ICC) are the pacemakers in

gastrointestinal (GI) muscles, and also transduce inputs from the enteric nervous system. In the intestine, slow wave generation has been linked to the presence of a layer of ICC within the Auerbach's plexus region^[1-3]. ICC are mesenchymal cells that have precursor cells in common with smooth muscle cells, and possess the Kit tyrosine kinase membrane receptor as their special characteristics^[4-6]. Although there are many researches on ICC now^[5-7], the procedure in isolation and culture of ICC still has many difficulties that should be studied and discussed. Their morphologic changes during short-term culture *in vitro* have no clear explanation by now. In this study, the method of dissociation and culture of ICC were established and discussed. The process of their morphologic changes *in vitro* was observed at least for 30 d. The method of ICC culture should help us make deeper research of ICC in future.

MATERIALS AND METHODS

Experimental animals

The BALB/C mice (15-20 d old) of either sex were from the Experimental Animal Center of Daping Hospital, Chongqing. Animals were not given food for 24 h prior to the experiment. Mice were killed by cervical dislocation. The ileal segment about 10 cm proximal to the ileocecal junction was removed. The muscularis propria was gently peeled from the mucosa and placed in Ca-free Hank's balanced salt solution (Hyclone) with 1% antibiotic-antimycotic (Sigma). Subsequently, the segment was washed thrice through the duct with Ca^{2+} -free Hank's balanced solution (1% antibiotic-antimycotic). The segment was opened flat by cutting along the mesenteric line and pinned flat with the mucosa facing the dissecting wood board. The dissected muscle was carefully cut into small pieces (1-2 mm³) for enzymatic digestion.

Enzymatic dissociation

The muscle pieces were incubated at 37 °C in collagenase-based dissociation solution containing 1.3 mg/mL collagenase (type II, Sigma), 2 mg/mL bovine serum albumin (BSA, Sigma), 2 mg/mL trypsin inhibitor (Sigma), and 0.27 mg/mL ATP (Sigma), 10 mL of calcium-containing Hank's balanced salt solution (Hyclone). The pH was adjusted to 7.0 with 0.1 mol/L NaOH. After 30 min at 37 °C without shaking the water bath, the tissue was bluntly triturated with pipette every 3 min until single cells were obtained for approximately 10 min. After passing through the sieve (size: #200), all cell suspension was layered on the surface of a 200 g/L Ficoll density cushion and spun at 15 r/min for 15 min. The cell band located at the interface was transferred to a new container

and resuspended with M199 medium with 10% fetal bovine serum (Hyclone), 1% antibiotic to the desired density (about 2×10^6). The suspension was plated into Falcon petri dishes (with collagen-coated coverslips) on the bottoms. The cells were maintained in 50 mL/L CO_2 at 37 °C.

Observation of ICC under light microscope

ICC were observed under Olympus inverted microscope, with 100 $\times$, 200 $\times$ or 400 $\times$ power. The images were captured with Olympus color video camera and then directly recorded in the computer.

Immunofluorescence labeling of ICC

Cultured cells were prepared for immunofluorescence labeling by fixation in acetone (4 °C, 10 min). After fixation, the cells were incubated in normal goat serum for 1 h (10% in PBS), and then at 4 °C with a rat monoclonal antibody specific for Kit protein (ACK2, 5 mg/mL) in PBS overnight. Immunoreactivity was detected using fluorescein isothiocyanate (FITC)-conjugated secondary antibody (FITC-anti-rat, Zhongshan, China; diluted 1:100 in PBS, 1 h, room temperature). Control cultures were prepared in a similar manner, but ACK2 was omitted from the incubation solution. The cells were examined with a LEICA TCS SP2 (Germany) confocal microscope with an excitation wavelength appropriate for FITC (488 nm). All images were captured and recorded in the computer.

RESULTS

Identification of ICC cultured *in vitro*

We performed immunofluorescence analysis to determine the morphology of cells that displayed Kit immunoreactivity. The cells showed fusiform cell bodies, prominent nuclei, and multiple thin processes extending from the nuclear region on light micrograph. ICC, with this morphology expressed Kit-like immunoreactivity, could form network of each other as shown on the fluorescence micrograph (Figure 1).

Morphologic changes of ICC

After 24 h in culture, most cells showed fusiform cell bodies, large and prominent nuclei, and multiple, short and thin processes extending from the nuclear region (Figure 2A). Cells with this morphology were easily distinguished from the smooth muscle cells.

After 72 h in culture, cells with more and longer processes extending from the nuclear region could be observed

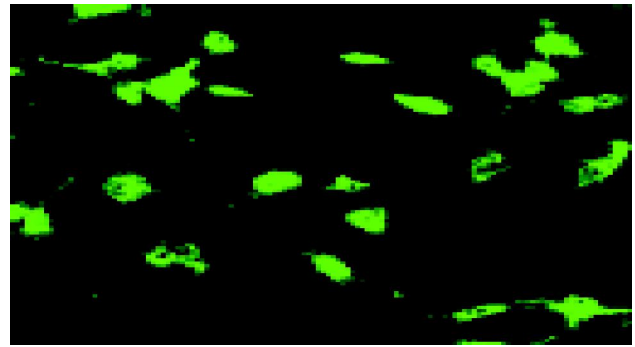


Figure 1 C-kit immunoreactivity on fluorescence micrographs of ICC cultured *in vitro* (400 $\times$).

