P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2135-2136
World Journal of Gastroenterology
 ISSN 1007-9327

• REVIEW •

# Gastroesophageal reflux disease at the turn of *millennium*

Lee-Guan Lim, Khek-Yu Ho

**Lee-Guan Lim, Khek-Yu Ho**, Department of Medicine, National University Hospital, Singapore
**Correspondence to:** Associate Professor Khek-Yu Ho, MBBS (Syd Hons 1) MD FRACP FAMS, Department of Medicine, National University Hospital, Lower Kent Ridge Road, 119074 Singapore. mdchoky@nus.edu.sg
**Telephone:** +65-67724353 **Fax:** +65-67794112
**Received:** 2003-07-04 **Accepted:** 2003-07-14

## Abstract

Gastroesophageal reflux disease (GERD) has been an area of active research in the Asia-Pacific region in the recent years. This article outlines some of the interesting research findings. It comprises three parts. The first part dealt with recent data on the changing epidemiology of GERD in Asia. The second part summarized published studies on the relationship between GERD and *Helicobacter pylori*, relevant to the Asia-Pacific region. The last part discussed some of the recent advances in the treatment of GERD.

Lim LG, Ho KY. Gastroesophageal reflux disease at the turn of *millennium*. *World J Gastroenterol* 2003; 9(10): 2135-2136
http://www.wjgnet.com/1007-9327/9/2135.asp

## INTRODUCTION

Gastroesophageal reflux disease (GERD) has been an area of dynamic research in the Asia Pacific region in the last few years. This article outlines some of the interesting research findings.

## CHANGING EPIDEMIOLOGY OF GERD IN THE ASIA PACIFIC REGION

There was little information on GERD in the Asia Pacific region until recently. A cross-sectional survey of randomly selected adults in Singapore provided evidence that reflux-type symptoms were uncommon in the Asian population in the early 90' s[1]. Of 700 persons evaluated, only 2 % reported having heartburn more than once a month. A similar study among a random sample of 5 000 adult residents in Shanghai and Beijing showed a point prevalence of symptomatic GERD of 6 %[2].

These prevalence rates were lower than those of Western populations. Endoscopic esophagitis was also less common among Asians than their Western counterparts. One of the evidences came from a comparative study of consecutive English patients and Singaporean patients seen for upper abdominal discomfort[3]. Reflux *esophagitis* was found in 25 % of the English patients and only 6 % of the Singaporean patients ($P<0.005$). The most important risk factor for *esophagitis* was race. An endoscopic study conducted among 16 606 patients in Southern China supported the Singaporean finding, showing a similarly low frequency of endoscopic esophagitis of 4 %[4]. It also showed that esophagitis, when present, was often mild with the vast majority of cases being Los Angeles grade A or B. Severe esophagitis and large hiatal hernia were rare in Asians.

In the past few years, there has been an increase in the frequency of GERD in Asia. In a re-survey[5] of community residents who were interviewed in an earlier study in 1994[1], there was a more than 4-fold increase in the frequency of heartburn. This trend could not be explained by genetic factors *per se*. It also did not appear to be related to lifestyle changes such as smoking, alcohol consumption, or changes in body weight. Among a consecutive series of 9 000 patients who had diagnostic esophagogastroduodenoscopy, the frequency of endoscopic *esophagitis* was also increasing ($P<0.001$) while that of duodenal ulcer was decreasing ($P<0.005$), from 1992 to 1999[6].

The lower frequency of GERD in Asian populations in the early 90' s was unlikely to be solely caused by the known extrinsic risk factors. Genetic factors were probably involved as Asians have a smaller parietal cell mass and a lower acid output compared with Caucasians. The lower prevalence of *hiatus hernia* and smaller body mass index in the Asian population might also have accounted for the lower prevalence of GERD in Asia[7]. The cause of the opposing time trend of GERD and duodenal ulcer disease in Asia was unclear but might be related to the declining rate of *Helicobacter pylori* (*H pylori*) infection, or lifestyle changes, such as increased dietary fat intake.

However, similarities exist in Asian and Western patients with GERD. Interestingly, pathogenic factors of reflux esophagitis in Asians were found to include lower esophageal sphincter competence, esophageal peristaltic contractility, and esophageal acid exposure[8], which were identical to results in Western studies. Elderly Chinese patients were found to have more severe gastroesophageal reflux and esophageal lesions compared with their younger counterparts[9].

## GERD AND *HELICOBACTER PYLORI*

Although the relationship between *H pylori*, peptic ulcer disease and gastric malignancy is well established, the link between *H pylori* and GERD remains controversial.

In a systemic review of 20 studies[10], the prevalence of *H pylori* infection in subjects with GERD was significantly lower than that in those without GERD. Geographical location was a strong contributor to the heterogeneity between studies. Although the prevalence of *H pylori* in the general population was found to be higher in the East, patients from the Far East with reflux disease had a lower prevalence of *H pylori* infection than patients from Europe and North America.

Since associations do not prove causality, a more pertinent question is whether eradication of *H pylori* increases the risk of GERD. Hamada and colleagues[11] addressed this question by comparing the prevalence of new onset reflux *esophagitis* among 286 patients who underwent *H pylori* eradication therapy with that of 286 age- and disease-matched *H pylori*-positive controls who did not undergo eradication therapy. Within 3 years of follow-up, 18 % of those who had successful eradication of *H pylori* developed reflux *esophagitis* and this prevalence was higher than the 0.3 % recorded among those without therapy. Reflux esophagitis, when present was mild in most cases. The presence of *hiatal hernia* and severe corpus gastritis was closely related to the development of reflux esophagitis after *H pylori* eradication therapy. The data suggested that increased gastric acid secretion after *H pylori* eradication might only be one of the several factors responsible

for the increased risk of GERD following *H pylori* eradication. On the other hand, in a post hoc analysis of 8 prospective double blind US trials of *H pylori* therapy for patients with active duodenal ulcers or a history of duodenal ulcers[12], no difference was found in the likelihood of developing new GERD symptoms or *esophagitis* in individuals cured of *H pylori* infection compared with those with persistent infection. There was no association of *H pylori* eradication with worsening symptoms in those with preexisting GERD. The likelihood for patients who were successfully cured of their *H pylori* disease to experience a worsening of their GERD symptoms was less than that for those with persistent infection (odds ratio: 0.47, 95 % confidence interval: 0.24-0.91). However, this study had its limitations. Although the overall number of subjects included in the analysis was large, the numbers of patients in some of the subgroup analyses were small. In addition, follow-up was less than 2 months in 7 of the 8 studies included in the analysis. Nevertheless, this study suggested that *H pylori* eradication should not be withheld for fear of causing or worsening GERD. The findings in this study that patients with preexisting GERD were less likely to develop worse symptoms must not be taken to mean that patients with GERD improved after *H pylori* eradication. At present, the treatment of *H pylori* in patients with GERD remains controversial.

## TREATMENT OF GERD

In a study investigating the healthcare-seeking behavior of Asian subjects with heartburn, the decision to medicate and to seek medical advice was linked to symptom severity, but not to ethnicity[13].

The mainstay of treatment for GERD is acid suppression. Proton pump inhibitors provide the most rapid symptomatic relief and the highest healing rates for *esophagitis*. Omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole had all been demonstrated to improve GERD symptoms and to heal *esophagitis*. Interestingly, antireflux therapy has been shown to decrease bronchial hyper-responsiveness and improve pulmonary function in asthmatic patients with GERD[14].

For patients with GERD who do not like the idea of taking long-term proton pump inhibitors, Nissen fundoplication, which was modified to the laparoscopic technique in 1991, is an option. However, its association with significant morbidity and its mortalilty rate of 0.2 % prompted the birth of innovative endoscopic techniques.

The Stretta procedure, which involves radiofrequency induction of localized thermal energy to lower *esophageal sphincter* or *cardia*, has been shown in a multicentre randomized double-blind sham-controlled trial[15] to improve heartburn symptom scores and physical quality of life scores. There was no bleeds, perforations or deaths in this study. Another technique, known as the gatekeeper system, has the unique advantage of allowing addition or removal of implants as necessary and was shown to improve symptoms and decrease requirement for anti-reflux medication[16]. A third option, transesophageal endoscopic plication, resulted in significant improvement in lower *esophageal sphincter* pressure and post-procedure 24-hour *esophageal* pH[17]. Finally, endoscopic implantation of inert materials such as Enteryx has been shown to improve symptom scores, quality of life and 24-hour *esophageal* pH, with reduction in the use of acid suppression 6 months after treatment[18].

## REFERENCES


1 **Ho KY**, Kang JY, Seow A. Prevalence of gastrointestinal symptoms in a multi-racial Asian population, with particular reference to reflux-type symptoms. *Am J Gastroenterol* 1998; **93**: 1816-1822

2 **Pan GZ**, Xu GM, Ke MY, Han SM, Guo HP, Li ZS, Fang XC, Zou DW, Lu SC, Liu J. Epidemiological study of symptomatic gastroesophageal reflux disease in China: Beijing and Shanghai. *Chin J Dig Dis* 2000; **1**: 2-8

3 **Kang JY**, Ho KY. Different prevalences of reflux oesophagitis and hiatus hernia among dyspeptic patients in England and Singapore. *Eur J Gastroenterol Hepatol* 1999; **11**: 845-850

4 **Wong WM**, Lam SK, Hui WM, Lai KC, Chan CK, Hu WH, Xia HH, Hui CK, Yuen MF, Chan AO, Wong BC. Long-term prospective follow-up of endoscopic oesophagitis in southern Chinese - prevalence and spectrum of the disease. *Aliment Pharmacol Ther* 2002; **16**: 2037-2042

5 **Ho KY**, Lim LS, Goh WT, Lee JMJ. The prevalence of gastrooesophageal reflux has increased in Asia: A longitudinal study in the community. *J Gastro Hepatol* 2001; **16**(Suppl): A132

6 **Ho KY**, Gwee KA, Yeoh KG, Lim SG, Kang JY. Increasing frequency of reflux esophagitis in Asian patients. *Gastroenterology* 2000; **118**: A5704

7 **Ho KY.** Gastroesophageal reflux disease is uncommon in Asia: evidence and possible explanations. *World J Gastroenterol* 1999; **5**: 4-6

8 **Ho KY**, Kang JY. Reflux esophagitis patients in Singapore have motor and acid exposure abnormalities similar to patients in the Western hemisphere. *Am J Gastroenterol* 1999; **94**: 1186-1191

9 **Huang X**, Zhu HM, Deng CZ, Porro GB, Sangaletti O, Pace F. Gastroesophageal reflux: the features in elderly patients. *World J Gastroenterol* 1999; **5**: 421-423

10 **Raghunath A**, Pali A, Hungin S, Wooff D, Childs S. Prevalence of *Helicobacter pylori* in patients with gastro-oesophageal reflux disease: systematic review. *BMJ* 2003; **326**: 737

11 **Hamada H**, Haruma K, Mihara M, Kamada T, Yoshihara M, Sumii K, Kajiyama G, Kawanishi M. High incidence of reflux oesophagitis after eradication therapy for *Helicobacter pylori*: impacts of hiatal hernia and corpus gastritis**.** *Aliment Pharmacol Ther* 2000; **14**: 729-735

12 **Laine L**, Sugg J. Effect of *Helicobacter pylori* eradication on development of erosive esophagitis and gastroesophageal reflux disease symptoms: A post hoc analysis of eight double blind prospective studies. *Am J Gastroenterol* 2002; **97**: 2992-2997

13 **Ho KY**, Kang JY, Seow A. Patterns of consultation and treatment for heartburn: findings from a Singaporean community survey. *Aliment Pharmacol Ther* 1999; **13**: 1029-1033

14 **Jiang SP**, Liang RY, Zeng ZY, Liu QL, Liang YK, Li JG. Effects of antireflux treatment on bronchial hyper-responsiveness and lung function in asthmatic patients with gastroesophageal reflux disease. *World J Gastroenterol* 2003; **9**: 1123-1125

15 **Corley DA**, Katz P, Wo J, Stefan A, Patti m, Rothstein RI, Edmundowicz SA, Kline M, Mason R, Wolfe MM. Radiofrequency energy to the gastroesophageal junction for treatment of GERD (the Stretta procedure): A randomized sham-controlled multicentre clinical trial. *Gastrointest Endos* 2002; **55**: AB100

16 **Fockens P**, Bruno MJ, Hirsch DP, Lei A, Boeckxstaens GE, Tytgat GN. Endoscopic augmentation of the lower esophageal spincter: Pilot study of the gatekeeper reflux repair system in patients with GERD. *Gastrointest Endosc* 2002; **55**: AB257

17 **Swain CP**, Park P, Kjellin T, Gong F, Kadairkamanathan SS, Appleyard M. Endoscopic gastroplasty for gastroesophageal reflux disease. *Gut* 2002; **46**(Sppul): TH3

18 **Lehman GA**, Aisenberg J, Cohen LB, Deviere J, Ganz RA, Haber GB, Hagenmuller F, Johnson DA, Neuhaus H, Ortner MAE, Eters JH, Ponchon T, Rey JF, Hieston K, Silverman D, Visor J. Enteryx solution: A minimally invasive injectable treatment for GERD. International multicentre trial results. *Gastrointest Endosc* 2002; **55**: AB101


**Edited by** Xu XQ

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2137-2139
World Journal of Gastroenterology
 ISSN 1007-9327

• REVIEW •

# *Helicobacter pylori* infection and micronutrient deficiencies

Javed Yakoob, Wasim Jafri, Shahab Abid

**Javed Yakoob, Wasim Jafri, Shahab Abid,** Section of Gastroenterology, Department of Medicine, Aga Khan University Hospital, Stadium Road, Karachi, Pakistan
**Correspondence to:** Javed Yakoob, MBBS, PhD. Section of Gastroenterology, Department of Medicine, Agha Khan University Hospital, Stadium Road, Karachi-74800, Pakistan. yakoobjaved@hotmail.com
**Telephone:** +92-21-48594661 **Fax:** +92-21-4934294
**Received:** 2003-06-05 **Accepted:** 2003-08-19

## Abstract

It is known that deficiencies of micronutrients due to infections increase morbidity and mortality. This phenomenon depicts itself conspicuously in developing countries. Deficiencies of iron, vitamins A, E, C, B12, *etc* are widely prevalent among populations living in the third world countries. *Helicobacter pylori* (*H pylori*) infection has a high prevalence throughout the world. Deficiencies of several micronutrients due to *H pylori* infection may be concomitantly present and vary from subtle sub-clinical states to severe clinical disorders. These essential trace elements/ micronutrients are involved in host defense mechanisms, maintaining epithelial cell integrity, glycoprotein synthesis, transport mechanisms, myocardial contractility, brain development, cholesterol and glucose metabolism. In this paper *H pylori* infection in associaed with various micronutrients deficiencies is briefly reviewed.

Yakoob J, Jafri W, Abid S. *Helicobacter pylori* infection and micronutrient deficiencies. *World J Gastroenterol* 2003; 9 (10): 2137-2139
http://www.wjgnet.com/1007-9327/9/2137.asp

## INTRODUCTION

*Helicobacter pylori* (*H pylori*) is a gram negative, microaerophilic human pathogen which colonizes the gastric mucosa. Infection with *H pylori* leads to gastritis and is associated with the development of peptic ulcer disease, gastric carcinoma and lymphoma[1]. *H pylori* may be acquired at any age, and the infection persists for years once acquired. The age specific prevalence of *H pylori* infection is higher in developing countries and particularly in lower socioeconomic group[2]. In developing countries *H pylori* infection occurs early in life, and hypochlorhydria commonly seen in the malnourished predisposes them to repeated gastrointestinal infection, persistent diarrhea and malnutrition[3]. *H pylori* infection usually causes both acute and chronic inflammatory cell infiltration, leading to an increase in reactive oxygen species (ROS) which have been shown to accumulate in *H pylori* gastritis[4]. Excessive production of reactive oxygen metabolites (ROMs) by phagocytic cells is thought to contribute to mucosal lesions produced by *H pylori* infection. These are highly reactive compounds capable of combining with DNA in a number of potentially genotoxic ways[5]. Reactive oxygen species can react with the lipid-bilayers releasing peroxidation products such as malondialdehyde. These processes could lead to alterations in the structure of DNA facilitating mutations and carcinogenesis.

Nutrition is a critical determinant of the outcome of host microbe interactions through a modulation of the immune response. "Micronutrient" or "trace elements" are generally defined as constituting less than 0.01 % of body mass and are needed in much smaller amounts. Trace minerals and vitamins are essential for life and include iron (Fe), zinc (Zn), copper (Cu), nickel (Ni), *etc*. They act as essential cofactors of enzymes and as organizers of the molecular structures of the cell, e.g. mitochondria and its membrane. Deficiencies of micronutrients influence immune homeostasis and thus affect infection-related morbidity and mortality. Micronutrients like β carotene, vitamin C, selenium, copper and others are powerful antioxidants and have a significant impact on infection related morbidity in humans. Subclinical deficiencies are known to impair biological and immune functions in the host. Antioxidants play a part in gastric mucosal defense by protecting against damage caused by excessive oxygen derived free radicals. β-carotene and α-tocopherol are lipophilic and have been shown to suppress the oxidation induced by either lipophilic or hydrophilic radical species[6]. In addition, they could act as anti-carcinogens through their ability to prevent the formation of N-nitrosamines which are important in the development of gastric carcinoma[7]. These vitamins are the major oxidant scavengers in biomembranes in contrast to vitamin C, which is mainly responsible for scavenging free radicals in the aqueous phase. However, compensatory mechanisms may become defective while gastric inflammation develops from normal to chronic gastritis and finally to gastric atrophy/intestinal metaplasia, perhaps due to reduced infiltration of inflammatory cells, loss of gastric gland cells and increased ROM production.

## IRON

Iron deficiency anemia affects all groups of the under privileged population in most developing countries. Iron is an essential growth factor for *H pylori*, which contains Fe in their outer membrane protein and a system for intracellular storage of iron, consisting of ferritin like molecules pfr and napA[8]. Patients with *H pylori* associated iron deficiency anemia (IDA) would have involvement of both antral and corporal mucosa when compared with controls (90 % *vs* 42.7 %; $P$=0.0001)[9]. Iron deficiency anemia associated with *H pylori* gastritis is characterized by a concomitant increase in median intragastric pH value >3 and lowering of intragastric concentrations of ascorbic acid. A significant percentage (43 %) of *H pylori* positive IDA patients presented atrophic changes in the gastric body, and the remaining had a superficial gastritis extended to the fundic mucosa, in contrast with *H pylori* positive controls[10]. *H pylori* eradication has also been shown to improve the absorption of other nutrients besides iron, and produce more rapid and complete clinical responses in patients with iron deficiency anemia[11].

## COPPER

Copper is involved in the function of several enzymes. It is required for infant growth, host defense mechanisms, bone strength, red and white cell maturation, iron transport, *etc*. Acquired deficiency is mainly seen in infants. However, it has been diagnosed also in malnourished children and adults[12]. A

gene, copA, associated with copper transport, has been isolated from *H pylori* UA802. The adenosine triphosphatase-derived copper-transporting mechanism is employed by various *H pylori* strains[13]. As a cofactor in various redox enzymes and an essential trace metal required for the synthesis of metalloproteins, copper plays a role in the pathogenesis of *H pylori*. *H pylori* has a differential effect on some gastric mucosal scavenger enzymes of ROMs, namely mitochondrial and cytoplasmic superoxide dismutases reflected by a large increase in the cytokine inducible manganese superoxide dismutase and a decrease in the constitutive copper/zinc superoxide dismutase[14].