(Figure 2B). Some cells began to form a characteristic network of each other.

After 7 d in culture, cells with more multiple and longer processes could be observed under phase-contrast microscope (Figure 2C). More characteristic networks could be found and the morphologic changes could be observed even after 30 d.

DISCUSSION

In GI tract, ICC are intimately connected with smooth muscle cells, fibrocytes and nerve fasciculi. Some methods can isolate and culture ICC^[5-7]. Many difficulties should be overcome in the procedure of culturing ICC. However, no detailed research on the method of ICC culture is available before this study. In this study, we found that following factors should be discussed in the procedure of ICC culture.

Preventing contamination

If the culture medium is contaminated, few cells can be adhered to the coverslips at first, and no cells could be obtained after 3 d. The sources of infection mainly include *E. coli*, staphylococci, eumycetes, etc. In the study, no food was given for at least 12-24 h prior to the experiment. It is helpful to make the small intestine clean. In the procedure of isolating ICC, sufficient washing of the small intestine should be equally important to reduce contamination. Moreover, antibiotics (1% antibiotic-antimycotic was used in the study) should be added in the washing solution and culture medium. At last, strict aseptic manipulation should be mentioned in the process of cell culture.

Shortening the time *in vitro* and minimizing zero Ca^{2+} exposure

Solution containing no Ca^{2+} solution is very important to



Figure 2 Morphologic changes of cultured ICC after 24 h (A), 72 h (B), and 7 d (C).

decrease the connection of ICC to other cells and matrix. For good dissociation of tissue, it is unavoidable to expose ICC to solution containing no Ca^{2+} ^[5-8]. However, when it is re-exposed to normal Ca^{2+} levels, overshoot of cellular Ca^{2+} levels would occur. We suggest that tissues exposed to low Ca^{2+} levels before placement in medium M199 (about 2.5 mmol/L Ca^{2+}) should be helpful to reduce the overshoot of Ca^{2+} . To get the desired quantity of ICC, shortening the time *in vitro* and minimizing exposure to solution containing no Ca^{2+} should be mentioned.

Appropriate enzyme digestion

Enzyme digestion is necessary in releasing ICC from tissues. Collagenase was used to isolate ICC in this study, while some other enzymes have been used either alone or in combination in other researches^[5]. BSA and inhibitor of trypsin can be added in the solution to retain collagenase activity. We found that collagenase worked better in the presence of Ca^{2+} . Besides the component of enzyme solution, appropriate time of the enzyme digestion should also be mentioned. We found that insufficient digestion would get few single cells, while sufficient digestion would not get desired quantity of ICC as well. In this study, 30-min digestion at 37 °C was conducted to isolate ICC.

Changing the culture medium

In the study, the culture medium was changed after 24 h in culture. Then the medium was changed every 3-4 d. Twenty-four hours would be enough for ICC to adhere to the coverslips. Changing the culture medium in time can provide nutrient for ICC.

Stem cell factor is essential to maintain ICC in culture

Stem cell factor (SCF) is the ligand of the c-kit (CD117). The signal stimulated by c-kit/SCF is essential to maintain the phenotype of ICC^[8-10]. In the culture medium M199, murine recombinant SCF was added at the concentration of 5 ng/mL in our study. Both the results in our study and other works showed that SCF is essential to obtain and maintain ICC in good condition^[8,11-13]. In the study, we found that the c-kit with SCF was expressed on ICC in a dose-dependent manner.

In the study, the isolated ICC could contact each other. Multiple networks of ICC could also be found after about 7 d in culture, suggesting that the specific network of ICC is essential to maintain and realize its phenotype function.

It has been confirmed that c-kit only emerges on ICC and mast cells (~5%) in GI^[10]. We consider that almost all c-kit-stained cells should be ICC. In the study, ICC were identified by immunofluorescence with c-kit-specific antibody ACK2.

After ICC were plated in M199 medium, the cells became round and could be adhered to the coverslips within

24 h in culture. The cell body was generally triangular or stellar and with a large nucleus surrounded by little cytoplasm. In the first 3 d, there were generally 3-5 primary processes, which could branch into secondary and tertiary processes. In the study, the isolated ICC could contact each other and form multiple networks within 7 d. These specific networks of ICC are the same as *in vivo*, suggesting that it is essential to maintain and realize its phenotype and function.

The detailed method of ICC culture can contribute to its deeper research.

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• BRIEF REPORTS •

Analyses of prognostic indices of chronic liver failure caused by hepatitis virus

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Abstract

AIM: To analyze the related indices about the prognoses of chronic liver failure caused by hepatitis virus.

METHODS: Retrospectively reviewed 320 cases of chronic liver failure caused by hepatitis viruses. An improved group and an ineffective group (IG) were made to compare and analyze their clinical manifestations, laboratory examination indices and complications. Logistic regression was also carried out.

RESULTS: There were significant differences ($P < 0.05$) between the improved group and the IG upon such indices as age, bilirubin, prothrombin time, albumin, alpha fetoprotein, the size of liver and complications ($P < 0.05$). The regression formula was as follows: $P = 1/(1+e^{-\gamma})$ ($\gamma = 1.7262 - 0.0948X_1 + 2.9846X_2 + 0.6992X_3 + 1.6019X_4 + 2.0398X_5$). (Note: X_1 -Prothrombin activity; X_2 -digestive tract hemorrhage; X_3 -hepatic encephalopathy; X_4 -hepatorenal syndrome; X_5 -pulmonary infection.).

CONCLUSION: Laboratory examination such as bilirubin, prothrombin time and alpha fetoprotein can be regarded as indices of the prognoses of chronic liver failure caused by hepatitis. Moreover, the regression equation can evaluate prognoses more comprehensively and direct our treatments.

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Key words: Chronic liver failure; Hepatitis; Prognostic indices; Laboratory indices; Complications; Regression equation

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INTRODUCTION

Hepatitis B virus (HBV) infection is very common in China^[1-3]. Fulminant hepatitis (FH) refers to liver diseases that have severe states and poor prognoses, with a mortality rate of more than 50%^[4]. In China, the majority of FH is chronic liver failure caused by hepatitis virus (CLFHV) (accounting for 85% of all FH), with the most common cause of HBV infection^[5]. Because of the chronic underlying pathological changes (most commonly chronic hepatitis or liver cirrhosis), when liver failure led by necrosis of mass liver cell develops, treatments become more difficult. In order to improve our knowledge about the judgment of the prognoses of CLFHV, we conducted a retrospective study on the potential prognostic influential factors on 320 cases of CLFHV patients to investigate into the ways of predicting the prognoses of CLFHV.

MATERIALS AND METHODS

The 320 CLFHV patients who met the clinical diagnostic grouping criteria made jointly by infectious diseases branch, parasitic diseases branch and liver diseases branch of Chinese Medical Association (2000, Xi'an)^[6] were admitted to our hospital from January 2000 to June 2003. Two hundred and eighty-eight cases were male (90%) and 32 were female (10%), with an average age of 43.00 ± 12.24 years (ranged from 15 to 77 years). Three hundred and one cases were single HBV infected, two were hepatitis E virus (HEV) infected and one with undetermined cause. There were 16 cases who were multi-infected: three were HBV+hepatitis C virus (HCV) infected, two were HBV+hepatitis D virus (HDV) infected, 10 were HBV+HEV infected and one was HBV+hepatitis A virus (HAV)+HDV infected.

We divided those 320 patients into two groups: 123 cases were in the effective group (EG) (improved in symptoms and signs on the whole or significantly, with liver function rebuilt or significantly improved) and 197 cases were in the ineffective group (IG) (discharged by themselves without improving or died in hospital). We compared their laboratory indices, B-ultrasonography, complications and such potential prognoses-influencing factors. Among them, the value of bilirubin was taken at the highest time in our hospital. The results were analyzed by SPSS 10.0 software. It would be significant if $P < 0.05$.

RESULTS

Age

The average age in EG was 39.67 ± 11.13 years and the counterpart in IG was 45.02 ± 12.50 years, with significant difference ($P < 0.05$).

Table 1 Comparison of laboratory indices of the two groups (mean±SD)

Items	Ineffective group	Effective group	Value of <i>P</i>
Glutamate-pyruvate transaminase (ALT)	196.70±291.74 U/L	215.54±349.53 U/L	>0.05
Glutamic-oxalacetic transaminase (AST)	214.05±234.75 U/L	207.93±233.69 U/L	>0.05
Alkaline phosphatase (ALP)	137.42±74.60 U/L	150.61±44.94 U/L	>0.05
Total bilirubin (Tbil)	612.332±195.943 μmol/L	506.624±184.454 μmol/L	<0.05
Direct bilirubin (Dbil)	356.837±119.196 μmol/L	306.827±112.52 μmol/L	<0.05
Serum albumin (ALB)	32.80±6.04 g/L	35.17±5.46 g/L	<0.05
Cholesterol (CHOL)	1.601±3.146 mmol/L	1.721±0.884 mmol/L	>0.05
Cholinesterase (CHE)	3 453.69±1 820.24 U/L	3 198.16±1 794.42 U/L	>0.05
Blood glucose (GLU)	4.609±3.932 mmol/L	4.650±2.303 mmol/L	>0.05
Blood urea nitrogen (BUN)	10.33±13.72 mmol/L	4.87±3.60 mmol/L	<0.05
Creatinine (Cr)	159.26±170.88 mmol/L	85.37±37.71 mmol/L	<0.05
Alpha fetoprotein (AFP)	111.46±177.53 ng/mL	170.45±230.25 ng/L	<0.05
Blood ammonia (NH ₃)	101.80±54.03 μmol/L	75.23±38.71 μmol/L	<0.05
Prothrombin time (PT)	35.63±14.37 s	27.14±11.04 s	<0.01
Prothrombin activity (PTA)	23.44±9.36%	32.56±9.51%	<0.01

Laboratory indices

There were significant differences between those two groups on such indices as bilirubin, serum albumin (ALB), blood clotting functions, alpha fetoprotein (AFP), creatinine (Cr) and blood ammonia (NH₃), while there were no significant differences on aminotransferase, cholesterol (CHOL) or blood glucose (GLU) (Table 1).

Complications and the result of B-ultrasonography

There were more significant differences on complications and liver size between the two groups (Tables 2 and 3).