## VITAMIN B12

The mechanisms of vitamin B12 malabsorption caused by *H pylori* infection are unclear but following are the possibilities: a) The diminished acid secretion in *H pylori* induced gastritis may lead to a failure of critical splitting of vitamin B12 from food binders and its subsequent transfer to R binder in the stomach. b) A secretory dysfunction of the intrinsic factor. c) Decreased secretion of ascorbic acid from the gastric mucosa and increased gastric pH[15,16]. Annibale *et al*, studied the prevalence of *H pylori* infection in pernicious anemia patients and have demonstrated that almost two thirds of pernicious anemia patients had evidence of *H pylori* but only those with an active *H pylori* infection had distinctively functional and histological features[17]. These findings support the hypothesis that *H pylori* infection could play a triggering role in a subgroup of pernicious anemia patients, and suggest the possibility that *H pylori* is involved in the early stages of PA that lead to severe corpus atrophy. The later progress of gastritis seems to be dependent on factors other than *H pylori*, most likely "autoimmune" mechanisms[18]. *H pylori* may also be involved in the pathogenesis of pernicious anemia via antigenic mimicry as antibodies directed against the H+, K+- adenosine-triphosphate protein that has been found in high numbers of patients with *H pylori* infection[19]. Food cobalamin malabsorption may occur without gastric atrophy or achlorhydria. Malabsorption can respond to antibiotics, but only in some patients[20].

## VITAMIN A

Vitamin A has effects on important determinants of immune function and epithelial cell integrity such as gene expression, cellular proliferation and differentiation and also glycoprotein synthesis. Loss of integrity of the epithelial lining of mucus membranes in a vitamin A deficient state could explain its close association with increased susceptibility to infections particularly of gastrointestinal, respiratory and genitourinary tracts especially in children and pregnant women[21]. Even mild or subclinical vitamin A deficiency could induce keratinizing metaplasia of the epithelium and depletes goblet cells from mucosal linings thus causing xerosis of the membrane[22]. The xerotic surfaces form potential sites for increased bacterial adherence thus leading to bacterial colonization. The antimicrobial enzyme lysozyme depends on vitamin A for its synthesis. A decrease in T cell number with no change in proliferative activity has been demonstrated in children suffering from mild xerophthalmia due to vitamin A deficiency. *H pylori* infection and low β-carotene in plasma contribute to the increased risk of gastric atrophy, indicating that *H pylori* infection might be associated with low plasma β-carotene[23].

## VITAMIN E

Vitamin E is composed of a group of compounds termed tocopherols and tocotrienols. α-tocopherol is the major active form in the human body, accounting for 95 % of vitamin E and is the most effective lipid soluble anti-oxidant in biomembranes. It acts as the major chain breaking antioxidant and is able to interfere with the propagation of lipid peroxidation. It plays an immune modulatory part and is capable of increasing natural killer cell activity. Concentrations of α-tocopherol in *H pylori* negative subjects were higher in the corpus than in the antrum or duodenum[6]. This distribution of α-tocopherol is reversed in the presence of antral *H pylori* infection. These findings may reflect a mobilization of antioxidant defenses to the sites of maximal inflammation in the stomach.

## VITAMIN C

Vitamin C exists as ascorbic acid (AA) or dehydroascorbic acid. The stomach secretes ascorbic acid across the gastric mucosa into the gastric juice against a concentration gradient. Ascorbic acid is the reduced form of the vitamin and can act as a potent antioxidant, and is able to scavenge ROS in gastric mucosa. This has been proposed as one means by which it exerts an anti-carcinogenic effect. Ascorbic acid may also prevent formation of N-nitroso compounds in gastric juice by scavenging nitrite. It has been observed that diets poor in foods containing AA were associated with an increased risk of gastric cancer[24]. Wei-cheng *et al* showed that presence of *H pylori* infection at the baseline and smoking were strongly associated with progression to dysplasia or gastric cancer, whereas the risk of progression was decreased by 80 % among subjects with baseline ascorbic acid levels in the highest tertile compared with those in the lowest tertile[25]. A number of studies have demonstrated that gastric juice but not gastric mucosal AA levels were reduced in the presence of *H pylori* gastritis and that successful eradication restored the juice/plasma AA ratio[23, 26]. The lower plasma AA concentration in *H pylori* positive subjects could be due to reduced bioavailability, active secretion from plasma to gastric juice in attempts to restore the positive gastric juice/plasma ratio or both[27]. In some studies no difference was found in the gastric juice AA concentration between patients with antral-limited gastritis and *H pylori* negative healthy controls, while lower AA levels were observed in patients with gastric body involvement and increased pH[28]. These observations suggest that AA, which is very unstable in the presence of increased pH, is converted to the less active form of dehydroascorbic acid, in the presence of gastric damage extending to the corporal mucosa with consequent hypochlorhydria[29,30]. It has been demonstrated that eradication of *H pylori* could lead to a reduction in ROS activity in gastric mucosa[31]. Ascorbic acid has also been shown to inhibit *H pylori* urease activity and growth *in vitro*[32]. *H pylori* infection associated low gastric juice-ascorbic acid levels return to normal after successful eradication of the infection[33]. A study of antibiotic treatment failure showed that compliant patients in whom *H pylori* infection did not clear had lower baseline plasma and gastric juice vitamin C concentrations than patients whose infection was cleared[26].

In developing countries micronutrient deficiencies facilitated by *H pylori* infection are a clinical and public health problem. It is essential to define the precise extent of the problem. Several micro and macronutrient deficiencies could be concomitantly present in the population with several other deficits. They will require correction to achieve significant effects on the over all health of the population.

## REFERENCES

1 **Huang JQ**, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between *Helicobacter pylori* seropositivity and gastric cancer. *Gastroenterology* 1998; **114**: 1169-1179

2 **Graham DY**. *Helicobacter pylori*: its epidemiology and its role in duodenal ulcer disease. *J Gastroenterol Hepatol* 1991; **6**: 105-113
3 **Gilman RH**, Partanen R, Brown KH, Spira WM, Khanam S, Greenberg B, Bloom SR, Ali A. Decreased gastric acid secretion and bacterial colonization of the stomach in severely malnourished Bangladeshi children. *Gastroenterology* 1988; **94**: 1308-1314
4 **Farinati F**, Della Libera G, Cardin R, Molari A, Pelabani M, Rugg M, DiMario F, Naccarato R. Gastric antioxidant, nitrites and mucosal lipoperoxidation in chronic gastritis and *Helicobacter pylori* infection. *J Clin Gastroenterol* 1996; **22**: 275-281
5 **Guyton KZ**, Kensler TW. Oxidative mechanisms in carcinogenesis. *Br Med Bullet* 1993; **49**: 523-544
6 **Sies H**, Stahl W. Vitamin E and C, β-carotene and other carotenoids as antioxidants. *Am J Clin Nutr* 1995; **62**: 1315S-1321S
7 **Hwang H**, Dwyer J, Russel RM. *H pylori* infection, food preservation and gastric cancer risk: are there new roles for preventive factors? *Nutr Rev* 1994; **52**: 75-83
8 **Dundon WG**, Polenghi A, DelGuidice G, Rappuoli R, Montecucco C. Neutrophil-activating protein (HP-NAP) versus ferritin (Pfr): comparison of synthesis in *Helicobacter pylori*. *FEMS Microbiol Lett* 2001; **199**: 143-149
9 **Annibale B**, Capurso G, Delle Fava G. Consequences of *Helicobacter pylori* infection on the absorption of micronutrients. *Digest Liver Dis* 2002; **34**: S72-77
10 **Annibale B**, Capurso G, Lahner E, Passi S, Ricci R, Maggio F, Delle Fava G. Concomitant alterations in intragastric pH and ascorbic acid concentration in patients with *Helicobacter pylori* gastritis and associated iron deficiency anemia. *Gut* 2003; **52**: 496-501
11 **Annibale B**, Marignani M, Monarca B, Antonelli G, Marcheggiano A, Martino G, Mandelli F, Caprilli R, Delle Fave G. Reversal of Iron deficiency anemia after *Helicobacter pylori* eradication in patients with asymptomatic gastritis. *Ann Intern Med* 1999; **131**: 668-672
12 **Olivares M**, Uauy R. Copper as an essential nutrition. *Am J Clin Nutr* 1996; **63**: 791S-796S
13 **Ge Z**, Jiang Q, Taylor DE. Conservation and diversity of the *Helicobacter pylori* copper-transporting ATPase gene (copA) sequence among *Helicobacter species* and *Campylobacter* species detected by PCR and RFLP. *Helicobacter* 1996; **1**: 112-117
14 **Gotz JM**, Thio JL, Verspaget HW, Offerhaus GJ, Biemond I, Lamers CB, Veenendaal RA. Treatment of *Helicobacter pylori* infection favorably affects gastric mucosal superoxide dismutases. *Gut* 1997; **40**: 591-596
15 **Del Corral A**, Carmel R. Transfer of cobalamin from the cobalamin-binding protein of egg-yolk to R binder of human saliva and gastric juice. *Gastroenterology* 1990; **98**: 1460-1466
16 **Appelmelk BJ**, Simoons-Smit I, Negrrini R, Moran AP, Aspinall GO, Forte JG, DeVries T, Quan H, Verboom T, Maaskantt JJ, Ghiara P, Kuipers EJ, Bloemena E, Tadema TM, Townsend RR, Tyagarajan K, Crothers JM Jr, Monteiro MA, Savio A, De Graaff J. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. *Infect Immun* 1996; **64**: 2031-2040
17 **Annibale B**, Lahner E, Bordi C, Martino G, Caruana P, Grossi C, Negrini R, Delle Fava G. Role of *Helicobacter pylori* infection in pernicious anemia. *Digest Liver Dis* 2000; **32**: 756-762
18 **Varis O**, Valle J, Siurala M. Is *Helicobacter pylori* involved in the pathogenesis of the gastritis characteristic of pernicious anemia? Comparison between pernicious anemia relatives and duodenal ulcer relatives. *Scand J Gastroenterol* 1993; **28**: 705-708
19 **Claeys D**, Faller G, Appelmelk BJ, Negrini R, Kirchner T. The gastric $H^+K^+$-ATPase is a major autoantigen in chronic *Helicobacter pylori* gastritis with body mucosa atrophy. *Gastroenterology* 1998; **115**: 340-347
20 **Cohen H**, Weinstein WM, Carmel R. Heterogeneity of gastric histology and function in food cobalamin malabsorption: absence of atrophic gastritis and achlorhydria in some patients with severe malabsorption. *Gut* 2000; **47**: 638-645
21 **Christian P**, Schulze K, Stolzfus RJ, West KP Jr. Hyporetinolemia, illness symptoms and acute phase protein response in pregnant women with and without night blindness. *Am J Clin Nutr* 1998; **67**: 1237-1243
22 **Reddy V**, Rao VM, Jyothi A, Reddy M. Conjunctival impression cytology for assessment of vitamin A status. *Am J Clin Nutr* 1989; **50**: 814-817
23 **Tsugane S**, Kabuto M, Imai H, Goy F, Tai Y, Hanaoka T, Sugano K, Watanabe S. *Helicobacter pylori*, dietary factors and atrophic gastritis in five Japanese populations with different gastric cancer mortality. *Cancer Causes Control* 1993; 4: 297-305
24 **Block G**. Epidemiologic evidence regarding vitamin C and cancer. *Am J Clin Nutr* 1991; **54**: S1310-S1314
25 **Weicheng Y**, Zhang L, Gail MH. Gastric dysplasia and gastric cancer *Helicobacter pylori*, serum vitamin C and other risk factors. *J Natl Cancer Instit* 2000; **92**: 1607-1611
26 **Ruiz B**, Rood JC, Fontham ETH, Malcom GT, Hunter FM, Sobhan M, Johnson WD, Correa P. Vitamin C concentration in gastric juice before and after anti- *Helicobacter pylori* treatment. *Am J Gastroenterol* 1994; **89**: 533-539
27 **Woodward M**, Tunstall-Pedoe H, McColl KEL. *Helicobacter pylori* infection reduces systematic availability of dietary vitamin C. *Eur J Gastroenterol Hepatol* 2001; **13**: 233-237
28 **Zhang ZW**, Patchett SE, Perrett D, Katelaris PH, Domizio P, Farthing MJG. The relationship between gastric vitamin C concentrations, mucosal histology and CagA seropositivity in the human stomach. *Gut* 1998; **43**: 322-326
29 **Sobala GM**, Schorah CJ, Shires S, Lynch DA, Gallacher B, Dixon MF, Axon AT. Effect of eradication of *Helicobacter pylori* on gastric juice ascorbic acid concentrations. *Gut* 1993; **34**: 1038-1041
30 **Waring AJ**, Drake IM, Schorah CJ, White KL, Lynch DA, Axon AT, Dixon MF. Ascorbic acid and total vitamin C concentrations in plasma gastric juice, and gastrointestinal mucosa: effects of gastritis and oral supplementation. *Gut* 1996; **38**: 171-176
31 **Goodman KJ**, Correa P, Tengana Aux HJ, Delany JP, Collazos T. Nutritional factors and *Helicobacter pylori* infection in Colombian children. *J Paedr Gastroentrol Nutr* 1997; **25**: 507-515
32 **Nilius M**, Bode G, Lehnhardt G, Malfertheiner P. *In vitro* inhibition of *Helicobacter pylori urease*: Biochemical and ultrastructural analysis. *Eur J Clin Invest* 1991; **21**: 551-557
33 **Phull PS**, Green CJ, Jacyna MR. A radical view of the stomach: The role of oxygen-derived free radical and anti-oxidants in gastroduodenal disease. *Eur J Gastroenterol Hepatol* 1995; **7**: 265-274


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2140-2142
World Journal of Gastroenterology
 ISSN 1007-9327

• REVIEW •

# Pathophysiological aspects of diverticular disease of colon and role of large bowel motility

Gabrio Bassotti, Fabio Chistolini, Antonio Morelli

**Gabrio Bassotti, Fabio Chistolini, Antonio Morelli,** Clinica di Gastroenterologia ed Epatologia, Dipartimento di Medicina Clinica e Sperimentale, Università di Perugia, Italy
**Correspondence to:** Dr. Gabrio Bassotti, Strada del Cimitero, 2/a, 06131 San Marco (Perugia), Italy. gabassot@tin.it
**Telephone:** +39-75-5847570 **Fax:** +39-75-5847570
**Received:** 2003-06-16 **Accepted:** 2003-07-14

## Abstract

Colonic diverticular disease (diverticulosis) is one of the most common gastrointestinal disorders in Western countries. This disorder is strictly related to aging and fibre intake, and still bears a discrete amount of morbidity. Numerous etiological co-factors have to date been implicated in the pathogenesis of the disease, yet the supporting evidence is still far from absolute. The present review considers the pathophysiology of colonic diverticular disease, with a special emphasis on factors related to abnormal colonic motility.

Bassotti G, Chistolini F, Morelli A. Pathophysiological aspects of diverticular disease of colon and role of large bowel motility. *World J Gastroenterol* 2003; 9(10): 2140-2142
http://www.wjgnet.com/1007-9327/9/2140.asp

## INTRODUCTION

Diverticular disease of the colon (diverticulosis) 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.

It is commonly thought that an altered motility of the large bowel may have a major pathophysiological role, even though it is probable that multiple factors (anatomic features intrinsic to the colon, alterations in colonic wall with aging, dietary fiber, motor dysfunction, abnormal intraluminal pressures, and possibly genetic influences) interact in ill-defined relationships to play a greater or lesser role in the genesis of colonic diverticul[3,4].

This article will review the pathogenetic factors of colonic diverticular disease (diverticulosis), with an emphasis on those related to large bowel motility.

## ANATOMIC FACTORS

Diverticul are usually found in the left (sigmoid, descending) colon on either mesenteric side of the antimesenteric teniae in Western countries[5], and occur at weak points in the circular muscle layer, where the blood vessels supply the mucosa[6], suggesting that increased intraluminal pressure might play a role in their formation. The presence of right-sided diverticula is conversely more frequently seen in Eastern populations[7]. An impaired structure of the colonic wall has been described in patients with diverticular disease. In fact, both *in vitro*[8] and *in vivo*[9] studies showed that these patients displayed an increased colonic compliance in the affected segments. This might be due to a pathological accentuation of the physiological differences among colonic segments[10], that has been ascribed to qualitative than quantitative changes in collagen[11], since the content of the latter remained unaltered, with respect to controls, in the entire colonic wall[12] and in the muscle layers[13]. However, the available studies measured colonic compliance by means of latex balloons that constitute a suboptimal method for this measurement. More precise studies with up to date systems (i.e., polyethylene bags, which intrinsic compliance is negligible) are needed. Colonic diverticula were also prevalent in young patients with connective tissue disease[14,15].

Previous studies showed that a consistent anatomic finding in colonic diverticular disease was the presence of thickening of the bowel wall muscle layers[16,17]. However, no evidence of hyperplasia or hypertrophy of the muscle cells has been found (although this finding has been challenged[18]). There is an enormous increase in elastin deposition in the teniae, that leads to shortening of this layer with thickening of the circular muscle layer and produces the concertina-like folds in the inner muscle layer[12,19]. This deformity (also called myochosis) would narrow the lumen, allowing muscle contractions to obliterate the lumen and divide the bowel into isolated segments[4].

Unfortunately, available neurophysiopathological data are remarkably few in colonic diverticulosis. A study investigating the myenteric plexus in such patients was unable to demonstrate any morphological abnormality[20]. Another more recent study showed that cholinergic nerves were dominant in the left-sided diverticular colon, and that a decreased action of non-adrenergic non-cholinergic nerves by nitric oxide might be related to the high intracolonic pressures by colonic segmentation observed in such portions of the viscus[21]. Moreover, *in vitro* cholinergic stimulation of colons of patients with diverticular disease yielded increased low frequency and uncoordinated smooth muscle contractions in response to acetylcholine, compared with controls[22].

## AGING

Colonic diverticular disease showed a striking correlation with advancing age[23], and it was estimated that 50 % of the Western population approximately 70 years of age were affected[24], an important consequence of aging was that the properties of the colon wall changed with a decrease of the tensile strength[25]. The reason of this decrease is unknown, but it might be related to an increased cross-linking of collagen fibrils with age, which also became smaller, more numerous and more tightly packed in the left colon[9]. These changes seemed to be accentuated in diverticular disease[26]. Moreover, since elastin deposition continued throughout life in all layers of the colonic wall[27] (with predominance in the teniae in diverticular disease), the fibers lost some mechanical properties and became more distensible in diverticulosis[28].

## DIET

There is substantial evidences that colonic diverticulosis was related to civilization, industrialization, and a Western lifestyle and diet[29]. Dietary factors, and in particular low fiber intake,

have been considered as main pathogenic factors[30,31], as also shown by the observations that populations moving from rural to urban environments displayed an increased prevalence of the disease[32], and that vegetarians had a lower incidence of diverticular disease of the colon[33]. In addition, an interesting observation in rats showed that the animals whose mothers were fed on a fiber-deficient diet had an increased incidence of colonic diverticulosis[34].

The "protective" action of dietary fiber would make the stools bulkier, thereby increasing the colon size and decreasing intraluminal pressures (since according to Laplace's law, the pressure required to distend the wall is the greatest where the radius is small)[35], and reducing colonic transit time[36]. These hypotheses have been indirectly confirmed by the observations that typical African diets yielded rapid colonic transit times, bulkier stools and no need to strain[37], whereas those of Europeans resulted in almost double colonic transit times[38] and firmer and more viscous stools that increased intraluminal pressures and the need to strain. Of course, other dietary factors are likely to play a role in this area, as shown by the fact that a Western-type diet was implicated in the increased proline intake from the gut, that led to elastosis of the sigmoid colon[39], and that the prevalence of right-sided diverticular disease displayeds a strongly positive association with past meat consumption frequency[40].