Table 2 Comparison of complication distribution of two groups

Items	Ineffective group, <i>n</i> (%)	Effective group, <i>n</i> (%)	<i>P</i>
Alimentary tract hemorrhage	39 (19.8)	4 (3.3)	<0.01
Hepatorenal syndrome	53 (26.9)	3 (2.4)	<0.01
Primary peritonitis	130 (66.0)	47 (38.2)	<0.01
Hepatic encephalopathy	136 (69.0)	17 (13.8)	<0.01
Infection of biliary tract	117 (59.4)	64 (52.0)	>0.05
Pulmonary infection	34 (17.1)	5 (4.1)	<0.01

Table 3 Comparison of B-ultrasonography of two groups

Items	Ineffective group, <i>n</i> (%)	Effective group, <i>n</i> (%)	<i>P</i>
Liver shrinkage	105 (53.3)	39 (31.7)	<0.01
Enlarge of spleen	125 (63.5)	76 (61.8)	>0.05
Ascites	159 (80.7)	89 (72.4)	>0.05

Analyses of influencing factors

We made Logistic Stepwise Regression about those variable factors. We put together glutamate-pyruvate transaminase (ALT)/glutamic-oxalacetic transaminase (AST), total bilirubin (Tbil)/direct bilirubin (Dbil), prothrombin time (PT) and prothrombin activity (PTA); blood urea nitrogen (BUN), creatinine (Cr) and hepatorenal syndrome. Together with age, ALB, CHOL, cholinesterase (CHE), AFP, liver size, hemorrhage and hepatorenal syndrome, *etc.*, we got 19 variables as regression factors. By Forward method, we got the regression model as follows: $P = 1/(1+e^{-y})$; ($y = 1.7262 - 0.0948X_1 + 2.9846X_2 + 0.6992X_3 + 1.6019X_4 + 2.0398X_5$);

Note: X_1 -PTA (numeric), X_2 -digestive tract hemorrhage (0-none; 1-develops), X_3 -hepatic encephalopathy (0-none; 1-degree I; 2-degree II; 3-degree III; 4-degree IV), X_4 -hepatorenal syndrome (0-none; 1-develops), X_5 -pulmonary infection (0-none; 1-develops). When $P > 0.5$, the patient would get worse. Otherwise, the patient would improve. The coincidence of its rightness was 83.55%, which conformed to the reports abroad^[7,8].

DISCUSSION

The mortality rate of CLFHV is very high. The pathological underlyings are mass apoptosis^[9-11], necrosis or severe degeneration of liver cells, which lead to liver failure. There are abnormalities of many biochemical indices. The prognoses are related to the extent of liver cell necrosis, regeneration and whether complications will develop. Just as the statistical results of 320 cases in our study, PT and ALB, which represent synthetic functions of liver cells, Tbil and Dbil, which represent transportation function of liver cells^[12], all have significant correlations with the prognoses of FH. The increased Tbil and Dbil, prolonged PT^[13] and decreased ALB will suggest a poorer function of liver cells and a poorer prognosis. The older a patient is, poorer the ability of liver regeneration is, which is hard for recovery of liver function and leads to a poor prognosis. The increase of AFP in hepatitis is due to the acute reaction of liver cells after infected by viruses and this is also the regeneration label of liver cells after necrosis. A lower AFP suggests a poorer regeneration ability of liver cells and a poorer prognosis^[14,15]. The prognosis of FH is greatly correlated with the incidence, amount and severity of complications. The increased and more serious complications will lead to poorer prognoses. Especially when hepatic encephalopathy IV, renal failure^[16] or alimentary tract hemorrhage^[17,18] develops, a high mortality rate will occur.

The purpose of our multi-factors regression model is to evaluate the influence of many factors on the prognoses of FH more objectively and comprehensively. It is easy for calculation receiving information and is good for observation of the effects of treatments. We applied Forward method to delete those not significant variables and got five variable factors such as PTA, alimentary tract hemorrhage, hepatic encephalopathy, hepatorenal syndrome and pulmonary

infection, which had significant influence on the prognoses of CLFHV, and further suggested that blood clotting function and development of complications are the key determinants of survival of CLFHV. It has been generally accepted that PTA<40% is the affirmative threshold of necrosis of liver cells^[19]. The decreased PTA suggests a poorer liver synthetic function of blood coagulation factors and furthermore, the more severe necrosis of liver cells. While the other four variable factors were all complications, which further suggested the importance of the influence of complications on the prognoses of CLFHV. We should pay attention to the prevention and treatment of complications in treating CLFHV.

Heretofore, the treatment of FH has not matured, with a poor recovery rate. However, there are new treatments such as liver transplantation^[20-24], which has brought new hope to patients with CLFHV^[25-28]. During the course of diagnosis and treatment, the combination of dynamic observation of single indices and comprehensive judgment on prognoses can not only direct our treatments according to the change of condition, but also present objective criteria for timely liver transplantation to shorten the course of FH and relieve patients' burden.

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• CASE REPORT •

Encapsulating peritonitis and familial Mediterranean fever

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Abstract

AIM: To investigate the relationship between encapsulating peritonitis and familial Mediterranean fever (FMF).

METHODS: The patient had a history of type 2 diabetes and laparoscopic cholecystectomy was performed one year ago for cholelithiasis. Eleven months after the operation she developed massive ascites. Biochemical evaluation revealed hyperglycemia, mild Fe deficiency anemia, hypoalbuminemia and a CA-125 level of 2 700 IU. Ascitic evaluation showed characteristics of exudation with a cell count of 580/mm³. Abdominal CT showed omental thickening and massive ascites. At exploratory laparotomy there was generalized thickening of the peritoneum and a laparoscopic clip encapsulated by fibrous tissue was found adherent to the uterus. Biopsies were negative for malignancy and a prophylactic total abdominal hysterectomy and bilateral salpingo-oophorectomy were performed.

RESULTS: The histopathological evaluation was compatible with chronic nonspecific findings and mild mesothelial proliferation and chronic inflammation at the uterine serosa and liver biopsy showed inactive cirrhosis.

CONCLUSION: The patient was evaluated as sclerosing encapsulating peritonitis induced by the laparoscopic clip acting as a foreign body. Due to the fact that the patient had FMF the immune response was probably exaggerated.

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Key words: Encapsulating peritonitis; Familial Mediterranean fever; Ascites

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INTRODUCTION

Familial Mediterranean fever (FMF) is an immunological disorder characterized by recurrent abdominal pain and polyserositis and is common in Mediterranean countries. Episodes of exudative peritonitis, pleuritis, arthritis and rarely inflammation of the organs may occur. Unnecessary laparotomies exacerbate accumulation of fibrotic adhesions caused by the disease itself. Ascites has been reported in FMF patients only after the development of secondary amyloidosis, which is seen mainly in patients who are not taking colchicine^[1]. We hereby report an unusual cause of ascites in FMF and review the literature.

CASE REPORT

A 65-year-old female patient with a history of FMF for 20 years presented with massive ascites. The patient had a history of type 2 diabetes for 5 years which was regulated with oral antidiabetics and a laparoscopic cholecystectomy was performed one year ago for cholelithiasis. At the time of the operation she was not taking colchicine. There was no alcohol consumption or a history of hepatitis. Six months after the operation she felt a tenderness over the suprapubic region, and bloating developed all over the abdomen 11 mo after the operation. Besides the presence of dyspnea and anemia the abdomen was enlarged and she noticed a vaginal hemorrhagic discharge. An abdominal ultrasound revealed massive ascites, normal liver and splenic echogeneity and gynecological ultrasound did not show any abnormalities. Biochemical evaluation revealed a fasting blood glucose level of 220 mg/dL, normal renal function tests, mild Fe deficiency anemia, normal liver function tests, hypoalbuminemia, normal platelets and prothrombin time and a CA-125 level of 2 700 U/mL (interval: 0-25 U/mL). Other tumor markers were normal. Ascitic evaluation showed characteristics of exudative ascites with a cell count of 580/mm³ and a predominance of lymphocytes with no malignant cells. Serum ascitic albumin gradient was 1.0. Cultures for bacteria and *Mycobacterium tuberculosis* were negative. The patient was negative for serum markers of hepatitis B and C viruses, and autoantibodies. Portal Doppler ultrasound, upper gastrointestinal endoscopy and barium enema were normal. Abdominal CT showed minimal omental thickening and massive ascites (Figures 1A and B). We decided to perform an exploratory laparotomy in order to exclude tuberculous peritonitis and ovarian malignancy. At the operation there was thickening of the peritoneum in general and a laparoscopic clip encapsulated by fibrous tissue was found adherent to the uterus. Biopsies taken from the peritoneum, omentum, ovaries and fibrous tissue were negative for

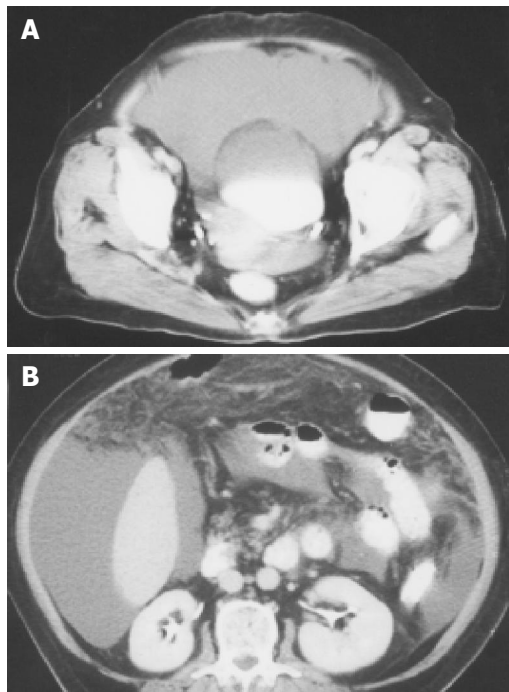


Figure 1 Abdominal CT. A: omental thickening; B: massive 8 ascites.

malignancy and a prophylactic total abdominal hysterectomy and bilateral salpingo-oophorectomy were performed. The histopathological evaluation of the peritoneum and omentum was compatible with chronic nonspecific findings and mild mesothelial proliferation. There was chronic inflammation at the uterine serosa and the liver biopsy showed inactive cirrhosis. In none of the biopsies taken from different organs there was a finding compatible with amyloidosis. The fibrous tissue surrounding the clip was accepted as a chronic reaction to the clip and the generalized thickening of the peritoneum was interpreted as a mild form of encapsulating peritonitis, which was probably induced by the foreign body namely the laparoscopic clip. Ascites developed again 10 d after the operation. Therefore, we started with 30 mg prednisolone and colchicine 1 mg/d and tapered the corticosteroids gradually in 6 wk. After this treatment ascites disappeared completely. In the early postoperative period the CA-125 level was still very high and decreased to normal levels gradually in three months with the disappearance of the ascites. The patient is still doing well after eight months of follow-up and there is no ascites.