## COLONIC MOTILITY

Abnormal intraluminal pressure and disordered colonic motility have been implicated as pathogenic factors in diverticulosis. The evidence for this belief (as discussed below) is far from absolute, however, there are several data suggesting that abnormal colonic motility may be considered as an important pathophysiologic mechanism. It is worth noting that most studies on colonic motility and myoelectrical activity were biased by poor patient selection, small numbers of patients recruited, heterogeneity of clinical conditions, recording techniques (only the rectosigmoid or even rectal motor activity was often obtained) and duration of recording periods (at best, less than two hours, whereas colonic motility displayed wide and important fluctuations around-the-clock[41]).

Concerning right-sided colonic diverticular disease, there is paucity of studies facing the motor aspects. Two investigations, featuring very brief recording periods, reported that patients with diverticulosis displayed higher motility indices with respect to controls, both basally and after pharmacologic stimulation[42, 43]. An increased motor activity was also observed in the segment with diverticula with respect to the non affected sigmoid colon.

Looking at studies in patients with "traditional" diverticulosis, most manometric investigations, as already stated, were carried out in the rectum and (at best) very distal sigmoid area. Therefore, it was possible that the portion of the viscus harboring diverticula might have not been studied at all. This might justify the fact that increased pressures were documented[44-47] or not found[48-51] with respect to controls and in response to pharmacological stimulation. Similar contrasting results were reported from myolectrical studies of the rectosigmoid area[52, 53]. However, more recent studies, carried out in the true sigmoid and descending colon (and, therefore, investigating colonic segments actually bearing diverticula) are available, which showed an increased motor activity in such patients (both symptomatic and asymptomatic) with respect to controls[54,55]. This abnormal motility was reduced following surgery[56]. More recently, we investigated colonic motility in patients with symptomatic uncomplicated diverticular disease, by means of a 24-hour manometric technique that allows studying most of the viscus, its daily fluctuations[57], and detection of the motor equivalents of mass movements, the so-called high-amplitude propagated contractions (HAPC)[58]. With respect to controls, patients with diverticulosis displayed a significantly overall increase of daily motor activity, except in the transverse colon (the segment not involved by diverticula), and of propulsive activity[59]. Interestingly, patients had several retropropagated HAPC (never observed in controls), and the motor activity of the affected segments, especially the sigmoid, was significantly higher than that of the unaffected ones (the transverse).

All the above observations seem to support a discrete role of colonic motility (with the probable concourse of other factors) as a pathophysiological mechanism in diverticular disease. Colonic motility is influenced by the aging process, as shown by the decrease of HAPC frequency with age, whereas segmental contractile activity increases[60]. Anatomical studies in experimental animal models showed that these changes might be related to aging of colonic smooth muscle[61].

The muscle thickening observed in affected bowel segments was thought to be obstructive, and to contribute to the delayed transit of feces[18]. Studies with intracolonic displacement tools suggested that an accentuation of segmentary motor activity (as observed in diverticular disease) might abolish oro-aboral progression of contents[62], thereby facilitating retropropulsion and drying of the semiliquid fecal matter. Moreover, reverse peristalsis, as observed in animal studies, might be a general response to distal obstruction associated with a narrowed terminal colon segment[63]. We have shown that a similar mechanism is present in diverticulosis, and this might have some pathophysiological relevance (for instance, a local nondominant pacemaker might take over in the "spastic" region, initiating an oral spreading of contractions along the less active proximal colonic segments).

## CONCLUSIONS

Although many evidences suggest that colonic diverticular disease is related to low-residue diet, the scarce effectiveness of dietary manipulations[64] and the complex relationships with other factors, among which abnormal colonic motility might play an important role, still make this disease a fascinating pathophysiological puzzle. Further studies are needed to understand the intrinsic mechanisms better and possibly, to give us useful insights for a better and more targeted therapeutic approach.

## REFERENCES


1 **Smith AN**. Diverticular disease of the colon. In Phillips SF, Pemberton JH, Shorter RG, eds. The large intestine: physiology, pathophysiology, and disease. *Raven Press, New York* 1991: 549-577

2 **Torsoli A**, Inoue M, Manousos O, Smith A, Van Steensel CJ. Diverticular disease of the colon: data relevant to management. *Gastroenterol Int* 1991; **4**: 3-20

3 **Simpson J**, Scholefield JH, Spiller RC. Pathogenesis of colonic diverticula. *Br J Surg* 2002; **89**: 546-554

4 **Simmang CL**, Shires GT. Diverticular disease of the colon. In Feldman M, Friedman LS, Sleisenger MH, eds. Sleisenger & Fordtran's gastrointestinal and liver disease, 7th edition. *Saunders, Philadelphia* 2002: 2100-2112

5 **Almy TP**, Howell DA. Diverticular disease of the colon. *N Engl J Med* 1980; **302**: 324-331

6 **Slack WW**. The anatomy, pathology, and some clinical features of diverticulitis of the colon. *Br J Surg* 1962; **50**: 185-190

7 **Chia JG**, Wilde CC, Ngoi SS, Goh PM, Ong CL. Trends of diverticular disease of the large bowel in a newly developed country. *Dis Colon Rectum* 1991; **34**: 498-501

8 **Smith AN**, Shepherd J, Eastwood MA. Pressure changes after balloon distension of the colon wall in diverticular disease. *Gut* 1981; **22**: 841-844

9 **Parks TG**. Rectal and colonic studies after resection of the sigmoid for diverticular disease. *Gut* 1970; **11**: 121-125
10 **Ford MJ**, Camilleri M, Wiste JA, Hanson RB. Differences in colonic tone and phasic response to a meal in the transverse and sigmoid human colon. *Gut* 1995; **37**: 264-269
11 **Bode MK**, Karttunen TJ, Makela J, Risteli L, Risteli J. Type I and III collagens in human colon cancer and diverticulosis. *Scand J Gastroenterol* 2000; **35**: 747-752
12 **Wess L**, Eastwood MA, Wess TJ, Busuttil A, Miller A. Cross linking of collagen is increased in colonic diverticulosis. *Gut* 1995; **37**: 91-94
13 **Whiteway J**, Morson BC. Elastosis in diverticular disease of the sigmoid colon. *Gut* 1985; **26**: 258-266
14 **Beighton PH**, Murdoch JL, Votteler T. Gastrointestinal complications of the Ehlers-Danlos Syndrome. *Gut* 1969; **10**: 1004-1008
15 **Eliashar R**, Eliashar R, Sichel JY, Biron A, Dano I. Multiple gastrointestinal complications in Marfan syndrome. *Postgrad Med J* 1998; **74**: 495-497
16 **Morson BC**. The muscular abnormality in diverticular disease of the sigmoid colon. *Br J Radiol* 1963; **36**: 385-392
17 **Hughes LE**. Postmortem survey of diverticular disease of the colon. II. The muscular abnormality of the sigmoid colon. *Gut* 1969; **10**: 344-351
18 **Raguse T**, Bubenzer J. Functional and morphological studies on diverticulosis of the large bowel. *Chir Forum Exp Klin Forsch* 1979; **3**: 138-143
19 **Whiteway J**, Morson BC. Pathology of the ageing – diverticular disease. *Clin Gastroenterol* 1985; **14**: 829-846
20 **Vuong NP**, Sezeur A, Balaton A, Malafosse M, Camilleri JP. Myenteric plexuses and colonic diverticulosis: results of a histological study. *Gastroenterol Clin Biol* 1985; **9**: 434-436
21 **Tomita R**, Fujisaki S, Tanjoh K, Fukuzawa M. Role of nitric oxide in the left-sided colon of patients with diverticular disease. *Hepatologastroenterology* 2000; **47**: 692-696
22 **Huizinga JD**, Waterfall WE, Stern HS. Abnormal response to cholinergic stimulation in the circular muscle layer of the human colon in diverticular disease. *Scand J Gastroenterol* 1999; **34**:363-368
23 **Cheskin LJ**, Bohlman M, Schuster MM. Diverticular disease of the elderly. *Gastroenterol Clin North Am* 1990; **19**: 391-403
24 **Manousos ON**, Truelove SC, Lumsden K. Prevalence of colonic diverticula and diverticulosis in general population of Oxford area. *Br Med J* 1967; **3**: 762-763
25 **Watters DA**, Smith AN, Eastwood MA, Anderson KC, Elton RA, Mugerwa JW. Mechanical properties of the colon: comparison of the features of the African and European colon *in vitro*. *Gut* 1985; **26**: 384-392
26 **Thomson HJ**, Busuttil A, Eastwood MA. Submucosal collagen changes in the normal colon and in diverticular disease. *Int J Colorectal Dis* 1987; **2**: 208-213
27 **Smith AN**. Colonic muscle in diverticular disease. *Clin Gastroenterol* 1986; **15**: 917-935
28 **Smith AN**, Shepherd J. The strength of the colon wall in diverticular disease. *Br J Surg* 1976; **63**: 666
29 **Mimura T**, Emanuel A, Kamm MA. Pathophysiology of diverticular disease. *Best Pract Res Clin Gastroenterol* 2002; **16**: 563-576
30 **Painter NS**, Burkitt DP. Diverticular disease of the colon: a deficiency disease of Western civilization. *BMJ* 1971; **2**: 450-454
31 **Mendeloff AI**. Thoughts on the epidemiology of diverticular disease. *Clin Gastroenterol* 1986; **15**: 855-877
32 **Segal I**, Solomon A, Hunt JA. Emergence of diverticular disease in the urban South African black. *Gastroenterology* 1977; **72**: 215-219
33 **Nair P**, Mayberry JF. Vegetarianism, dietary fibre and gastrointestinal disease. *Dig Dis* 1994; **12**: 177-185
34 **Wess L**, Eastwood M, Busuttil A, Edwards C, Miller A. An association between maternal diet and colonic diverticulosis in an animal model. *Gut* 1996; **39**: 423-427
35 **Painter NS**. The cause of diverticular disease of the colon, its symptoms and its complications. Review and hypothesis. *J R Coll Surg Edinb* 1985; **30**: 118-122
36 **Lupton JR**, Turner ND. Potential protective mechanisms of wheat bran fiber. *Am J Med* 1999; **106**: 24S-27S
37 **Walker AR**, Walker BF, Richardson BD. Bowel transit times in Bantu populations. *BMJ* 1970; **3**: 48-49
38 **Chaussade S**, Roche H, Khyari A, Couturier D, Guerre J. A new method for measuring colonic transit time. Description and validation. *Gastroenterol Clin Biol* 1986; **10**: 385-389
39 **Ludeman L**, Warren BF, Shepherd NA. The pathology of diverticular disease. *Best Pract Res Clin Gastroenterol* 2002; **16**: 543-562
40 **Lin OS**, Soon MS, Wu SS, Chen YY, Hwang KL, Triadafilopoulos G. Dietary habits and right-sided colonic diverticulosis. *Dis Colon Rectum* 2000; **43**: 1412-1418
41 **Narducci F**, Bassotti G, Gaburri M, Morelli A. Twenty four hour manometric recording of colonic motor activity in healthy man. *Gut* 1987; **28**: 17-25
42 **Sugihara K**, Muto T, Morioka Y. Motility study in right sided diverticular disease of the colon. *Gut* 1983; **24**: 1130-1134
43 **Sasaki D**, Kido A, Yoshida Y. An endoscopic method to study the relationship between bowel habit and motility of the ascending and sigmoid colon. *Gastrointest Endosc* 1986; **32**: 185-189
44 **Arfwidsson S**. Pathogenesis of multiple diverticula of the sigmoid colon in diverticular disease. *Acta Chir Scand* 1964; **342** (Suppl): 1-68
45 **Painter NS**, Truelove SC, Ardran GM. Segmentation and the localization of intraluminal pressures in the human colon, with special reference to the pathogenesis of colonic diverticula. *Gastroenterology* 1965; **49**: 169-177
46 **Parks TG**, Connell AM. Motility studies in diverticular disease of the colon. *Gut* 1969; **10**: 534-542
47 **Ritsema GH**, Thijn CJ, Smout AJ. Motility of the sigmoid in irritable bowel syndrome and colonic diverticulosis. *Ned Tijdschr Geneeskd* 1990; **134**: 1398-1401
48 **Weinreich J**, Andersen D. Intraluminal pressure in the sigmoid colon. II. Patients with sigmoid diverticula and related conditions. *Scand J Gastroenterol* 1976; **11**: 581-586
49 **Weinreich J**, Moller SH, Andersen D. Colonic haustral pattern in relation to pressure activity and presence of diverticula. *Scand J Gastroenterol* 1977; **12**: 857-864
50 **Leandro A**, Cecconello I, Habr-Gama A, de Olivereira e Silva A, Pontes JF. Gastrointestinal motility in normal subjects and patients with diverticulosis of the colon. *Arq Gastroenterol* 1984; **21**:157-163
51 **Viebig RG**, Pontes JF, Michelson NH. Electromanometry of the rectosigmoid in colonic diverticulosis. *Arq Gastroenterol* 1994; **31**: 135-144
52 **Kratzsch KH**. Results of electromyographic studies of the rectosigmoid. *Dtsch Z Verdau Stoffwechselkr* 1985; **45**: 45-51
53 **Suchowiecky M**, Clarke DD, Bhasker M, Perry RJ, Snape WJ Jr. Effect of secoverine on colonic myoelectric activity in diverticular disease of the colon. *Dig Dis Sci* 1987; **32**: 833-840
54 **Trotman IF**, Misiewicz JJ. Sigmoid motility in diverticular disease and the irritable bowel syndrome. *Gut* 1988; **29**: 218-222
55 **Cortesini C**, Pantalone D. Usefulness of colonic motility study in identifying patients at risk for complicated diverticular disease. *Dis Colon Rectum* 1991; **34**: 339-342
56 **Cortesini C**, Bruno L, Pantalone D. Motility effects of anterior resection of the rectum performed for diverticular disease. *Ital J Surg Sci* 1989; **19**: 369-373
57 **Bassotti G**, Crowell MD. Colon and rectum: normal function and clinical disorder. Manometry. In Schuster MM, Crowell MD, Koch KL, eds. Schuster Atlas of Gastrointestinal Motility in health and disease. Second Edition. *BC Decker Inc, Hamilton* 2002: 241-252
58 **Bassotti G**, Gaburri M. Manometric investigation of high-amplitude propagated contractile activity of the human colon. *Am J Physiol* 1988; **255**: G660-G664
59 **Bassotti G**, Battaglia E, Spinozzi F, Pelli MA, Tonini M. Twenty-four hour recordings of colonic motility in patients with diverticular disease. Evidence for abnormal motility and propulsive activity. *Dis Colon Rectum* 2001; **44**: 1814-1820
60 **Di Lorenzo C,** Flores AF, Hyman PE. Age related changes in colon motility. *J Pediatr* 1995; **127**: 593-596
61 **Butt WG**, Wang M, Kaufmann ST. Age-related changes in rat colon mechanics. *J Gastrointest Mot* 1993; **5**: 121-128
62 **Garcia-Olmo D**, Sanchez PC. Patterns of colonic motility as recorded by a sham fecaloma reveal differences among patients with idiopathic chronic constipation. *Dis Colon Rectum* 1998; **41**:480-489
63 **Brann L**, Wood JD. Motility of the large intestine of piebald-lethal mice. *Am J Dig Dis* 1976; **21**: 633-640
64 **Simpson J**, Spiller RC. Colonic diverticular disease. *Clin Evid* 2002; **7**: 398-405


**Edited by** Xu XQ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2143-2148
World Journal of Gastroenterology
 ISSN 1007-9327

• ESOPHAGEAL CANCER •

# Separation and identification of differentially expressed nuclear matrix proteins between human esophageal immortalized and carcinomatous cell lines

Xing-Dong Xiong, En-Min Li, Li-Yan Xu, Hai-Bin Chen, Ling Chen, Wei-Jia Cai, Ya-Li Han, Zhong-Ying Shen, Yi Zeng

**Xing-Dong Xiong, En-Min Li,** Department of Biochemistry and Molecular Biology, Medical College, Shantou University, Shantou 515031, Guangdong Province, China
**Xing-Dong Xiong, En-Min Li, Li-Yan Xu, Zhong-Ying Shen, Wei-Jia Cai,** Institute of Oncologic Pathology, Medical College, Shantou University, Shantou 515031, Guangdong Province, China
**Hai-Bin Chen,** Department of Histology and Embryology, Medical College, Shantou University, Shantou 515031, Guangdong Province, China
**Ling Chen,** Molecular Biological Center, Medical College, Shantou University, Shantou 515031, Guangdong Province, China
**Ya-Li Han,** Department of Biology, Shantou University, Shantou 515031, Guangdong Province, China
**Yi Zeng,** Institute of Virology, Chinese Academy of Preventive Medicine, Beijing 100052, China
**Supported by** the National Natural Science Foundation of China, No. 39900069, No.30170428; Natural Science Foundation of Guangdong Province, No.990799, No.010431; College Natural Science Foundation of Guangdong province, No.200033; Medical Scientific Foundation of Guangdong Province, No.A2001419 and Research and Development Foundation of Shantou University, No.L0004, No.L00012
**Correspondence to:** Dr. En-Min Li, Department of Biochemistry and Molecular Biology, Medical College, Shantou University, 22 Xinling Road, Shantou 515031, Guangdong Province, China. nmli@stu.edu.cn
**Telephone:** +86-754-8532720 +86-754-8900847
**Received:** 2002-11-19 **Accepted:** 2003-03-11

## Abstract

**AIM:** To separate and identify differentially expressed nuclear matrix proteins (NMPs) between the immortalized human esophageal epithelial cell line (SHEE) and the malignantly transformed esophageal carcinoma cell line (SHEEC), and to provide new ways for finding specific markers and the pathogenesis of esophageal carcinoma.

**METHODS:** SHEE and SHEEC cell lines were used to extract NMPs. The quality of NMPs was monitored by Western blot analysis including DNA topoisomerase IIα, proliferation cell nuclear antigen (PCNA) and histone. NMPs of SHEE and SHEEC were analyzed by two-dimensional electrophoresis (2-DE), silver staining and PDQuest6.2 image analysis software. Three spots in which the differentially expressed NMPs were more obvious, were selected and analyzed with matrix-assisted laser desorption/ionization time of flying mass spectrometry (MALDI- TOF-MS) and database search.

**RESULTS:** Western blot analysis revealed that DNA topoisomerase IIα and PCNA were detected, and the majority of histones were deleted in NMPs of SHEE and SHEEC. After 2-DE image analysis by PDQuest6.2 software, the 2-DE maps were detected with an average of 106±7.1 spots in SHEE and 132±5.0 spots in SHEEC. Most of them were matched one another ($r$=0.72), only 16 protein spots were found differing in intensity. Three NMPs including cytoskeletal tropomyosin, FK506-binding protein 6, similar to retinoblastoma binding protein 8 were preliminarily identified by MALDI- TOF-MS.

**CONCLUSION:** These differentially expressed NMPs may play an important role during malignant transformation from SHEE to SHEEC. Their separation and identification will contribute to searching for specific markers and probing into the pathogenesis of esophageal carcinoma.

Xiong XD, Li EM, Xu LY, Chen HB, Chen L, Cai WJ, Han YL, Shen ZY, Zeng Y. Separation and identification of differentially expressed nuclear matrix proteins between human esophageal immortalized and carcinomatous cell lines. *World J Gastroenterol* 2003; 9(10): 2143-2148
http://www.wjgnet.com/1007-9327/9/2143.asp

## INTRODUCTION

Nuclear matrix (NM) represents the insoluble structural framework of nucleus which removes membrane lipid, soluble protein and chromatin. According to many investigations, nuclear matrix has been shown to play an important role not only in maintaining the structure of nucleus, but also in chromatin/chromosome construction, DNA replication, gene expression and regulation (RNA synthesis, RNA splicing and RNA transportation)[1]. Recently changes of the composition, structure and function of NMPs in the generation and development of tumors have been more and more concerned. Separation and identification of tumor associated NMPs have been a new way to search for tumor specific markers and to study tumor pathogenesis. Nowadays several tumor specific NMPs have been separated and identified from hepatocellular carcinoma[2], colon cancer[3] and prostate cancer[4], etc. Some of them (such as NMP22) have been applied to clinical diagnosis and therapy[5], but studies of the separation and identification of esophageal carcinoma specific NMPs have not been carried out.

Esophageal carcinoma is one of the most common malignant tumors in China[6-27]. In recent years, it has been increasingly concerned about the roles of human papilloma virus (HPV) in esophageal carcinogenesis[28-32]. In our previous work, we transfected human embryonic esophageal mucosal cells with HPV18 E6E7 genes, and established an immortalized epithelial cell line SHEE[33,34]. The SHEE cells were further exposed to the tumor promoter (12-O-tetradecanoyl-phorbol-13-acetate, TPA) to induce malignant transformation from which a human embryonic esophageal epithelial carcinoma cell line SHEEC was then established[35,36]. These studies not only provided the evidence for the close relationship between HPV and esophageal carcinogenesis, but also established a reliable model for studying the molecular mechanisms of esophageal carcinogenesis. In the present study, the differentially expressed NMPs between SHEE and SHEEC were investigated by Western blot, 2-DE and MALDI-TOF-MS, and three esophageal carcinoma associated NMPs were preliminarily identified. The separation and identification of these proteins may contribute to searching for specific markers and studying the pathogenesis of esophageal carcinoma.

## MATERIALS AND METHODS

### Cell culture

SHEE and SHEEC were cultured in MEM medium (Gibco) supplemented with 100 ml/L fetal bovine serum (100 u/ml penicillin, 100 u/ml streptomycin) and incubated at 37 ℃ in humidified atmosphere of 50 ml/L $CO_2$. Cells were harvested when they grew into a full monolayer and kept at -70 ℃ until use.

### Extraction of NMPs

The method used was modified from Fey *et al*[37]. Cultured cells were extracted by cytoskeleton (CSK) buffer (100 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM PIPES pH6.8, 300 mM sucrose, 0.5 % triton X-100, 1.2 mM PMSF) for 5 min at 4 ℃. After centrifugation at 650 g for 5 min, the supernatants contained cytoplasmic proteins. The pellets were resuspended in digestion buffer (same as CSK buffer except with 50 mM NaCl instead of KCl) containing 400 μg/ml DNase I and 100 μg/ml RNase A. Enzyme digestion was carried out for 20 min at room temperature and terminated by adding cold ammonium sulfate to a final concentration of 0.25 M. After centrifugation at 1 000 g for 10 min, the pellets were then solubilized in disassembly buffer (8 M urea, 20 mM Mes pH6.6, 0.1 mM $MgCl_2$, 1 mM EGTA, 1 % 2-mercaptoethanol, 1 mM PMSF) and dialyzed overnight at 4 ℃ against 1 000 volumes of assembly buffer (150 mM KCl, 5 mM $MgCl_2$, 0.125 mM EGTA, 25 mM imidazole hydrochloride pH7.1, 2 mM dithiothreitol, 0.2 mM PMSF). The samples were centrifuged at 200 000 g for 100 min. The protein concentration of supernatants containing NMPs was determined by Bradford method and then precipitated in 5 volumes of absolute ethanol. The dried pellets were resuspended in electrophoresis sample buffer. The sample aliquots were stored at -70 ℃ until use.

### Western blot

The experimental procedures were referred to *Molecular cloning*[38]. Briefly, the proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. Nonspecific reactivity was blocked by incubation overnight at 4 ℃ in buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 2 % Tween-20, 4 % bovine sreum albumin). The membrane was then incubated with primary antibody including mouse anti-human histone (Roche), mouse anti-human DNA topoisomerase IIα. (Roche) and mouse anti-human PCNA (Dako). The secondary antibody (Zymed) was used to detect bound primary antibody. Reactive protein was detected by Western blot luminol reagent (Santa Cruz) and exposed to X-films (Kodak). The X-films were scanned and analyzed with Kodak 1-D 3.5 software (Kodak).

### Two-dimensional electrophoresis

To separate NMPs, the 2-DE procedures were referred to Xiong *et al*[39]. Briefly, 2-DE was carried out by using the Mini-PROTEAN II 2-D apparatus (Bio-Rad). 90 μg of the NMPs was mixed with the rehydration solution to a total volume of 125 μl. After rehydration, the isoelectric focusing (IEF) and equilibration, and the IPG strips (pH4-7, 7 cm) were placed on a 1.0 mm thick, 10 % SDS-PAGE gel. On electrophoresis, the SDS-PAGE gels were stained with PlusOne™ silver staining kit (Phamacia).

### Image acquisition and analysis

Image scanning for the silver-stained 2-D gels was performed with EDAS290 digital camera system (Kodak) and image analysis with the PDQuest 6.2 software (Bio-Rad). To obtain reliable results, three gels were employed for each cell line. After the background subtraction, spot detection and match, one standard gel for each cell line was obtained. These standard gels were then matched to yield information about the spots of differentially expressed NMPs.

### Protein identification by MALDI-TOF-MS

Three spots in which the differentially expressed protein was more obvious in each cell line were cut out from the gel. The gel pieces were treated by a series of steps including silver-removal, reduction, alkylation and in-gel digestion with trypsin. The peptide mass maps were generated by Applied Biosystems Voyager System 6192 MALDI-TOF-mass spectrometry (ABI, USA). Peptide masses were analyzed using the MS-Fit search program (http:// prospector.ucsf.edu/ucsfhtml4.0u/ msfit.htm).

## RESULTS

### Evaluation of NMPs quality

The NMPs extracted from SHEE and SHEEC were evaluated by Western blot, and the aim of evaluation was to monitor whether some NMPs were lost and whether other non-NMPs components were mixed. In recent years, many studies proved that DNA topoisomerase IIα and PCNA were the major components of NMPs[40,41], the existence of histone in nucleosome was the major soluble protein of nucleus. In our study, 170 kD DNA topoisomerase IIα was detected in the nuclear protein fraction of SHEE and SHEEC at the same position of NMPs fraction, but the density in NMPs fraction was weaker than that in the nuclear protein fraction (Figure 1). This agreed with other reports[40]. PCNA (36kD) was detected in the NMPs fraction of SHEE and SHEEC, and this showed PCNA was the major protein of NMPs (Figure 2). Moreover, histone was seen at 23 kD and 12-15 kD in the nuclear protein fraction (Mw of histone: H1:23 kD, H2A:14.5 kD, H2B: 13.8 kD, H3:15 kD, H4:11.8 kD) and almost no histone was detected at the same position of NMPs (Figure 3). This showed majority of histone was removed during extraction and there was almost no histone in the NMPs fraction of SHEE and SHEEC. According to these results, the NMPs fractions extracted from SHEE and SHEEC almost deleted the histone and retained the major NMPs components including DNA topoisomerase IIα and PCNA, so the NMPs were pure in high quality.

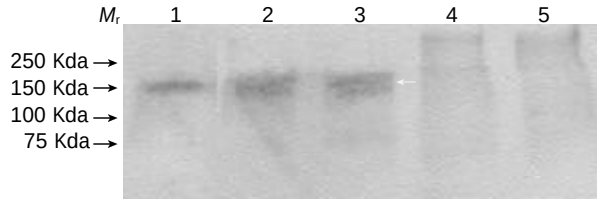



**Figure 1** Western blot analysis of DNA topoisomerase IIα. Lane 1, nuclear matrix fraction of SHEE. Lane 2, total crude nuclear protein fraction of SHEEC. Lane 3, total crude nuclear protein fraction of SHEE. Lane 4, cytoplasmic protein fraction of SHEEC. Lane 5, cytoplasmic protein fraction of SHEE. $M_r$, molecular weight standard.

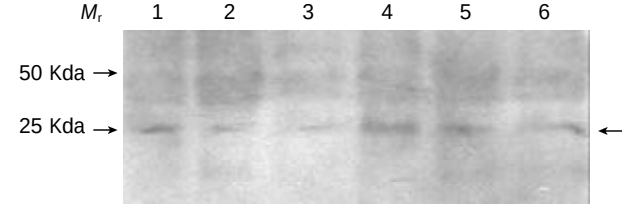



**Figure 2** Western blot analysis of PCNA. Lane 1 to Lane 3, nuclear matrix fraction of SHEEC. Lane 4 to Lane 6, nuclear matrix protein of SHEE. $M_r$, molecular weight standard.

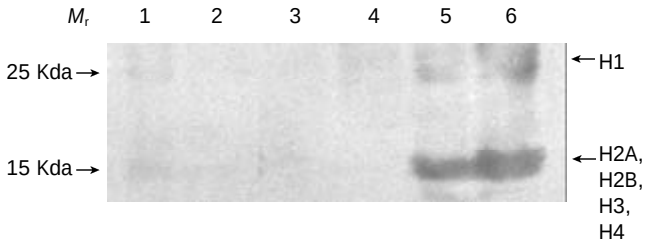



**Figure 3** Western blot analysis of histone. Lane 1, nuclear matrix fraction of SHEEC. Lane 2, nuclear matrix protein of SHEE. Lane 3, cytoplasmic protein fraction of SHEEC. Lane 4, cytoplasmic protein fraction of SHEE. Lane 5, total crude nuclear protein fraction of SHEEC. Lane 6, total crude nuclear protein fraction of SHEE. *M*r, molecular weight standard.

### *2-D map and image analysis*

To obtain a higher electrophoretic resolution, IPG strips (pH4-7) were selected to separate the NMPs of SHEE and SHEEC in our study because the p*I* range of NMPs was mainly in acid pH. Three pairs of gels from different batches of SHEE and SHEEC were analyzed by using the software PDQuest6.2. There were 106±7.1 and 132±5.0 protein spots observed in SHEE and SHEEC respectively. Most of them were matched one another ($r$=0.72), only 16 protein spots were found differing in intensity. These 16 protein spots belonged to 4 types. Namely A: one protein spot was detected in SHEE (No.2). B: Seven protein spots were detected only in SHEEC (No.3, 4, 9, 11, 12, 14, 16), C: One protein spot was expressed higher in SHEE (No.1), D: Seven protein spots were expressed higher in SHEEC (No.5, 6, 7, 8, 10, 13, 15). According to the standard molecular weight and pH gradient of IPG strips, the positions of these 16 protein spots were estimated with the software PDQuest6.2 (Table 1). Three protein spots (No.4, 14, 16) which belonged to type B and had a higher intensity were selected and analyzed with MALDI-TOF-MS (Figure 4).

### *MALDI-TOF-MS analysis and protein identification*

These three protein spots (No.4, 14, 16) were cut out from the gels and analyzed with MALDI-TOF-MS. Peptide mass fingerprint (PMF) of each protein spot was then generated (Figure 5). By searching the NCBInr protein database with the MS-Fit search program, we identified these three proteins combined with the searching results. The characteristics of the protein, the number and intensity of peptide matching peak, the sequence coverage of matching peptide, as well as the theoretical and approximate values of $M_r$ and p*I*, the identified protein names, accession numbers, as well as the sequence coverages, the theoretical $M_r$ and p*I* values for each protein spot are listed in Table 2.

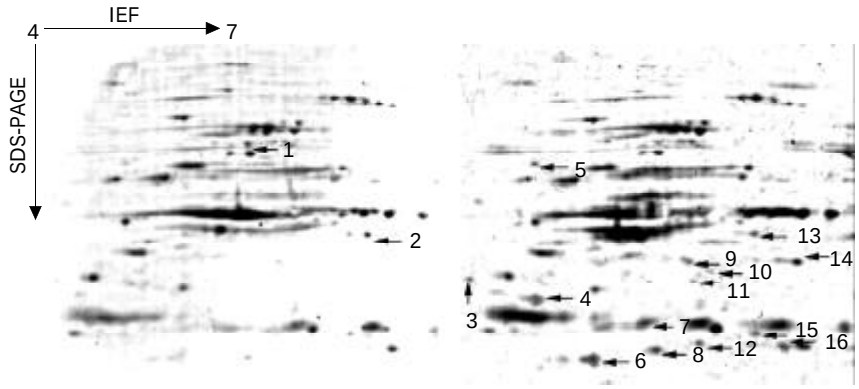



**Figure 4** Differentially expressed NMP spots observed in SHEE (left) and SHEEC (right) two-dimensional gels (IPG dry strips: pH 4-7, 7 cm). The arrows show differentially expressed protein spots. Three protein spots (No.4, 14, 16) were selected and analyzed with MALDI-TOF-MS.

**Table 1** Differentially expressed protein spots between SHEE and SHEEC

| Spot No. | Type | Experimental $M_r$ | Experimental p*I* | Spot No. | Type | Experimental $M_r$ | Experimental p*I* |
|---|---|---|---|---|---|---|---|
| 1 | C | 73.0 | 5.55 | 9 | B | 38.2 | 5.79 |
| 2 | A | 45.1 | 6.60 | 10 | D | 35.8 | 5.96 |
| 3 | B | 34.0 | 4.10 | 11 | B | 33.5 | 5.83 |
| 4 | B | 30.0 | 4.60 | 12 | B | 23.1 | 5.88 |
| 5 | D | 62.5 | 4.50 | 13 | D | 44.6 | 6.32 |
| 6 | D | 21.0 | 5.03 | 14 | B | 37.6 | 6.66 |
| 7 | D | 26.2 | 5.45 | 15 | D | 24.6 | 6.33 |
| 8 | D | 22.2 | 5.53 | 16 | B | 22.7 | 6.54 |

**Table 2** Proteins identified by MALDI-TOF-MS

| Spot No. | Accession No.(gi) | Theoretical $M_r$ | Theoretical p*I* | Intensity matched | Length (aa) | Name of protein |
|---|---|---|---|---|---|---|
| 4 | 37424 | 27975 | 4.8 | 44 % | 239 | Cytoskeletal tropomyosin |
| 14 | 17149849 | 37227 | 6.9 | 29 % | 327 | FK506-binding protein 6 |
| 16 | 13647876 | 26775 | 5.7 | 54 % | 230 | Similar to retinoblastoma binding protein 8 |

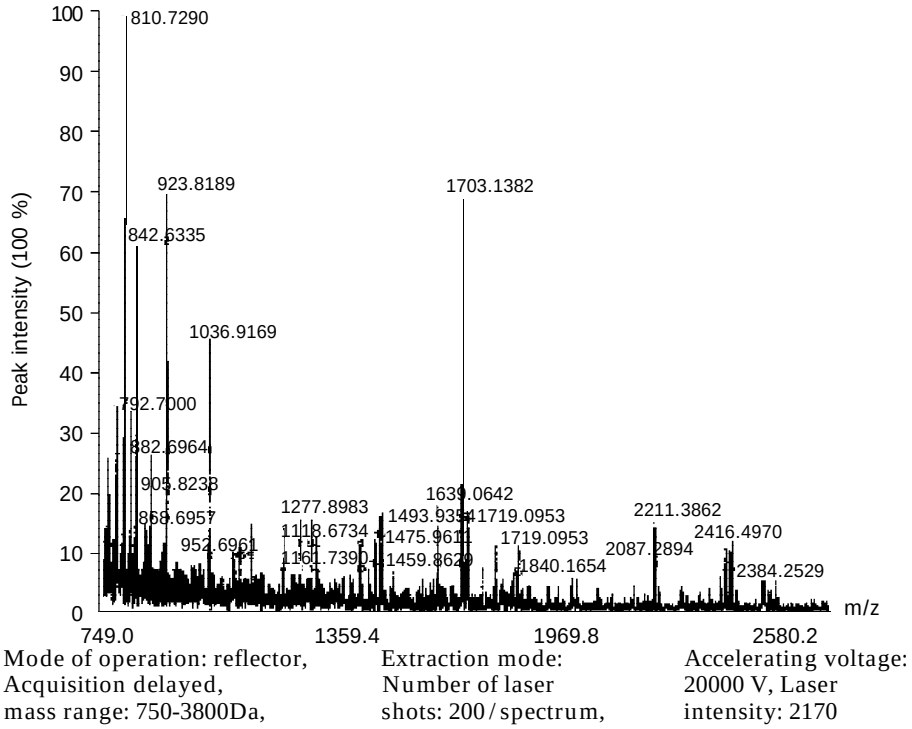



**Figure 5** MALDI-TOF mass spectrum map of protein spot 16.

## DISCUSSION

In the present study, we studied the differentially expressed NMPs extracted from SHEE and SHEEC by Western blot, 2-DE and MALDI-TOF-MS, and preliminarily identified three differentially expressed NMPs. According to their characteristics, these three proteins were connected with diseases such as colon carcinoma and myofibroblastic tumor, etc, but they were not related to esophageal carcinoma.

Cytoskeletal tropomyosin (tropomyosin 3, cytoskeletal (TPM3)) belongs to the tropomyosin family. There were at least 5 isoforms in tropomyosins including TPM1, TPM2, TPM3, TPM4, TPM5 which were produced by a tissue-specific alternative mRNA splicing mechanism[42]. The tropomyosins were a group of actin-binding proteins which served to mediate the effect of $Ca^{2+}$ on the actin-myosin interaction[43]. Actin is one of the major components of NMPs. Owing to active cell division during the course of malignant transformation, cytoskeletal tropomyosin connected with actin in the nucleus becomes overexpressed. Zeng and his associates[44] found that tropomyosin was localized in the nuclear matrix and chromosome scaffold, and dispersively distributed in the interphase nuclei and metaphase chromosomes. Many studies have proved that cytoskeletal tropomyosin is related to the generation and development of tumors. Martin-Zanca and his associates[45] found the oncogene-Onc D (named later "trk" (tropomyosin(t)-receptor(r) -kinase(k)) in colon carcinoma. Sequence analysis for the oncogene Onc D found that it was generated by a somatic rearrangement of two genes, one of which coded for cytoskeletal tropomyosin (TPM3) and the other for a tyrosine-specific protein kinase. The amino end of TPM3 has been found to be fused with tyrosine protein kinase domain and has become a new oncogenic protein. These results indicate that cytoskeletal tropomyosin is related to the generation and development of colon carcinoma. Furthermore, Lawrence and his associates[46] reported that TPM3-ALK (anaplastic lymphoma kinase, ALK) and TPM4-ALK fusion genes occurred in inflammatory myofibroblastic tumors. In our study, cytoskeletal tropomyosin (TPM3) showed overexpression in the NMPs fraction of SHEEC cells, indicating that cytoskeletal tropomyosin as a oncoprotein might be related to esophageal carcinoma. But its characteristics and functions in esophageal carcinoma remain to be further studied.

FK506-binding protein 6 (FKBP6) is one member of FKBP family. The FKBP family proteins were those directly binding to the immunosuppressant drug FK506[47]. So far there are several FKBP family members, namely FKBP6, FKBP12, FKBP13, FKBP25 and FKBP52. These FKBP family members differ in their subcellular localization. FKBP12 and FKBP52 are localized in the cytosol, FKBP13 in the endoplasmic reticulum, and FKBP25 in the nucleus. Like other FKBP members, FKBP6 had some peptidyl-prolyl cis-trans isomerase activity and a drug binding site[48]. Owing to its possession of cis-trans isomerase activity, FKBPs are thought to be essential for protein folding during protein synthesis. FKBP6 gene is localized in chromosome 11 and consists of nine exons. Meng and his associates[49] found FKBP6 gene was deleted in Williams syndrome (WS), and this might contribute to certain defects such as hypercalcemia and growth delay in WS. In our study, FKBP6 was overexpressed in the NMPs fraction of SHEEC. This indicated that FKBP6 as an active NMP might play a role during malignant transformation of the immortalized human esophageal epithelial cells.