DISCUSSION

Encapsulating peritonitis has been described in the literature as peritonitis chronica fibrosa incapsulata, sclerosing peritonitis, sclerosing encapsulating peritonitis^[2] and more severe forms as abdominal cocoon^[3]. The pathogenesis and etiology is not well known. Prolonged treatment with beta-adrenergic blockers, sarcoidosis, systemic lupus erythematosus, ventriculoperitoneal and peritoneovenous shunting, intraperitoneal instillation of drugs, and chronic ambulatory peritoneal dialysis^[4] have been accused as causative agents.

Clinical picture is characterized by recurrent episodes of intestinal obstruction and abdominal mass but there may

also be ascites^[5]. For diagnosis, radiological evaluation is the mainstay but radiological findings are nonspecific and surgical confirmation is mostly necessary^[6].

In the literature there is one case of encapsulating peritonitis in periodic disease, which had abdominal pain and a pseudocystic appearance in USG and CT, and surgical intervention was necessary for the diagnosis^[7]. There is another report about previously unpublished peritoneal complications of familial paroxysmal polyserositis^[8]. Our case is the third report of encapsulating peritonitis in association with FMF. It is interesting that this patient presented with ascites, but there were no signs of intestinal obstruction. This was probably due to the fact that the history of ascites was not very prolonged and adhesions have not been formed yet. The absence of amyloidosis but the presence of ascites has led us to look for another cause of ascites in FMF. The patient had stopped to take colchicine for the past two years because she did not have any abdominal attacks, but this may have led to the development of an exaggerated immunological response to the foreign body in the peritoneal cavity, namely laparoscopic clip. This laparoscopic clip acted as a chronic stimulus in the cavity and caused the chronic peritoneal inflammation and this was the triggering point for the encapsulating peritonitis. The resection of the clip together with the uterus eliminated this chronic stimulus and the immunosuppression of immunologic events of FMF with corticosteroids and colchicine has led to the disappearance of the ascites. It is well known that there are abnormalities of suppressor T lymphocytes, altered metabolism of lipoxygenase products of arachidonic acid and absence of a normal inhibitor of the complement-derived anaphylatoxin C5a^[9].

This case confirmed the hypothesis that the elevation of CA-125, which is seen in peritoneal tuberculosis^[10,11] and ovarian and endometrial malignancies^[12,13], may also be due to the chronic peritoneal irritation. The regression of the level of CA-125 after the medical treatment is another evidence for this fact.

In summary encapsulating peritonitis although rare can also be due to some reason of chronic peritoneal irritation, and immunological disorders like FMF may facilitate the development of encapsulating peritonitis.

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• CASE REPORT •

TT virus infection and pancreatic cancer: Relationship or accidental coexistence

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Abstract

TT virus (TTV) was first isolated in 1997 from the patient with acute post-transfusion hepatitis. This fact led to the conclusion that the virus was hepatotropic and could be one of the causative agents of acute hepatitis. Afterwards, however, the virus was found in other human tissues and serological studies revealed that it was widespread. Multiple tropisms of TTV and the fact of its high incidence in general population are considered to indicate no medical significance of TTV in human pathology. Here we present a report of two cases of TTV infection in patients who developed pancreas cancer. The patients were hospitalized at the Department of Infectious Diseases due to hepatitis of unknown origin. Since serological and virological markers of common primary and secondary hepatotropic viruses were negative, TTV-DNA was found in serum and was believed to be the only causative agent with probable hepatotropic action. The patients later developed pancreas cancer and they underwent operation. The relationship is difficult to confirm, however the cases we present should be treated as a preliminary report and a comment on the real role of TTV in human pathology.

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Key words: TT virus; Hepatitis; Pancreas cancer; Hepatotropic viruses

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INTRODUCTION

The medical significance of TT virus (TTV) has not been completely explained. The virus was first isolated in 1997 in a Japanese patient with acute posttransfusion hepatitis and considered a transfusion-transmitted and hepatotropic one. The virus was named TT both due to initials of the first patient with detectable TTV-DNA in serum and as an

abbreviation of the word “transfusion-transmitted”.

The exact taxonomy of this unenveloped circular DNA virus has not been established. TTV is related to *Circoviridae*, although there are some opinions, that it should belong to a new family called *Circinoviridae*^[1].

The analysis of TTV infected patients with symptoms, signs and laboratory results characteristic of acute hepatitis have revealed that TTV is likely to be associated with human liver pathology. The fact that the virus was frequently found in the liver tissue of patients with hepatitis supports this thesis. However, the liver is probably not the only target of the virus. It was also identified in the bone marrow, lymph nodes, pulmonary tissue, spleen, kidney, muscles and pancreas. This indicates that the virus can replicate in these tissues and that the accurate assessment of the virus tropism is difficult^[1].