Similar to retinoblastoma binding protein 8 (STRBP8), it contains 230 amino acid residues. Its sequences are the same as residues 668-897 of retinoblastoma binding protein 8 (RBP8), and so it is named STRBP8. So far there are not any reports about the function of STRBP8. Moreover, RBP8 is localized in the nucleus. It might interact with some tumor suppressive factors including CtBP1, Rb1 and BRCA1, and was involved in transcription regulation and tumor suppression[50,51]. The interaction with CtBP1 requires a short amino acid motif PLDLS (residues 490-494) of RBP8, whereas the Rb1 tumor suppressor binding to a LXCXE motif is located within the N-terminal region of RBP8 (residues 153-157). Unlike RBP8, STRBP8 has no Rb1 binding motif (sequence LXCXE) and CtBP1 binding motif (sequence PLDLS). The results indicate that STRBP8 possibly loses the function of tumor suppression and on the contrary possesses carcinogenetic effect. The fact that STRBP8 was overexpressed in SHEEC in our study, showed that STRBP8 as a new oncoprotein might

be involved in the malignant transformation of SHEE.

Although three NMPs have been found to be associated with esophageal carcinoma in the present study, but whether they would become specific markers of esophageal carcinoma needs to be further studied. In addition, the generation and development of tumors are the extraordinary pathological complex phenomena, and the process of coordinated interaction and programed development of many proteins. It will help elucidate the process of tumor generation and development to study the network relationship of these tumor associated proteins. Therefore, the network functional relationship of these differentially expressed NMPs needs to be further studied.

## REFERENCES


1 **Pederson T**. Thinking about a nuclear matrix. *J Mol Biol* 1998; **277**: 147-159
2 **Yoon GS**, Lee H, Jung Y, Yu E, Moon HB, Song K, Lee I. Nuclear matrix of calreticulin in hepatocellular carcinoma. *Cancer Res* 2000; **60**: 1117-1120
3 **Brunagel G**, Vietmeier BN, Bauer AJ, Schoen RE, Getzenberg RH. Identification of nuclear matrix protein alterations associated with human colon cancer. *Cancer Res* 2002; **62**: 2437-2442
4 **Lakshmanan Y**, Subong EN, Partin AW. Differential nuclear matrix protein expression in prostate cancers:correlation with pathologic stage. *J Urol* 1998; **159**: 1354-1358
5 **Oge O**, Atsu N, Kendi S, Ozen H. Evaluation of nuclear matrix protein 22 (NMP22) as a tumor marker in the detection of bladder cancer. *Int Urol Nephrol* 2001; **32**: 367-370
6 **Xiong XD**, Xu LY, Shen ZY, Cai WJ, Luo JM, Han YL, Li EM. Identification of differentially expressed proteins between human esophageal immortalized and carcinomatous cell lines by two-dimensional electrophoresis and MALDI-TOF-mass spectrometry. *World J Gastroenterol* 2002; **8**: 777-781
7 **Hou J**, Lin PZ, Chen ZF, Ding ZW, Li SS, Men FS, Guo LP, He YT, Qiao CY, Guo CL, Duan JP, Wen DG. Field population-based blocking treatment of esophageal epithelia dysplasia. *World J Gastroenterol* 2002; **8**: 418-422
8 **Hao MW**, Liang YR, Liu YF, Liu L, Wu MY, Yang HX. Transcription factor EGR-1 inhibits growth of hepatocellular carcinoma and esophageal carcinoma cells lines. *World J Gastroenterol* 2002; **8**: 203-207
9 **Shen ZY**, Shen WY, Chen MH, Shen J, Cai WJ, Zeng Y. Mitochondria, calcium and nitric oxide in the apoptotic pathway of esophageal carcinoma cells induced by As203. *Int J Mol Med* 2002; **9**: 385-390
10 **Xu M**, Jin YL, Fu J, Huang H, Chen SZ, Qu P, Tian HM, Liu ZY, Zhang W. The abnormal expression of retinoic acid receptor-β, p53 and Ki67 protein in normal, premalignant and malignant esophageal tissues. *World J Gastroenterol* 2002; **8**: 200-202
11 **Deng LY**, Zhang YH, Xu P, Yang SM, Yuan XB. Expression of IL 1β converting enzyme in 5-FU induced apoptosis in esophageal carcinoma cells. *World J Gastroenterol* 1999; **5**: 50-52
12 **Wang AH**, Sun CS, Li LS, Huang JY, Chen QS. Relationship of tobacco smoking, CYP1A1, GSTM1 gene polymorphism and esophageal cancer in Xi'an. *World J Gastroenterol* 2002; **8**: 49-53
13 **Shen ZY**, Xu LY, Li EM, Cai WJ, Chen MH, Shen J, Zeng Y. Telomere and telomerase in the initial stage of immortalization of esophageal epithelial cell. *World J Gastroenterol* 2002**; 8**: 357-362
14 **Shen ZY**, Shen WY, Chen MH, Shen J, Cai WJ, Yi Z. Nitric oxide and calcium ions in apoptotic esophageal carcinoma cells induced by arsenite. *World J Gastroenterol* 2002; **8**: 40-43
15 **Shen ZY**, Shen J, Li QS, Chen CY, Chen JY, Zeng Y. Morphological and functional changes of mitochondria in apoptotic esophageal carcinoma cells induced by arsenic trioxide. *World J Gastroenterol* 2002; **8**: 31-35
16 **Shen ZY**, Xu LY, Li C, Cai WJ, Shen J, Chen JY, Zeng Y. A comparative study of telomerase activity and malignant phenotype in multistage carcinogenesis of esophageal epithelial cells induced by human papillomavirus. *Int J Mol Med* 2001; **8**: 633-639
17 **Yu GQ**, Zhou Q, Ivan D, Gao SS, Zheng ZY, Zou JX, Li YX, Wang LD. Changes of p53 protein blood level in esophageal cancer patients and normal subjects from a high incidence area in Henan, China. *World J Gastroenterol* 1998; **4**: 365-366
18 **Li J**, Feng CW, Zhao ZG, Zhou Q, Wang LD. A preliminary study on ras protein expression in human esophageal cancer and precancerous lesions. *World J Gastroenterol* 2000; **6**: 278-280
19 **Liu XL**, Xiao B, Yu ZC, Guo JC, Zhao QC, Xu L, Shi YQ, Fan DM. Down-regulation of Hsp90 could change cell cycle distribution and increase drug sensitivity of tumor cells. *World J Gastroenterol* 1999; **5**: 199-208
20 **Zhang LJ**, Chen KN, Xu GW, Xing HP, Shi XT. Congenital expression of mdr-1 gene in tissues of carcinoma and its relation with pathomorphology and prognosis. *World J Gastroenterol* 1999; **5**: 53-56
21 **Gao SS**, Zhou Q, Li YX, Bai YM, Zheng ZY, Zou JX, Liu G, Fan ZM, Qi YJ, Zhao X, Wang LD. Comparative studies on epithelial lesions at gastric cardia and pyloric antrum in subjects from a high incidence area for esophageal cancer in Henan, China. *World J Gastroenterol* 1998; **4**: 332-333
22 **Shen ZY**, Tan LJ, Cai WJ, Shen J, Chen C, Tang XM, Zheng MH. Arsenic trioxide induces apoptosis of oesophageal carcinoma *in vitro*. *Int J Mol Med* 1999; **4**: 33-37
23 **Shen ZY**, Shen J, Cai WJ, Hong C, Zheng MH. The alteration of mitochondria is an early event of arsenic trioxide induced apoptosis in esophageal carcinoma cells. *Int J Mol Med* 2000; **5**: 155-158
24 **Gu ZP**, Wang YJ, Li JG, Zhou YA. VEGF165 antisense RNA suppresses oncogenic properties of human esophageal squamous cell carcinoma. *World J Gastroenterol* 2002; **8**: 44-48
25 **Su M**, Lu SM, Tian DP, Zhao H, Li XY, Li DR, Zheng ZC. Relationship between ABO blood groups and carcinoma of esophagus and cardia in Chaoshan inhabitants of China. *World J Gastroenterol* 2001; **7**: 657-661
26 **Wu MY**, Chen MH, Liang YR, Meng GZ, Yang HX, Zhuang CX. Experimental and clinicopathologic study on the relationship between transcription factor Egr-1 and esophageal carcinoma. *World J Gastroenterol* 2001; **7**: 490-495
27 **Xiao ZF**, Yang ZY, Zhou ZM, Yin WB, Gu XZ. Radiotherapy of double primary esophageal carcinoma. *World J Gastroenterol* 2000; **6**: 145-146
28 **Chen HB**, Chen L, Zhang JK, Shen ZY, Su ZJ, Cheng SB, Chew EC. Human papillomavirus 16 E6 is associated with the nuclear matrix of esophageal carcinoma cells. *World J Gastroenterol* 2001; **7**: 788-791
29 **Lavergne D**, de-Villiers EM. Papillomavirus in esophageal papillomas and carcinomas. *Int J Cancer* 1999; **80**: 681-684
30 **Xu LY**, Li EM, Xiong HQ, Cai WJ, Shen ZY. Study of neutrophil gelatinase associated lipocalin (NGAL) gene overexpression in the progression of malignant transformation of human immortalized esophageal epithelial cell. *Shengwu Huaxue Yu Shengwu Wuli Jinzhan* 2001; **28**: 839-843
31 **Ma QF**, Jiang H, Feng YQ, Wang XP, Zhou YA, Liu K, Jia ZL. Detection of human papillomavirus DNA in squamous cell carcinoma of the esophagus. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 1218-1224
32 **Zhang J**, Yan XJ, Yan QJ, Duan J, Hou Y, Su CZ. Cloning and expression of HPV16 L2 DNA from esophageal carcinoma in E. coli. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 273-278
33 **Shen ZY**, Xu LY, Chen XH, Cai WJ, Shen J, Chen JY, Huang TH, Zeng Y. The genetic events of HPV-immortalized esophageal epithelium cells. *Int J Mol Med* 2001; **8**: 537-542
34 **Shen ZY**, Cen S, Cai WJ, Teng ZP, Shen J, Hu Z, Zeng Y. Immortalization of human fetal esophageal epithelial cells induced by E6 and E7 genes of human papillomavirus 18. *Zhonghua Shiyan He Linchuang Bingduxue Zazhi* 1999; **13**: 121-124
35 **Shen ZY**, Cai WJ, Shen J, Xu JJ, Cen S, Teng ZP, Hu Z, Zeng Y. Human papilloma virus 18E6E7 in synergy with TPA induced malignant transformation of human embryonic esophageal epithelial cells. *Bingdu Xuebao* 1999; **15**: 1-5
36 **Shen Z**, Cen S, Shen J, Cai W, Xu J, Teng Z, Hu Z, Zeng Y. Study of immortalization and malignant transformation of human embryonic esophageal epithelial cells induced by HPV18 E6E7. *J Cancer Res Clin Oncol* 2000; **126**: 589-594
37 **Fey EG**, Penman S. Nuclear matrix proteins reflect cell type of origin in cultured human cells. *Proc Natl Acad Sci U S A* 1988; **85**: 121-125

38 **Sambrook J**, Fritsch EF, Maniatis T. *Molecular cloning A Laboratory Manual, 3th ed*. Jin DY, Li MF, trans-ed, Beijing, China: *Sci Pub* 1998: 888-898

39 **Xiong XD**, Xu LY, Shen ZY, Han M, Niu YD, Han YL, Li EM. An optimized protocol for two-dimensional gel electrophoresis. *Shantou Daxue Xuebao* 2002; **17**: 5-9

40 **Fernandes DJ**, Qiu J, Catapano CV. DNA topoisomerase II isozymes involved in anticancer drug action and resistance. *Adv Enzyme Regul* 1995; **35**: 265-281

41 **Balajee AS**, May A, Bohr VA. Fine structural analysis of DNA repair in mammalian cells. *Mutat Res* 1998; **404**: 3-11

42 **Reinach FC**, MacLeod AR. Tissue-specific expression of the human tropomyosin gene involved in the generation of the trk oncogene. *Nature* 1986; **322**: 648-650

43 **MacLeod AR**, Houlker C, Reinach FC, Talbot K. The mRNA and RNA-copy pseudogenes encoding TM30nm, a human cytoskeletal tropomyosin. *Nucleic Acids Res* 1986; **14**: 8413-8426

44 **Zeng XL**, Jiao MD, Xing M, Wang XG, Hao S. Tropomyosin is localized in the nuclear matrix and chromosome scaffold of Physarum polycephalum. *Cell Res* 1999; **9**: 61-69

45 **Martin-Zanca D**, Hughes SH, Barbacid M. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. *Nature* 1986; **319**: 743-748

46 **Lawrence B**, Perez-Atayde A, Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL, Pinkus GS, Xiao S, Yi ES, Fletcher CD, Fletcher JA. TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. *Am J Pathol* 2000; **157**: 377-384

47 **Fruman DA**, Bierer BE, Benes JE, Burakoff SJ, Austen KF, Katz HR. The complex of FK506-binding protein 12 and FK506 inhibits calcineurin phosphatase activity and IgE activation-induced cytokine transcripts, but not exocytosis, in mouse mast cells. *J Immunol* 1995; **154**: 1846-1851

48 **Jin YJ**, Burakoff SJ. The 25-kDa FK506-binding protein is localized in the nucleus and associates with casein kinase II and nucleolin. *Proc Natl Acad Sci U S A* 1993; **90**: 7769-7773

49 **Meng X**, Lu X, Morris CA, Keating MT. A novel human gene FKBP6 is deleted in Williams syndrome. *Genomics* 1998; **52**: 130-137

50 **Schaeper U**, Subramanian T, Lim L, Boyd JM, Chinnadurai G. Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif. *J Biol Chem* 1998; **273**: 8549-8552

51 **Yu X**, Baer R. Nuclear localization and cell cycle-specific expression of CtIP, a protein that associates with the BRCA1 tumor suppressor. *J Biol Chem* 2000; **275**: 18541-18549


**Edited by** Wu XN and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2149-2153
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

# 15d-PGJ$_2$ inhibits cell growth and induces apoptosis of MCG-803 human gastric cancer cell line

Yun-Xian Chen, Xue-Yun Zhong, Yan-Fang Qin, Wang Bing, Li-Zhen He

**Yun-Xian Chen,** Department of Hematology, the First Affiliated Hospital of Sun-Yat-Sen University, Guangzhou 510080, Guangdong Province, China
**Xue-Yun Zhong, Yan-Fang Qin, Wang Bing, Li-Zhen He,** Department of Pathology, Medical College, Jinan University, Guangzhou 510632, Guangdong Province, China
**Supported by** Science Fund of Guangdong Province, No. 015012, Science Fund of Guangzhou, 2001-Z-01-2 and the State 973 Projects, 2002ccc0400
**Correspondence to:** Dr. Xue-Yun Zhong, Department of Pathology, Medical College, Jinan University, Guangzhou 510632, Guangdong Province, China. tzxy@jnu.edu.cn
**Telephone:** +86-20-85220252
**Received:** 2003-04-08 **Accepted:** 2003-05-19

## Abstract

**AIM:** To investigate the influence of peroxisome proliferator-activated receptor γ (PPARγ) ligand, 15-deoxy-△12, 14-prostaglandin $J_2$ (15dPGJ$_2$) on the proliferation and apoptosis of MCG-803 human gastric cancer cell lines.

**METHODS:** Cell proliferation was measured by $^3$H-TdR assay. Apoptosis was determined by ELISA and TUNEL staining. Protein and mRNA level of bcl-2 family and COXs were measured by Western blotting and Northern blotting respectively. $PGE_2$ production was examined by RIA.

**RESULTS:** 15dPGJ$_2$ inhibited cell growth and induced apoptosis of MCG-803 cells. The COX-2 and bcl-2/bax ratios were decreased following 15dPGJ$_2$ treatment. The $PGE_2$ production in supernatants was also decreased. These changes were in a dose-dependent manner.

**CONCLUSION:** 15dPGJ$_2$ may be a useful therapeutic agent for the treatment of gastric cancer.

Chen YX, Zhong XY, Qin YF, Bing W, He LZ. 15d-PGJ$_2$ inhibits cell growth and induces apoptosis of MCG-803 human gastric cancer cell line. *World J Gastroenterol* 2003; 9(10): 2149-2153
http://www.wjgnet.com/1007-9327/9/2149.asp

## INTRODUCTION

Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor, provided a strong link between lipid metabolism and the regulation of gene transcription[1,2]. Recent studies showed that PPARγ was expressed at high levels in human colon cancer cells[3-5]. Ligand activation of PPARγ in human colon cancer cells caused a reduction of growth[3,4]. In contrast, two independent groups demonstrated that activation of PPARγ promoted the development of colon tumors in mice[6,7]. Thus the roles of PPARγ activation in the growth of colon tumors are controversial. In addition to colon cancer, PPARγ activation induced growth arrest in human liposarcoma, prostate cancer and breast cancer[8-10]. These results suggest that PPARγ activation may be implicated in the proliferation and apoptosis of malignant tumor cells.

Gastric cancer is the most common malignant tumor of gastrointestinal tract in the world. The survival rate in gastric cancer is poor. Although PPARγ expression has been studied in various epithelial cancer cell lines such as colon, prostate and breast, no information is available as to whether PPARγ is involved in the regulation of gastric cancer cell survival. In the present study, we investigated the effect of natural PPARγ ligand, 15dPGJ$_2$ on the growth and apoptosis of gastric cancer cell lines. We have further examined the role of bcl-2 family in the regulation of 15dPGJ$_2$-induced apoptosis. In addition, the change of cyclooxyenase (COXs) and the rate-limiting enzyme in the synthesis of prostaglandins (PGs) have been investigated in the process since COXs is recently considered as a promoter of human gastrointestinal cancer.

## MATERIALS AND METHODS

### *Cell line and reagents*

MCG-803 gastric cancer cell was kindly provided by Cancer Institute, Zhongshan University. Cells were maintained in RPMI-1640 medium and supplemented with 10 % new fetal bovine serum. Antibodies used in this study were obtained from Santa Cruz Biotech. The apoptosis ELISA kit and RIA kit were purchased from Sigma.

### *[$^3$H] Thymidine incorporation*

Cells were planted in 96-well plates and grown for 24 h after they were serum starved for 48 h. They were treated with 15dPGJ$_2$ for 48 h and pulsed with 5 μCi of [$^3$H] thymidine for 4 h. We counted the radioactivity in Beckman L5 counter after washing the cells and stopping the reaction with 5 % trichloroacetic acid and solubilizing the cells in 0.5 % of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and repeated at least three times.

### *TUNEL*

TUNEL assay was performed using the apoptosis detection system.15dPGJ$_2$ (0, 10, 30 μM) was added to the culture medium for 48 h. Cells were fixed with 4 % paraformaldehyde in PBS overnight at 4 ℃. The samples were washed three times in PBS and permeabilized by 0.2 % Triton X-100 in PBS for 15 min on ice. After washed twice, cells were equilibrated at room temperature for 15-30 min in equilibration buffer (200 mmol/L potassium cacodylate, 0.2 mmol/L dithiothreitol, 0.25 g· $L^{-1}$ bovine serum albumin, and 2.5 mmol/L cobalt chloride in 25 mmol/L Tris-HCL, pH 6.6) and incubated in the presence of 5 μmol/L pluorescein-12-dUTP, 10 μmol/L dATP, 100 μmol/L ethylenediaminetetraacetic acid (EDTA), and terminal deoxynucleotidyl transferase at 37 ℃ for 1.5 h in dark. The tailing reaction was terminated by 2×standard saline citrate (SSC). The samples were washed three times in PBS and analyzed by fluorescence microscopy. At least 1000 cells were counted, and the percentage of TUNEL-positive cells was determined.

### *Detection of apoptotic DNA fragmentation*

MCG-803 cells were grown in 96-well culture plates. The cells

were incubated with various doses of $15dPGJ_2$ for 24 h. Apoptotic DNA fragmentation was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Sigma. This assay was based on a quantitative Sandwich enzyme-immunoassay directed against cytoplasmic histone-associated DNA fragments. Briefly, the cells were incubated in 200 μL lysis buffer provided with the kit, the lysates were centrifuged, and 20 μL supernatant containing cytoplasmic histone-associated DNA fragments were reacted overnight at 4 ℃ in streptavidin-coated microtitrator wells with 80 μL immunoreagent mixture containing biotinylated anti-histone antibody and peroxidase-conjugated anti-DNA antibody. After washED, the immunocomplex-bound peroxidase was probed with 2, 2' -azino-di [3-ethylbenzthiazoline sulfonate] for spectrophotometric detection at 405 nm.

### *RIA for PGE2*

The amounts of immunoreactive $PGE_2$ in supernatants were determined by radioimmunoassay (RIA) using a commercially available RIA kit according to the manufacturer' s instructions. Briefly, to each polypropylene RIA tube were added 100 μL each of anti-$PGE_2$, $^{125}$I-$PGE_2$ , and $PGE_2$ or the sample. Immune complexes were precipitated 24 h later with 1 ml of 16 % polyethylene glycol solution, and a gamma counter determined the radioactivity in the precipitate. There was no nonspecific interference of the assay by the components of the sample. Determinations were carried out in triplicate and the means and standard deviations were obtained.

### *Western blot analysis*

The cells were lysed in lysis buffer containing 25 mM Hepes, 1.5 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 g/L eupeptic (pH7.8) at 4 ℃ with sonication. The lysates were centrifuged at 15 000×g for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer (42 mM Tris-HCl, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol and 0.002 % bromophenol blue) was then added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-polyacrylamidel gel. Proteins were transferred to nitrocellulose and incubated respectively with antibodies against COX-1, COX-2, PPARγ, Bcl-2, bax, bcl-$x_l$ and β-actin, and then with peroxidase-conjugated secondary antibodies. Detection was performed with enhanced chemiluminescence reagent.

### *Northern blot*

Total mRNA was isolated and stored at -80 ℃ until use. The synthetic DNA oligonucleotide probes complemented to COX-1, COX-2, PPARγ, Bcl-2, bax, bcl-$x_l$ and β-actin mRNA were labeled using terminal deoxynucleotidyltransferase [Boehringer]. Thirty mg of RNA per sample was separated on 1 % agarose-formaldehyde gels and transferred to nylon membranes. The blots were hybridized overnight at 42 ℃, washed in 0.2×SSC containing 0.1 % SDS at 50 ℃ for 10 min, and subjected to autoradiography.

### *Statistical analysis*

Data were presented as the mean ± standard error of the mean, unless otherwise indicated. Multiple comparisons were made for significant differences using analysis of variance, followed by individual comparisons with the Bonferroni post-test. Comparisons between two groups were made with the Student *t* test. *A P*<0.05 was considered significant.

## RESULTS

### *15dPGJ2 inhibited growth of MCG-803 cells*

To evaluate the effect of $15dPGJ_2$ on the growth of MCG-803 cells, $15dPGJ_2$ (1,10,30 μM) was added to the culture medium for 48 h. Cell growth was determined by $^3$H-dRT assay (Figure 1). $15dPGJ_2$ inhibited MCG-803 cell growth in a dose-dependent manner.

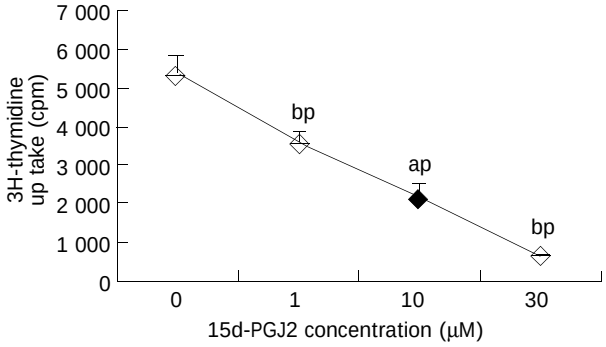



**Figure 1** Effect of 15d-$PGJ_2$ on cell growth by $H^3$-TdR assay (48 h). MCG-803 cells were incubated with various concentrations of 15d-$PGJ_2$ for 48 h. The value is represented as mean±SEM ($n$=3). $^aP$<0.05, $^bP$<0.01 *versus* control group.

### *15dPGJ2 induced apoptosis in MCG-803 cells*

There are several methods to evaluate apoptosis. In this study, we used ELISA and TUNEL to check for apoptosis of MCG-803 cells after 24 h treatment with $15dPGJ_2$. The percentage of TUNEL-positive cells increased from 13.7±1.5 % to 48.3±2.9 % (Table 1). The same results were obtained by ELISA. The effect of 15dPGJ2 at concentrations from 1 μmol/L to 30 μmol/L on DNA fragmentation in MCG-803 cells is shown in Figure 2. 15dPGJ2 was found to significantly induce DNA fragmentation after the onset of incubation and this effect was in a dose-dependent manner.

**Table 1** TUNEL assay performed using apoptosis detection system

| 15d-$PGJ_2$ (μM) | Control | 10 | 30 |
|---|---|---|---|
| Percentage of TUNEL-positive cells | 13.7±1.5 % | 34.9±2.7 %$^{bp}$ | 48.3±2.9 %$^{bp}$ |

$15dPGJ_2$ (0, 10, 30 μM) was added to the culture medium for 48 h. The percentage of TUNEL-positive cells was determined. $^aP$<0.05, $^bP$<0.01 compared with respective controls. The value is represented as mean ±SEM ($n$=3). $^bP$<0.01 *versus* corresponding control groups.

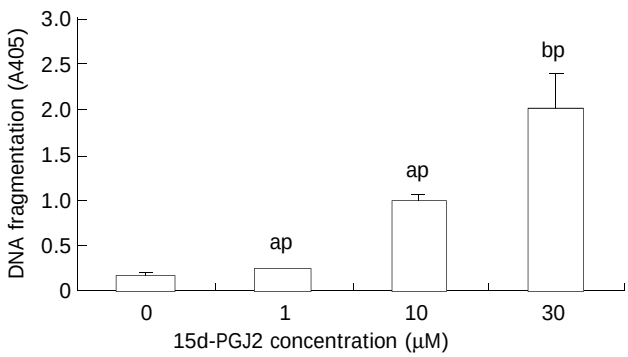



**Figure 2** Effect of 15d-PGJ2 on DNA fragmentation in MCG-803 cells. Cytoplasmic histone-associated DNA fragments were determined using a commercial ELISA kit. Culture of MCG-803 cells for 24 h in the presence of 15d-PGJ2 resulted in dose

dependent DNA fragmentation. $^aP<0.05$, $^bP<0.01$ compared with the controls. The value is represented as mean±SEM ($n$=3).

### *PPAR mRNA and protein expression in MCG-803 cells increased by 15dPGJ2*

Since 15dPGJ$_2$ is a potent PPAR agonist in the events leading to cell apoptosis, we have investigated its effects on the expression of PPARγ mRNA and protein. MCG-803 cells were treated with 15dPGJ$_2$ in the range of 1-30 μM for 6 h. Figure 4 shows that PPARγ mRNA expression was significantly increased in a dose-dependent manner. A Western blot analysis further showed that the mRNA levels encoding PPARγ upon treatment with 15dPGJ$_2$ could be related to the variation of the corresponding protein (Figure 3).

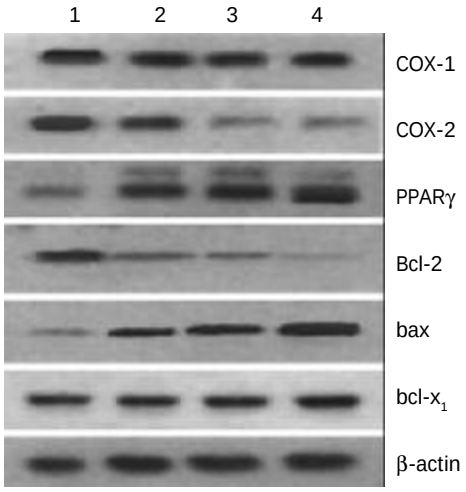



**Figure 3** COX-1, COX-2, PPARγ, bcl-2, bax, and bcl-XL protein levels in MCG-803 cells treated with 15d-PGJ2. Cell lysates were collected and processed at 6 h. The whole cellular protein was electrophoresed in SDS-PAGE gel. Western blot was performed using antibodies against COX-1, COX-2, PPARγ,bcl-2, bax, bcl-XL. β-actin was used as a lane-loading control. (1) control, (2) 1 μM, (3) 10 μM, (4) 30 μM.

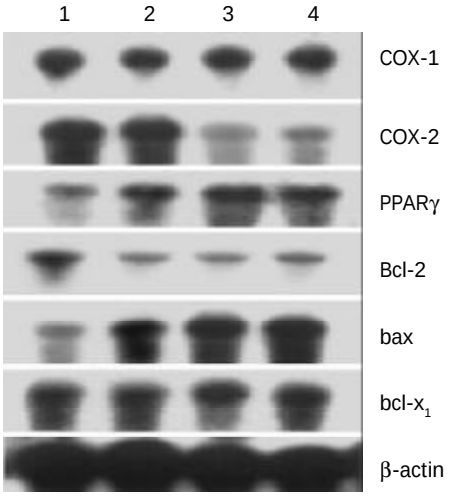



**Figure 4** COX-1, COX-2, PPAR-γ, bcl-2, bax, bcl-XLmRNA levels in MCG-803 cells treated with 15d-PGJ2 for 6 h by Northern blot. (1) control, (2) 1 μM, (3) 10 μM, (4) 30 μM.

### *Effect of 15dPGJ2 on bcl-2, bcl-XL and bax expression in MCG-803 cells*

To further elucidate the mechanism of 15dPGJ$_2$ induced apoptosis in MCG-803 cells, we evaluated the involvement of bcl-2 family in the apoptosis process by Western blot and Northern blot analysis. The protein and mRNA of bax were increased and bcl-2 decreased in a dose-dependent manner 6 h after 15dPGJ$_2$ treatment (Figures 3, 4). No change was observed in protein and mRNA expression of bcl-X$_L$ (Figures 3, 4).

### *Effect of 15dPGJ2 on COXs expression and PGE2 production in MCG-803 cells*

To assess the role of COXs in 15dPGJ$_2$ induced apoptosis, we first examined the changes of COX-1 and COX-2 in MCG-803 cells treated with 15dPGJ$_2$. As shown in Figures 3 and 4, the COX-2 protein increased 6 h after 15dPGJ$_2$ treatment and no change was observed in protein expression of COX-1. In Northern blot analysis, similar results were obtained. The expression of COX-1 mRNA was not changed and COX-2 mRNA was not regulated by 15dPGJ$_2$ in dose dependent manners. We used specific RIA to measure the production of PGE$_2$ as COX$_s$ catalyzed the rate-limiting step in the biosynthesis of prostaglandins. Parallel to the inhibition of COX-2, decrease of PGE$_2$ was found in culture medium of HCG-803 cells stimulated by 15dPGJ$_2$ (Figure 5). These results further confirmed that COX-2, not COX-1 activity was changed by 15dPGJ$_2$.

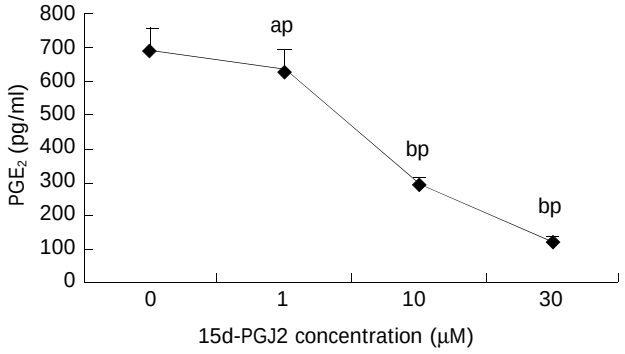



**Figure 5** Effect of 15d-PGJ$_2$ on PGE2 production by RIA (24 h). MCG-803 cells were incubated at various concentrations of 15d-PGJ$_2$ for 24 h. The value is represented as mean ±SEM ($n$=3). $^aP<0.05$ and, $^bP<0.01$ *versus* corresponding control groups.

## DISCUSSION

Peroxisome proliferator-activated receptor (PPAR) γ, a member of the steroid nuclear hormone receptor superfamily[11], has been known to trigger adipocyte differentiation and lipid storage by regulating the expression of genes critical for adipogenesis[12]. PPARγ functioned as a ligand-dependent transcription factor[13], which upon heterodimerization with the retinoid X receptor (RXR)[14], bound to specific response elements termed peroxisome proliferator response elements (PPRE). This in turn regulated the expression of target genes[15,16]. Activation of this receptor has been implicated in tumor promotion, cellular differentiation and apoptosis[17]. There are a number of naturally occurring agents that activate PPARγ. Some J-series prostaglandins have been found to bind to PPARγ in low micromolar range[18,19]. The PGD2 derivative, 15-deoxy-12, 14-PGJ2 (15d-PGJ2) was a high affinity ligand (*K*d=300 nM) that demonstrated anti-inflammatory[20,21] and anti-neoplastic activity[22].

The anti-neoplastic activity of PPARγ ligand prompted us to determine whether 15dPGJ$_2$ would trigger MCG-803 cells to undergo apoptosis. The results from Western blotting and Northern blotting have demonstrated that the PPARγ gene and protein expression were clearly observed in MCG-803 cells. 15dPGJ$_2$ stimulated the expression of PPARγ mRNA and its product. Coincubation of MCG-803 cells with 15dPGJ$_2$ potently inhibited cell growth and induced apoptosis in a dose-

related fashion. The dose-dependent suppression of cell growth in cancer cells was also reported after a number of synthetic PPARγ ligands such as thiazolidinediones (TZDs) and non-steroidal anti-inflammatory drugs (NSAIDs) treatment[9]. These results suggest that activation of the PPARγ pathway by $15dPGJ_2$ induces MCG-803 cells to undergo apoptosis.

In this study, the well-known important mediator of apoptosis, bcl-2 family was examined in MCG-803 cells treated with $15dPGJ_2$. In bcl-2 family, bcl-2 and bcl-$X_L$ were anti-apoptotic, whereas bax, bad and bak were pro-apoptotic[23]. The ratio of anti-apoptotic factor to pro-apoptotic factor in the cells was assumed to be a critical mechanism in maintaining normal homeostasis. A higher concentration of bax compared with bcl-2 enhanced susceptibility to apoptosis. Nevertheless, the cell continued to survive if bcl-2 predominated over bax. One of the most significant problems in the treatment of cancer was the resistance of cancer cells to chemotherapy-induced apoptosis. Of gastric cancers, 72 % bcl-2 overexpression and 33-40 % bax frameshift mutation were observed[24-26]. A higher ratio of bcl/bax strongly blocked gastric cancer cell apoptosis by inhibiting cytochrome C release from mitochondria and Caspase-3 activation[27-31]. Our data indicated that $15dPGJ_2$ led to a dramatic decrease in bcl-2 and increase in bax. The bcl-$X_L$ remained unchanged after $15dPGJ_2$. In addition, the decreased ratio of bcl/bax was detected prior to commencement of cell apoptosis after 6 h treatment. These results suggested a direct effect of PPAR pathway on bcl/bax expression. Therefore we concluded that the change of bcl-2 family might play a key role in $15dPGJ_2$-induced apoptosis in MCG-803 cells.

Cyclo-oyxgenase (COX) was the rate-limiting enzyme that catalyzed the initial step in biosynthesis of prostaglandins (PG) from arachidonic[32,33]. COX is encoded by two separate genes, COX-1 and COX-2, both of which participate in formation of a variety of eicosanoids. COX-1 is expressed constitutionally in most tissues and has been proposed to be a housekeeping gene involved in cytoprotection of gastric mucosa, vasodilation in the kidney, and control of platelet aggregation. In contrast, COX-2 is an inducible immediate early gene that is upregulated by various stimuli including mitogens, cytokines, growth factors, and tumor promoters. Accumulating evidence has shown that increased $PGE_2$ levels via overexpression of the inducible COX-2 isoform were important in the development of human cancer[34]. Previous studies have demonstrated that COX-2 expression was aberrantly increased in various human epithelial cancers, including those of colorectal[35,36], esophageal[37], gastric[38], lung[39] and bladder origin[40]. Data in this study also showed apparent COX-2 expression in MCG-803 cells. These findings suggest that cellular upregulation of COX-2 and $PGE_2$ may be a common mechanism in epithelial carcinogenesis.

In gastrointestinal system, it has been reported that COX-2 overexpression led to the inhibition of apoptosis or altered cell cycle kinetics in epithelial cells[41,42]. Numerous epidemiological studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) decreased incidence of gastrointestinal cancers and COX-2 was recognized as a major target of NSAIDs. Inhibition of COX-2 by NSAIDs or COX-2-specific inhibitors caused cell death in cancer cells[43-49], indicating that COX-2 was an important molecular target for prevention and treatment in gastrointestinal cancers. In this study, a crucial issue yet to be resolved was whether COX-2 inhibition played a role in the induction of apoptosis by $15dPGJ_2$ in human gastric carcinoma. We found decreased expression of COX-2 mRNA and protein as well as $PGE_2$ production in MCG-803 after $15dPGJ_2$ treatment. Our current study demonstrated that the decrease in COX-2 expression occurred prior to apoptosis, suggesting that down-regulation of COX-2 might be an upstream event of $15dPGJ_2$-induced apoptosis. These changes may imply that $15dPGJ_2$ interferes with the expression of COX-2 and $PGE_2$ production, thereby contributing to induced apoptosis in MCG-803 cells.

The premise is that COX-2 inhibitor has antitumor effect is based on the assumption that prostaglandins and other COX-2 generated downstream mediators promote tumor cell proliferation, survival, and angiogenesis in an autocrine and/or paracrine manner[50-53]. It was also reported that selective cyclo-oxygenase-2 inhibitor induced apoptosis and down-regulated bcl-2 expression in cancer cells[54]. Sheng H also found modulation of apoptosis and Bcl-2 expression by $PGE_2$ in human colon cancer cells[55]. However, Hsu AL reported that the cyclo-oxygenase-2 inhibitor celecoxib induced apoptosis by blocking Akt activation in human prostate cancer cells independent of Bcl-2[56]. In this study no data proved that Bcl-2 was one of the downstream mediators when COX-2 was inhibited by $15dPGJ_2$. The precise mechanisms on the molecular interaction of COX and bcl-2 expression in $15dPGJ_2$ induced gastric apoptosis should be established by more profound analysis.

In summary, our data demonstrate that 15dPGJ2 inhibited the growth of human gastric cancer cells (MCG803). It is proposed that the decrease of bcl-2/bax ratio and COX-2 be responsible for the apoptotic process in MCG803 cells. Furthermore, PPARγ ligand is a new strategic approach to provide new anticancer therapies.