The 10-300 times higher virus concentration in the liver than in the serum is one of the most evident indication of its hepatotropic nature^[2]. However, TTV-DNA is likely to either integrate with a host genome or be present in hepatocytes in the episomal form^[3-5].

Since the exact significance of TTV in human pathology has not been fully explained and its biological action probably involves integration with the human genome, some authors undertook studies on its possible role in the oncogenesis. Due to the association of TTV infection with liver diseases most of them focused on hepatocellular carcinoma.

We present here two case reports of patients infected with TTV who developed pancreas cancer. Since the coexistence of pancreatic cancer and TTV infection has not been reported to date, we found these cases interesting and worth investigating.

CASE REPORTS

Case 1

The patient, a 60-year-old male was admitted to our Department of Infectious Diseases of Medical University of Lublin due to moderate abdominal pain localized in the right upper quadrant and elevated aminotransferase levels. History revealed that he was hospitalized due to myocardial infarction 5 mo earlier, and therefore he did not travel abroad and stayed away from ill individuals, and refrained from the use of alcohol or drugs.

On the basis of epidemiological data and results of biochemical laboratory tests, infectious hepatitis was suspected. On admission the bilirubin level was 3.44 mg/dL, AST level - 222 UL and ALT - 372 UL (the normal level is up to 37 and 40 UL, respectively). Other routine biochemical serum examinations were normal. Morphology revealed no

pathology. Both chest X-ray and abdomen ultrasonography were normal.

Serological and virologic markers of common primary hepatotropic viruses A, B and C, including anti-HAV IgM, HBs Ag, HBe Ag, anti-HBe, anti-HBc, HBV-DNA, anti-HCV, and HCV-RNA, were negative. Moreover, negative results of serological tests excluded Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infection. To find the cause of liver inflammation the test for autoantibodies was performed and no antinuclear (ANA), antimitochondrial antibodies (AMA) and anti smooth muscles antibodies (ASMA) were found. Finally markers of hepatitis G virus (HGV) and TTV infections were tested. The test for HGV was negative, however, using qualitative test based on polymerase chain reaction method, TTV-DNA was found in the patient's serum. The patient still had abdominal pain, and both alkaline phosphatase (AP) and gamma glutamyl transpeptidase (GGTP) increased to the alarming value, 516 UL (normal range 37-111 UL) and 1 223 UL (normal range 11-43 UL), respectively. CT scan was performed, first without and then using the radiographic contrast (Ultravist). The examination showed irregular enlargement of the pancreas head up to 4 cm in diameter, whereas CT density before contrast administration was 42-50 HU, and after it 80-104 HU. Simultaneously the widening of the common biliary tract and intrahepatic biliary tracts was visible. CT examination was done 2 wk after ultrasonography. The patient was sent to the Surgical Department, where pancreas cancer was diagnosed and the patient underwent operation. Unfortunately it was not possible to test TTV presence in pancreas tissue.

Case 2

The patient, a 44-year-old female, was admitted to the Department of Infectious Diseases of Medical University of Lublin due to dyspeptic symptoms and moderate pain in the right upper quadrant. The symptoms appeared two weeks before admission.

The history revealed no health problems and surgery or invasive diagnostic procedures in the past. In our opinion, the most important fact in the history was that the patient worked as a nurse in the surgical ward, so she was likely to be at risk of hepatotropic virus infection. On admission the biochemical parameters were as follows: bilirubin level -8.4 mg/dL, AST - 322 UL, ALT - 608 UL. An abdomen ultrasonography, performed on admission, revealed no pathology. Diagnostic procedures involved serological and virological markers of common primary hepatotropic viruses A, B and C. Anti-HAV IgM, HBs Ag, HBe Ag, anti-HBe, anti-HBc, HBV-DNA, anti-HCV, and HCV-RNA were negative. We also excluded EBV and CMV infections were also excluded.

The tests for autoantibodies were also negative. Antinuclear (ANA) and antimitochondrial antibodies (AMA) were absent. The only positive viral test was TTV-DNA, which was found using qualitative test based on the PCR method.

Although the hepatoprotective treatment had been introduced, the patient's condition aggravated. The bilirubin level reached 20.1 mg/dL whereas aminotransferase activity decreased (AST-178 and 310 UL). The level of serum

cholestasis markers was continuously increasing (GGTP reached 1 174 UL). To find the cause of increasing jaundice, we repeated the abdomen ultrasonography. It showed the presence of widened intrahepatic biliary ducts and the hyperechogenic area in the lower zone of pancreas head, suggesting neoplasm. The patient was sent to the Surgical Department. The final diagnosis of pancreas cancer was based on the histological examination of the specimen obtained during operation.

DISCUSSION

The cases of TTV infection and pancreas cancer coexistence presented here should arouse special interest and provoke discussion about the question asked in the title of this paper. Is there any relationship between TTV infection and oncogenesis in the pancreas or is the coexistence completely accidental? We have not found any reports concerning that problem.