## REFERENCES


1 **Forman BM**, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci* 1997; **94**: 4312-4317

2 **Kliewer SA**, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci* 1997; **94**: 4318-4323

3 **Saffaf P**, Mueller E, Jones D, King F, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, Spiegelman BM. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nature Med* 1998; **4**: 1046-1052

4 **Brockman JA**, Gupta RA, Dubois RN. Activation of PPARgamma leads to inhibition of anchorage-independent growth of human colorectal cancer cells. *Gastroenterology* 1998; **115**: 1049-1055

5 **Fajas L**, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPARgamma gene. *J Biol Chem* 1997; **272**: 18779-18789

6 **Lefebvre AM**, Chen I, Desreumaux P, Najib J, Fruchart JC, Geboes K, Briggs M, Heyman R, Auwerx J. Activation of the peroxisome proliferator-activated receptor g promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. *Nature Med* 1998; **4**: 1053-1057

7 **Saez E**, Tontonoz P, Nelson MC, Alvarez JGA, Ming T, Baird SM, Thomazy VA, Evans RM. Activators of the nuclear receptor PPARgamma enhance colon polyp formation. *Nat Med* 1998; **4**: 1058-1061

8 **Tontonoz P**, Singer S, Forman BM, Sarraf P, Fletcher JA, Fletcher CD, Burn RP, Mueller E, Altiok S, Oppenheim H, Evans RM, Spiegelman BM. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. *Proc Natl Acad Sci U S A* 1997; **94**: 237-241

9 **Kubota T**, Koshizuka K, Willamson EA, Asou H, Said JW, Holden S, Miyoshi I, Koe•er HP. Ligand for peroxisome proliferator-activated receptor gamma (troglitazone) has potent antitumor effect against human prostate cancer both *in vitro* and *in vivo*. *Cancer Res* 1998; **58**: 3344-3352

10 **Elstner E**, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, Shintaku P, Said JW, Heber D, Koe•er HP. Ligands for peroxisome proliferator-activated receptorgamma and retinoic acid

receptor inhibit growth and induce apoptosis of human breast cancer cells *in vitro* and in BNX mice. *Proc Natl Acad Sci U S A* 1998; **95**: 8806-8811

11 **Evans RM**. The steroid and thyroid hormone receptor superfamily. *Science* 1988; **240**: 889-895

12 **Spiegelman BM**. PPARgamma: adipogenic regulator and thiazolidinedione receptor. *Diabetes* 1998; **47**: 507–514

13 **Dreyer C**, Keller H, Mahfoudi A, Laudet V, Krey G, Wahli W. Positive regulation of the peroxisome beta-oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR). *Biol Cell* 1993; **77**: 67-76

14 **Kliewer SA**, Umesono K, Noonan D, Heyman R, Evans RM. Positive regulation of the peroxisome beta-oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR). *Nature* 1992; **358:** 771-774

15 **Lemberger T**, Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol* 1996; **12**: 335–363

16 **Tugwood JD**, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J* 1992; **11**: 433-439

17 **Vanden Heuvel JP**. Peroxisome proliferator-activated receptors (PPARs) and carcinogenesis. *Toxicol Sci* 1999; **47**: 1-8

18 **Yu K**, Bayona W, Kaline CB, Harding HP, Ravera CP, McMahon G, Brown M, Lazar MA. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J Biol Chem* 1995; **270**: 23975–23983

19 **Straus DS**, Glass CK. Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. *Med Res Rev* 2001; **21**: 185-210

20 **Ricote M**, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor g is a negative regulator of macrphage activation. *Nature* 1998; **391**: 79–82

21 **Jiang C**, Ting A, Seed B. PPARγ agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998; **391**: 82–86

22 **Ohta K**, Endo T, Haraguchi K, Hershman JM, Onaya T. Ligands for peroxisome proliferator-activated receptor-γ inhibit growth, and induce apoptosis of human papillary thyroid carcinoma cells. *J Clin Endocrinol Metab* 2001; **86**: 2170–2177

23 **Reed JC**. Mechanisms of Bcl-2 family protein function and dysfunction in health and disease. *Behring Inst Mitt* 1996; **97**: 72-100

24 **Susin SA**, Lorenzo HK, Zamzami N. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999; **397**: 441–446

25 **Lavoie JN**, Nguyen M, Marcellus RC, Brantou PE, Shore GC. E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. *J Cell Biol* 1998; **140**: 637–645

26 **Schlapbach R**, Fontana A. Differential activity of bcl-2 and ICE enzyme family protease inhibitors on Fas and puromycin-induced apoptosis of glioma cells. Biochim. *Biophys. Acta* 1997; **1359**: 174–180

27 **Wolter KG**, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997; **139:** 1281–1292

28 **Rosse T**, Olivier R, Monney L, Rager M, Conus S, Fellay I, Jansen B, Borner C. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* 1998; **391**: 496–499

29 **Vaux DL**. CED-4-the third horseman of apoptosis. *Cell* 1997; **90**: 389–390

30 **Zou H**, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 1997; **90**: 405–413

31 **Pan G**, Humke EW, Dixit VM. Activation of caspases triggered by cytochrome c *in vitro*. *FEBS Lett* 1998; **426**: 151–154

32 **Dubois RN**, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De, Putte LB. Cyclooxygenase in biology and disease. *FASEB J* 1998; **12**: 1063-1073

33 **Herschman HR**. Prostaglandin synthase 2. *Biochem Bioph Acta* 1996; **1299**: 125-140

34 **Taketo MM**. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). *J Natl Cancer Inst* 1998; **90**: 1609-1620

35 **Eberhart CE**, Coffery RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183-1188

36 **Hao X**, Bishop AE, Wallace M, Wang H, Willcocks TC, Maclouf J, Polak JM, Knight S, Talbot IC. Early expression of cyclo-oxygenase-2 during sporadic colorectal carcinogenesis. *J Pathol* 1999; **187**: 295-301

37 **Wilson KT**, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of inducible nitric oxide synthase and cylooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res* 1998; **58**: 2929-2934

38 **Ristimaki A**, Honkanen N, Jankala H. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res* 1997; **57**: 1276-1280

39 **Wolff H**, Ssukkonen K, Anttila S, Karjalainen A, Vainio H, Restimaki A. Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer Res* 1998; **58**: 4997-5001

40 **Mohammed SI**, Knapp DW, Bostwick DG, Foster RS, Khan KN, Masferrer JL, Woerner BM, Snyder PW, Koki AT. Expression of cyclooxygenase-2 (COX-2) in human invasive transitional cell carcinoma (TCC) of the urinary bladder. *Cancer Res* 1999; **59**: 5647-5650

41 **Tsujii M**, DuBois RN. Alterations in celluar adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* 1995; **83**: 493-501

42 **DuBois RN**, Shao J, Tsujii M, Sheng H, Beauchamp RD. G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res* 1996; **56**: 733-737

43 **Chan TA**, Morin PJ, Vogelstein B, Kinzler KW. Mechanisms underlying nonsteroidal anti-inflammatory drug-mediated apoptosis. *Proc Natl Acad Sci USA* 1998; **95**: 681-686

44 **Piazza GA**, Rahm AL, Krutzsch M, Sperl G, Paranka NS, Gross PH. Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res* 1995; **55**: 3310-3116

45 **Hanif R**, Pittas A, Feng Y, Koutsos ML, Qiao L, Staiano-Coico L. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 1996; **52**: 237-245

46 **Thompson HJ**, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. *Cancer Res* 1997; **57**: 267-271

47 **Jones MK**, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat Med* 1999; **5**: 1418-1423

48 **Williams CS**, Tsujii M, Reese J, Dey SK, Dubois RN. Host cyclooxygenase-2 modulates carcinoma growth. *J Clin Invest* 2000; **105**: 1589-1594

49 **Zhang X**, Morham SG, Langenbach R, Young DA. Malignant transformation and antineoplastic actions of nonsteroidal anti-inflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J Exp Med* 1999; **190**: 451-459

50 **Hla T**, Ristimaki A, Appleby S, Barriocanal JG. Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann N Y Acad Sci* 1993; **696**: 197-204

51 **Taketo MM**. Cyclooxygenase-2 inhibitors in tumorigenesis (part 1). *J Natl Cancer Inst* 1998; **90**: 1529-1536

52 **Hla T**, Bishop-Bailey D, Liu CH, Schaefers HJ, Trifan OC. Cyclooxygenase-1 and –2 isoenzymes. *Inst J Biochem Cell Biol* 1999; **31**: 551-557

53 **Prescott SM**, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. *Biochim Biophys Acta* 2000; **1470**: M69-78

54 **Liu XH**, Yao S, Kirschenbaum A, Levine AC. NS398, a selective cyclooxygenase-2 inhibitor, induces apoptosis and down-regulates bcl-2 expression in LNCaP cells. *Cancer Res* 1998; **58**: 4245-4249

55 **Sheng H**, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res* 1998; **58**: 362-366

56 **Hsu AL**, Ching TT, Wang DS, Song X, Rangnekar VM, Chen CS. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by block Akt activation in human prostate cancer cells independently of Bcl-2. *J Biol Chem* 2000; **275**: 11397-11403

**Edited by** Ma JY

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2154-2159
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

# Ets1 as a marker of malignant potential in gastric carcinoma

Yong Yu, Yi-Chu Zhang, Wen-Zhu Zhang, Li-Song Shen, Paul Hertzog, Trevor J Wilson, Da-Kang Xu

**Yong Yu, Yi-Chu Zhang**, Department of General Surgery, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
**Wen-Zhu Zhang**, Department of Pathology, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
**Li-Song Shen**, Laboratory Diagnostic Center, Shanghai Children's Medical Centre, Shanghai Second Medical University, Shanghai 200127, China
**Paul Hertzog, Trevor J Wilson, Da-Kang Xu,** Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash University, Vic 3168, Australia
**Correspondence to:** Da-Kang Xu, MD.PhD, Centre for Functional Genomics and Human Disease, Monash Institute of Reproduction and Development, Monash University, 27-31 Wright Street, Clayton, Vic 3168, Australia. dakangxu@med.monash.edu.au
**Telephone:** +61-3-9594-7229 **Fax:** +61-3-9594-7211
**Received:** 2003-06-30 **Accepted:** 2003-08-18

## Abstract

**AIM:** Ets1 proto-oncogene is a transcription factor involved in the activation of several genes of tumor invasion and metastasis. We aimed to determine the relationship between the extent and intensity of Ets1 expression and patients' clinicopathological factors in gastric carcinoma.

**METHODS:** Immunohistochemical analysis was performed for gastric tumor paraffin-embedded sections, followed by image analysis.

**RESULTS:** Ets1 was not expressed in the normal gastric epithelium and its surrounding cells. The percentage of Ets1 expressing cells detected increased significantly in both epithelial tumor and stromal cells from high T classification, lymph node metastasis positive, clinical advanced-stage groups ($P<0.001$). The level of Ets1 staining in epithelial tumor cells also reflected the degree of cell differentiation. The percentage of epithelial and stromal cells expressing Ets1 was significantly correlated with the presence of lymph node metastasis ($P=0.014$ and $P<0.001$ respectively). Ets1 expression was not observed in tissue samples from patients with benign gastric ulcers.

**CONCLUSION:** Ets1 protein expression in epithelial tumor cells reflects the degree of differentiation, and the percentage of Ets1 positive tumor and stromal cells correlates with lymph node metastasis. Thus Ets1 is a valuable marker of malignant potential in terms of invasiveness and metastasis of gastric carcinoma. It is also possible that inhibition of Ets1 is a potential avenue for therapy in gastric cancer.

Yu Y, Zhang YC, Zhang WZ, Shen LS, Hertzog P, Wilson TJ, Xu DK. Ets1 as a marker of malignant potential in gastric carcinoma. *World J Gastroenterol* 2003; 9(10): 2154-2159
http://www.wjgnet.com/1007-9327/9/2154.asp

## INTRODUCTION

Gastric cancer is a leading cause of cancer death in the world. The pathological stage of gastric cancer, including the depth of tumor penetration and the presence of metastases to lymph nodes or distal organs, remains the most important determinant in its prognosis. Usually, these parameters can be determined by microscopic examination of tissue sections from the primary neoplasm and lymph nodes. However, where only primary gastric carcinoma specimens are available for histopathological examination cannot always accurately predict metastasis and disease progression. Recently, from microarray gene expression profiling data[1], the occurrence and progression of cancer were suggested to be related to a series of genetic events affecting the structure and expression of a number of genes. These genes include potential oncogenes, tumor suppressors and those involved in angiogenesis and extracellular matrix (ECM) remodeling. The factors regulating these genes could thus be important markers for disease prognosis and potential therapeutic targets. Since most transcription factors are expressed at very low levels (-10 copies/cell), sensitive assays are required. One suitable approach is to use a specific antibody to detect these factors by immunohistochemical analysis. This allows analysis of the epithelial tumor cells and its surrounding stromal cells. In addition, this approach will enable further understanding of the molecular mechanisms of gastric carcinoma invasion and metastasis.

The Ets family of transcription factors is defined by a conserved DNA binding domain of approximately 85 amino acids. These proteins bind to specific purine-rich DNA sequences, with a core motif of GGAA/T, and have been shown to transcriptionally regulate a number of viral and cellular genes. Ets factors have been shown to be important in a number of biological processes, including cellular proliferation, differentiation, development, transformation, and apoptosis[2-6]. Indeed the Ets1 protein has recently been shown to regulate genes such as vascular endothelial growth factor(VEGF), urokinase plasminogen activator (uPA), matrix-metallopteases (MMPs) and integrin in a variety of cancer cell lines and tissues[7-12]. Many of these genes are involved in angiogenesis and extracellular matrix (ECM) remodeling, important events in the processes of cancer invasion and metastasis.

Microarray analysis from a number of epithelial tumor tissues or cell lines indicate that alterations in Ets1 expression are associated with lung carcinoma[13], breast carcinoma[14], pancreatic carcinoma[15], thyroid carcinoma[16] and oral squamous cell carcinoma[17]. However, it is not clear from these data which cells in the tumor expressed Ets1 or whether this expression was associated with invasive growth. Thus the purpose of this study was to analyze the relationship between Ets1 expression and the malignant behavior in gastric carcinoma, in particular to determine whether Ets1 may have a role in breaking through the muscularis mucosae by invasive tumors and metastasis. This will determine whether Ets1 has potential as a credible marker in predicting the metastatic potential of gastric cancer and assisting in elucidating its role in gastric carcinoma's malignant progression.

## MATERIALS AND METHODS

### *Samples and patients*

Since January 2001 to June 2002, we collected biopsies from 43 patients who underwent surgery for gastric neoplasia in the General Surgery Department of Xinhua Hospital, Shanghai Second Medical University. The data set for each patient

included sex, age, histological classification, clinical grade, depth of infiltration (T classification), presence of lymph node metastasis (N classification), distant metastasis (M classification), and clinical stage. Pathological assessments were determined by clinical pathologists according to WHO standards. T1 indicated infiltration of the *lamina propria* or submucosa, T2 infiltration of muscularis propria or the subserosa, T3 penetration of the serosa (visceral peritoneum), and T4 penetration of the serosa and infiltration of adjacent structures. Lymph node (N) classification was divided into no metastasis (N0) and metastasis in 1-6 (N1), 7-15 (N2) or >15 (N3) regional lymph nodes. M classification was M1 for presence or M0 for absence of distant metastasis. Clinical stage was determined by combination of these parameters as stage IA (T1N0M0), stage IB (T1N1M0), stage II (T1N2M0, T2N1M0 or T3N0M0), stage IIIA (T2N2M0, T3N1M0 or T4N0M0), stage IIIB (T3N2M0) and stage IV (TxN3M0, T4NxM0, TxNxM1). In addition, two samples of both benign gastric ulcer and breast cancer were used as controls.

Samples were fixed in formalin immediately after the operation and specially prepared for immunohistochemistry. Briefly, samples of no more than 2×1.5×0.2 cm were dehydrated at 4 ℃, and low temperature paraffin wax (<60 ℃) was used for embedding. 3 μm thick sections were de-waxed immediately before use with xylene (4 ℃) and rehydrated for immunohistochemistry.

### *Immunohistochemistry*

Endogenous peroxidase activity was quenched with 3 % hydroperoxide followed by incubation with 1 % bovine serum albumin to block any nonspecific binding. Primary antibodies used were anti-human Ets1 monoclonal antibody (Transduction Laboratories, Lexington, KY), rabbit anti-human Ets1 polyclonal antibody (Santa Cruz Biotechnology, CA) or rabbit anti-human cytokeratin polyclonal antibody (DAKO, Copenhagen, Denmark). Amplification of the primary antibody reaction was achieved by incubation with an appropriate secondary antibody [Goat anti-Rabbit or anti-mouse IgG, Gene Tech] conjugated to peroxidase. The binding was visualized by DAB and the sections were counterstained with hematoxylin. All incubations and washing were performed at room temperature.

### *Image analysis*

Intensities, percentages, and patterns of immunohistochemical staining for each sample slide were analyzed by KS4000 image analysis system (Zeiss Company, Germany) and recorded individually. For each sample, six representative images were collected at high magnification (×400). Image analysis determined the proportion of epithelial or stromal cells within the tumor expressing Ets1 and the percentage of cells with moderate and intense staining. The data were expressed as proportion of tumor.

### *Statistic analysis*

The data were presented as the mean ± standard deviation (SD) for each group. Statistical analysis was performed using analysis of variance (ANOVA) and Mann-Whitney non-parametric test. Intergroup difference was evaluated by Fisher' s test. $P<0.01$ was considered statistically significant.

## RESULTS

### *Patients characteristics*

Samples were obtained from 41 patients, 30 males and 10 females. The age range was from 37 to 87. The 41 patients were diagnosed with malignant tumor. The histological classification included adenocarcinoma (30), signet ring cell carcinoma (6), undifferentiated carcinoma (4) and gastrointestinal stromal tumor (GIST) (1). The samples were divided into two groups according to their depth of infiltration (WHO classification): 15 of T1 or T2, and 25 of T3 or T4. Specimens were also divided according to histological grade: grade I (highly differentiated carcinoma) for 7 samples, grade II (moderately differentiated carcinoma) for 19 samples, grade III (poorly differentiated carcinoma) for 14 samples. 26/40 patients demonstrated lymph node metastasis and only one patient had known distant metastasis (liver).

### *Expression of Ets1 in gastric carcinoma*

In our study, staining of Ets1 was observed in all malignant gastric tumor cells, including adenocarcinoma, signet ring cell adenocarcinoma and undifferentiated carcinoma, and the results are summarized in Table 1. Epithelial tumor cells stained for Ets1 were mainly in the nucleus and only rarely was staining observed in the cytoplasm (Figure 1A). No staining for Ets1 was observed in the non-neoplastic tissue or in tissues from a patient with benign gastric ulcer (Figure 1G). In contrast, antibodies to another Ets factor which is known to be epithelium-specific (Elf5), stained normal gastric epithelium, but lost its expression in gastric cancer (data not shown). Ets1 staining was also observed in newly formed blood vessel endothelial cells (Figure 1B) and interstitial cells in surrounding tissue (Figure 1C). Ets1 protein was localized in both cytoplasm and nucleus of the stromal cells. Furthermore intense staining for Ets1 was observed in invasive cells at the junction of normal tissue and malignant tissue (Figure 1D). Increased Ets1 protein expression observed by immunohistochemistry was confirmed by quantitation of Ets1mRNA levels by RT-PCR and Northern blot (data not shown). Immunohistochemistry of a breast cancer specimen also detected Ets1, but it was predominantly expressed in the cytoplasm (Figure 1E).

### *Relationship between percentage of Ets1 expressing cells and pathological assessment in gastric carcinoma*

**Age, sex and histological grade** Statistical analysis determined that there was no significant correlation between the expressions of Ets1 in gastric cancer and age, sex or WHO histopathological classifications. Although there was a trend in proportions of Ets1 positively stained epithelial cells from 26.9 % in grade I, increasing to 39.5 % in grade II and 32.8 % in grade III (Table 1), there was no significant correlation ($P=0.366$). A similar trend was also observed for staining of tumor stromal cells ($P=0.486$).