There are some reports mentioning the problem of correlation between TTV infection and the development of different neoplasm. Shiramizu *et al* carried out studies on the virus in the etiology of acute lymphoblastic leukemia (ALL). They performed the analysis of peripheral blood mononuclear cells and bone marrow cells, as well as cerebrospinal fluid and concluded that TTV was unlikely to be associated with ALL^[6]. Because TTV is suspected of being a potentially hepatotropic virus, Liwen *et al* analyzed patients with chronic hepatitis, liver cirrhosis, and primary liver cancer, comparing the results in these study groups with a control group of healthy individuals. The frequency of TTV infection in hepatocellular carcinoma ranged from 8.1% to 100%. The authors suggested that such a great variability made explicit conclusions concerning the correlation between TTV infection and both chronic hepatitis and liver cancer impossible^[7]. Similar results were obtained by Japanese^[8,9] and Italian researchers^[10]. Moriyama *et al*^[11] demonstrated that the virus had no impact on clinical and serological profile of HBV and HCV infection. TTV did not change the results of chronic hepatitis B therapy with lamivudine. These conclusions were supported by the results of molecular research carried out by Yamamoto *et al*. They confirmed that the TTV genome was not integrated into the host hepatocyte DNA, which is probably necessary to initiate potential neoplasm development^[12].

TTV co-infection was not associated with the presence of B-cell non-Hodgkin lymphoma. Cacoub *et al*^[13] found TTV-DNA in only 7% of patients. On the other hand, according to Garbuglia *et al*^[14], the significant prevalence of TTV DNA in lymphocytes circulating in the lymph nodes of both B-cell lymphomas and Hodgkin disease suggested TTV infection implication in the development of these lymphoproliferative disorders. Recently, Camci *et al*^[15] has reported the high prevalence of TTV in patients with various malignancies. The viral load in cancer patients is extremely high. It might result from the impaired immune reaction^[1,15]. Further studies are needed to explain whether the impairment is caused by neoplasm or by the virus itself.

The cases of coexistence of TTV infection and pancreas cancer presented above should be treated as a preliminary

report and the comment in the discussion about the real role of TTV in human pathology. Since the virus may infect the pancreatic tissue and the pathogenesis of pancreas cancer has not been completely cleared many factors, including infectious agent(s) should be considered in the oncogenesis study. Two case reports are not enough to draw any conclusions. We believe that further research on possible link between hepatitis or other pathology and TTV infection, is needed.

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Major meetings coming up

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www.ddw.org/
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13th World Congress of Gastroenterology
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www.wcog2005.org/
Montreal, Canada

**13th United European Gastroenterology
Week, UEGW**
October 15-20, 2005
www.uegf.org/
Copenhagen, Denmark

**American College of Gastroenterology
Annual Scientific Meeting**
October 28-November 2, 2005
www.acg.gi.org/
Honolulu Convention Center, Honolulu,
Hawaii

Events and Meetings in the upcoming 6 months

EASL 2005 the 40th annual meeting
April 13-17, 2005
www.easl.ch/easl2005/
Paris, France

**World Congress on Gastrointestinal
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June 15-18, 2005
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**Digestive Disease Week DDW 106th
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Events and meetings in 2005

**Canadian Digestive Disease Week
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February 26-March 6, 2005

www.cag-acg.org
Banff, AB

2005 World Congress of Gastroenterology
September 12-14, 2005
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42	OD ₂₆₀	A ₂₆₀	"OD" has been abandoned.
43	Oneg/L	One microgram per liter	At the beginning of a sentence
44	A ₂₆₀ nm ^b P<0.05	A ₂₆₀ nm ^a P<0.05	A should be in italic. In Table, no note is needed if there is no significance in statistics: ^a P<0.05, ^b P<0.01 (no note if P>0.05). If there is a second set of P value in the same table, ^c P<0.05 and ^d P<0.01 are used for a third set: ^e P<0.05, ^f P<0.01.
45	*F=9.87, [§] F=25.9, [#] F=67.4	¹ F=9.87, ² F=25.9, ³ F=67.4	Notices in or under a table
46	KM	km	kilometer
47	CM	cm	centimeter
48	MM	mm	millimeter
49	Kg, KG	kg	kilogram
50	Gm, gr	g	gram
51	nt	N	newton
52	l	L	liter
53	db	dB	decibel
54	rpm	r/min	rotation per minute
55	bq	Bq	becquerel, a unit symbol
56	amp	A	ampere
57	coul	C	coulomb
58	HZ	Hz	
59	w	W	watt
60	KPa	kPa	kilo-pascal
61	p	Pa	pascal
62	ev	EV	volt (electronic unit)
63	Jonle	J	joule
64	J/mmol	kJ/mol	kilojoule per mole
65	10×10×10cm ³	10 cm×10 cm×10 cm	
66	N·km	KN·m	moment
67	$\bar{x} \pm s$	mean±SD	In figures, tables or text narration
68	Mean±SEM	mean±SE	In figures, tables or text narration
69	im	im	intramuscular injection
70	iv	iv	intravenous injection
71	Wang et al	Wang et al.	
72	EcoRI	EcoRI	Eco in italic and RI in positive. Restriction endonuclease has its prescript form of writing.
73	Ecoli	E.coli	Bacteria and other biologic terms have their specific expression.
74	Hp	H pylori	
75	Iga	Iga	writing form of genes
76	igA	IgA	writing form of proteins
77	~70 kDa	~70 ku	