**Depth of infiltration (T) classification** Considering the importance of breaking through muscularis mucosae, we compared the groups with tumors confined to the mucosa/submucosa (15 samples, T1 or T2) and those tumors which had penetrated the serosa (25 samples, T3 or T4). Our data demonstrated that Ets1 was detected in a higher proportion of both epithelial and stromal cells in tumors from patients with more advanced disease. For example, 17.2±12.0 % of tumor epithelial cells from T1 or T2 tumors were positive for Ets1, whereas 45.6±17.9 % were positive for T3 or T4 tumours ($P<0.001$, Table 1). The proportion of Ets1 expressing tumor stromal cells also significantly correlated with the presence of more advanced disease T classification ($P<0.05$).

**Lymph node and distant metastasis** Similarly, grouping of patients according to the presence (N123) or absence (N0) of lymph node metastasis showed an increase in Ets1 positive tumor epithelial cells from 24.1±17.4 % (N0) to 40.8±20.8 % (N123, $P<0.05$). In addition, the proportion of Ets1 expressing tumor stromal cells was also significantly higher in patients with lymph node metastasis ($P<0.001$). Only one patient in our samples had known distant metastasis, but Ets1 was detected in both epithelial and stromal cells in tumor sections from this patient (71 % and 61 % respectively).

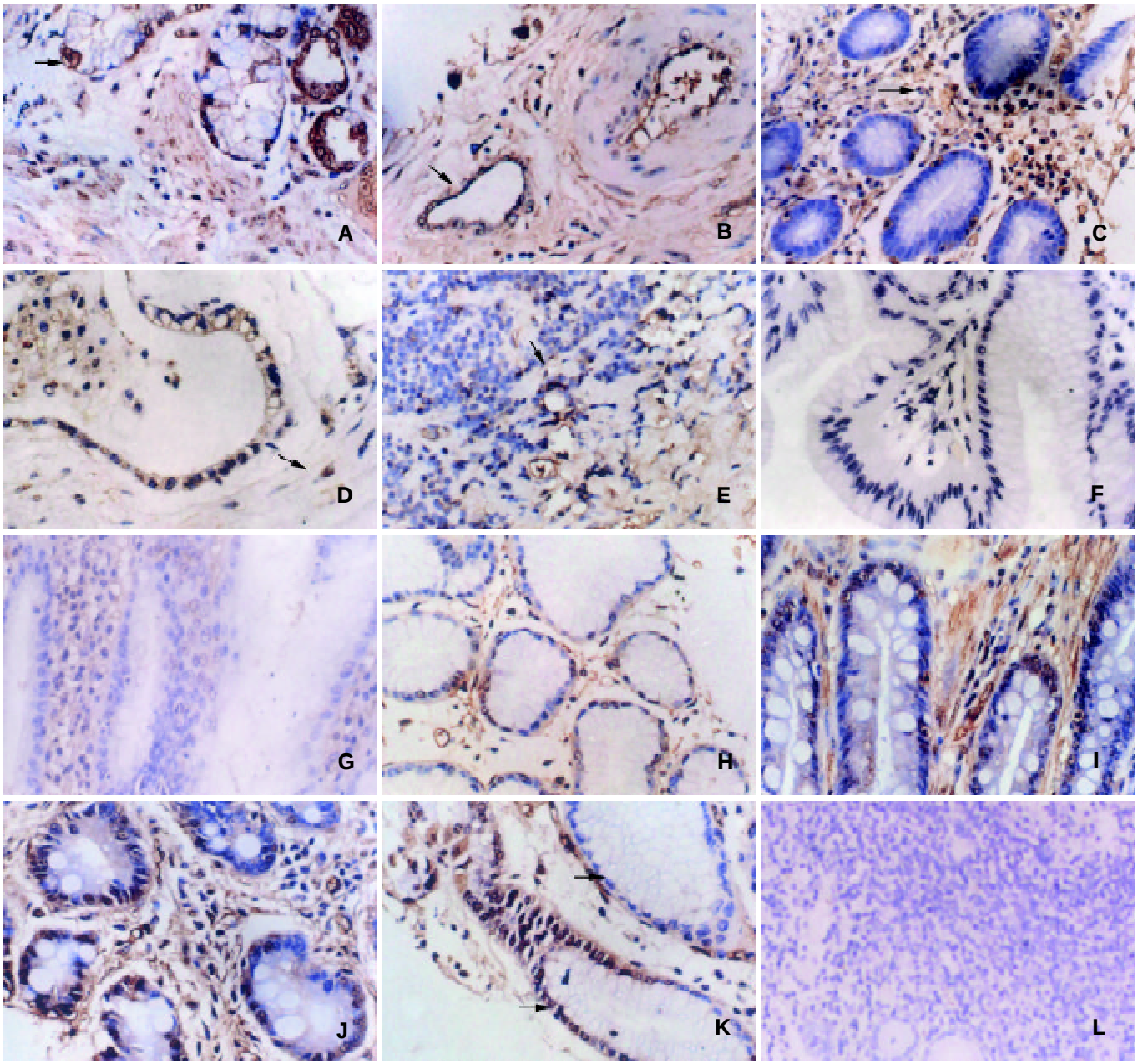



**Figure 1** A: Ets1 immunohistochemical stain in gastric cancer cells. The arrowed cell is a typical Ets1 positively stained cell. The area of stain was mainly, located in the nucleus of epithelial tumor cells. Ets1 protein was localized in both cytoplasm and nucleus of the stromal cells. B: Ets1 expression in vascular endothelial cells. This was an example of pooly differentiated gastric adenocarcinoma. Ets1 was present in endothelial cells of blood vessels (arrowed). C: Ets1 is expressed in interstitial cells. Photomicrograph of a typical gastric carcinoma. Ets1 positive stromal cells were detected in the interstitial tissue. D: The expression of Ets1 in polarity. Image of gastric cancer cells infiltrating to the muscular layer. Intense staining of Ets1 was observed at the junction of the infiltrating tumor cells and the serosa. We termed it the polarity expression. E: Ets1 expression in breast carcinoma. Example of breast carcinoma, Ets1 positive cells arrowed. F: Ets1 negative control staining. G: Ets1 is not expressed in glandular cells of benign gastric ulcer. H-J: Ets1 expression in tissues with different histopathological grades. Section of grade I(H), grade II(I) and grade III(J) gastric cancer showing increased positive cells and intensity of Ets1 staining with advanced disease. K: Ets1 expression is high in undifferentiated tumor cell. The image contained well differentiated and poorly differentiated tumor tissues. The number of positive cells and the intensity of the Ets1 staining were increased in undifferentiated tumor cells. L: Ets1 is not expressed in GIST. Photomicrograph of gastrointestinal stromal tumor (GIST). No Ets1 positive cancer cells were observed. (A, H, I, J, K original magnification ×400), (B, C, D, E, F, G original magnification ×100), (L, original magnification ×50).

**Clinical stage** When the T, N and M classifications were combined into the WHO defined clinical stages, there was a significant correlation between Ets1 staining in epithelial and stromal cells with the presence of more advanced disease ($P<0.001$ and $P<0.005$ respectively).

### *Relationship between Ets1 levels and clinical disease*

Our image analysis also enabled us to compare the signal intensity of Ets1 staining (Table 2-1,2), which reflected the levels of Ets1 (confirmed by RT-PCR, data not shown). When all the patients were considered together, the majority (24/40, 60 %) displayed moderate staining levels in tumor epithelial cells whereas 10/40 (25 %) displayed intense staining. Of these 10 patients with intense staining, however, 9 had tumors penetrating the mucosa (T3 or T4) and 8 had metastasis into the regional lymph nodes. The single patient with distant metastasis also displayed intense staining for Ets1. Similar high levels of Ets1 labelling were observed in stromal cells from

patients with advanced disease. In stromal cells 16/40 (40 %) displayed intense staining. These 16 patients with intense staining included a single patient with distant metastasis, while 11 of these 16 patients had tumors penetrating the mucosa (T3 or T4) and metastasis into the regional lymph nodes. Thus it appeared both the level of Ets1 and the proportion of positive cells were correlated with advanced gastric carcinoma.

**Table 1** Ets1 expression in tumor and stromal cells

| Factors | No. | Ets1 percentage[T] (mean±SD) | *P* value | Ets1 percentage[S] (mean±SD) | *P* value |
|---|---|---|---|---|---|
| Total patients | 40 | 35.0±21.1 | | 44.6±17.5 | |
| Gender | | | | | |
| Male | 30 | 38.0±22.5 | 0.115 | 45.2±17.3 | 0.704 |
| Female | 10 | 25.9±13.1 | | 42.7±18.7 | |
| Age | | | | | |
| >50 | 32 | 35.9±20.8 | 0.572 | 46.3±17.7 | 0.206 |
| <50 | 8 | 31.1±23.3 | | 37.5±15.7 | |
| Histological classification (WHO) | | | | | |
| Ad | 30 | 35.2±19.9 | 0.269 | 43.0±15.4 | 0.647 |
| Srcc | 6 | 43.3±26.4 | | 49.3±15.2 | |
| Uc | 4 | 21.1±19.6 | | 48.8±34.7 | |
| Grade (WHO) | | | | | |
| I | 7 | 26.9±26.7 | 0.366 | 37.3±18.3 | 0.486 |
| II | 19 | 39.5±19.7 | | 45.7±11.6 | |
| III | 14 | 32.8±19.8 | | 46.6±23.2 | |
| T (tumor) | | | | | |
| T1,2 | 15 | 17.2±12.0 | <0.001 | 35.3±17.7 | 0.008 |
| T3,4 | 25 | 45.6±17.9 | | 50.1±15.1 | |
| N (lymph node) | | | | | |
| N0 | 14 | 24.1±17.4 | 0.014 | 32.4±13.8 | 0.001 |
| N1,2,3 | 26 | 40.8±20.8 | | 51.1±15.8 | |
| M (metastasis) | | | | | |
| M0 | 39 | 34.0±20.5 | <0.001 | 44.1±17.5 | <0.001 |
| M1 | 1 | 71 | | 61 | |
| Clinical stage | | | | | |
| I, II | 18 | 21.8±15.2 | <0.001 | 36.2±18.1 | 0.005 |
| III, IV | 22 | 45.7±19.2 | | 51.4±13.9 | |

[T] refers to Ets1 expression in tumor cells [S] refers to Ets1 expression in stromal cells.

**Table 2-1** Intensity of Ets1 staining in tumor cell and distribution of patients

| Compartment | No. | Weak (+) | Moderate (++) | Intense (⩾+++) | *P* value |
|---|---|---|---|---|---|
| Tumor cells | 40 | 6 (15 %) | 24 (60 %) | 10 (25 %) | |
| T classification | | | | | |
| T1,2 | 15 | 2 (13.3 %) | 12 (80 %) | 1 (6.7 %) | 0.087 |
| T3,4 | 25 | 4 (16 %) | 12 (48 %) | 9 (36 %) | |
| LN metastasis | | | | | |
| Negative | 14 | 3 (21.4 %) | 9 (64.3 %) | 2 (14.3 %) | 0.438 |
| Positive | 26 | 3 (11.5 %) | 15 (57.7 %) | 8 (30.8 %) | |
| Distant metastasis | | | | | |
| Negative | 39 | 6 (15.4 %) | 24 (61.5 %) | 9 (23.1 %) | 0.214 |
| Positive | 1 | 0 (0 %) | 0 (0 %) | 1 (100 %) | |
| Clinical stage | | | | | |
| Stage I,II | 18 | 3 (16.7 %) | 13 (72.2 %) | 2 (11.1 %) | 0.183 |
| Stage III,IV | 22 | 3 (13.6 %) | 11 (50 %) | 8 (36.4 %) | |

**Table 2-2** Intensity of Ets1 staining in stromal cell and distribution of patients

| Compartment | No. | Weak (+) | Moderate (++) | Intense (⩾+++) | *P* value |
|---|---|---|---|---|---|
| Stromal cells | 40 | 3 (7.5 %) | 21 (52.5 %) | 16 (40 %) | |
| T classification | | | | | |
| T1,2 | 15 | 3 (20 %) | 7 (46.7 %) | 5 (33.3 %) | 0.066 |
| T3,4 | 25 | 0 (0 %) | 14 (56 %) | 11 (44 %) | |
| LN metastasis* | | | | | |
| Negative | 14 | 3 (21.4 %) | 6 (42.9 %) | 5 (35.7 %) | 0.048 |
| Positive | 26 | 0 (0 %) | 15 (57.7 %) | 11 (42.3 %) | |
| Distant metastasis | | | | | |
| Negative | 39 | 3 (7.7 %) | 21 (53.8 %) | 15 (38.5 %) | 0.463 |
| Positive | 1 | 0 (0 %) | 0 (0 %) | 1 (100 %) | |
| Clinical stage | | | | | |
| Stage I,II | 18 | 3 (16.7 %) | 8 (44.4 %) | 7 (38.9 %) | 0.129 |
| Stage III,IV | 22 | 0 (0 %) | 13 (59.1 %) | 9 (40.9 %) | |

## DISCUSSION

Gastric cancer is the most frequent malignancy of the gastrointestinal tract in China and the second most common cause of cancer-related death in the world[18]. The prognosis of patients with gastric cancer has been improving owing to the progress in diagnostic techniques and treatment methods for gastric cancer, but peritoneal dissemination is the main cause of recurrence after curative resection of advanced cancer. The prognosis of gastric cancer which has invaded as far as the gastric serosa was still poor with a 5-year survival of less than 35 %[19]. Among these malignant characteristics of gastric cancer cells, metastasis to the peritoneum is an especially complex phenomenon, which requires the involvement of many different genes in multiple steps for tumor cells. Although many aspects of gastric cancer metastasis await further clarification, adhesion molecules, apoptosis-related genes, and others have been reported to play an important role in peritoneal dissemination of gastric cancers[20], but details of the mechanism involved remain unclear. Since Ets1 has been shown to regulate many genes involved in angiogenesis and extracellular matrix (ECM) remodeling, events important in cancer metastasis, we analyzed the relationship between Ets1 expression and the malignant behavior in gastric carcinoma. This demonstrated that Ets1 expression was related to the pathological stage of gastric cancer, including tumor infiltration and presence of metastases to lymph nodes or distal organs. These data are consistent with other studies that have shown significant correlation between Ets1 expression in tumor cells and the presence of lymph node metastasis. But our data also demonstrated that Ets1 expression in cancer associated stromal cells was significantly correlated with the presence of lymph node metastasis. The proportion of Ets1 expressing cells was also associated with metastasis kinetics, expression of Ets1 within a few epithelial tumor cells in early disease, then expression in stromal, endothelial and other cells in advanced malignant disease where cancer has broken through muscularis mucosae. This suggested that Ets1 had a specific role in the regulation of genes involved in gastric invasion and metastasis, rather than carcinogenesis[2,3] in gastric carcinoma. It also indicated that Ets1 was a promising marker of malignant potential and a potential avenue for therapeutic intervention in gastric cancer.

The level of Ets1 expression in epithelial tumor cells was correlated with the progression of disease, perhaps associated with the degree of cancer cell differentiation. In addition, Ets1 expressing cells were located at the invasive front of the tumor. This is consistent with previous observations that Ets1 was associated with branching morphogenesis and organ formation in embryos[21] and embryonic stem cell differentiation (Xu *et al*.,

unpublished data). Other cell types in gastric cancer, such as stromal cells, significantly increased Ets1 expression which was correlated with penetration through the muscularis mucosae and the presence of lymph node metastasis. These data are important observations since the key approach for advancing our treatment of gastric carcinoma is to clarify the mechanism of infiltration, especially for the first step, namely penetrating the muscularis mucosae. Previously, the interrelated elements concerned with gastric carcinoma' s penetration of the extracellular matrix (ECM), angiogenesis and the surrounding microenvironment have not been connected.

Increased expression of genes encoding enzymes involved in degradation of the extracellular matrix (ECM), such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), and MMP-9 (type IV collagenase/gelatinase) has been identified in cancer from recent microarray data[22]. Hence, Ets1 is likely to contribute to tumor invasion and progression through activation of these enzymes. Indeed, expression of these ECM remodeling enzymes was detected concomitant with Ets1 mRNA in tumor cells and/or stroma cells.

Ets1 is also involved in angiogenesis, which is essential for tumor progression. In a non-vascularized tumor the growing tumor becomes hypoxic, thus the observation that Ets1 was induced by hypoxia via hypoxia-inducible factor-1 (HIF-1)[23] is important. VEGF and bFGF also induce Ets1 in endothelial cells. Ets1 was believed to confer an angiogenic phenotype to the endothelial cells through induction of the urokinase-type plasminogen activator (u-PA))[24-26] and MMPs, and also to regulate N-acetylglucosaminyl-transferase V (GnT-V), which has been associated with metastasis of tumors[27]. Thus the microenvironment of a growing tumor may induce Ets1 in tumor cells and/or stroma cells, which subsequently induces angiogenesis-related genes and ECM remodeling enzymes required for tissue invasion and metastasis. It is interesting to note that expression of Ets1 in both tumor and stroma was correlated with poor prognosis in human ovarian carcinoma[28].

Recent development in microarray technologies has resulted in extensive profiling of cancer and cancer metastases[29]. These approaches have generated a vast amount of new data to investigate the molecular mechanisms of cancer metastasis, however, many of these studies have not identified Ets1 as significantly increased. This is surprising given both our data and other data demonstrating increased Ets1 in lung carcinoma, breast carcinoma, pancreatic carcinoma, thyroid carcinoma, and oral squamous cell carcinoma[7-12]. We suggest that this anomaly is due to the sensitivity of microarray analysis, which is commonly used to detect genes with altered expression of more than 2 fold. As a transcription factor, 1-2 fold increased expression could have significant biological effects. It is interesting to note that one microarray study of gastric cancer cell lines reported that it did not find any transcription factor more than 2.0 fold upregulated from 20 k genes examined. However this study identified increased levels of defined Ets regulated genes, such as MMP and VEGF. It is also possible that mRNA status was not consistent with protein expression status or perhaps variation in cell types and Ets1 expression in the tumor sample concealed a more localized Ets1 increase. Thus we believe that our results from immunohistochemistry do not conflict with these microarray data.

In addition to the potential of Ets1 as a diagnostic marker, there is also potential for Ets1 as a potential therapeutic target for metastasis in gastric carcinogenesis. Since increased expression of Ets1 and subsequently its downstream target genes (MMPs, VEGF and HIF) contribute to invasiveness and metastasis of gastric carcinoma, inhibition of its expression is potentially therapeutic. Recently, we reported that inhibition of Ewing' s sarcoma associated EWS/FLI-1 transcription via sequence-specific transcriptional suppressor was sufficient to inhibit the transformed phenotype[30]. Thus specific inhibition of Ets1 function (or induction of Ets1) has potential for reducing metastasis in gastric carcinoma[31].

## REFERENCES


1 **Hasegawa S**, Furukawa Y, Li M, Satoh S, Kato T, Watanabe T, Katagiri T, Tsunoda T, Yamaoka Y, Nakamura Y. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res* 2002; **62**: 7012-7017

2 **Yordy JS**, Muise-Helmericks RC. Signal transduction and the Ets family of transcription factors. *Oncogene* 2000; **19**: 6503-6513

3 **Xu D**, Wilson TJ, Chan D, De Luca E, Zhou J, Hertzog PJ, Kola I. Ets1 is required for p53 transcriptional activity in UV-induced apoptosis in embryonic stem cells. *EMBO J* 2002; **21**: 4081-4093

4 **Wolvetang EJ**, Wilson TJ, Sanij E, Busciglio J, Hatzistavrou T, Seth A, Hertzog PJ, Kola I. ETS2 overexpression in transgenic models and in Down syndrome predisposes to apoptosis via the p53 pathway. *Hum Mol Genet* 2003; **12**: 247-255

5 **Li X**, Lu JY, Zhao LQ, Wang XQ, Liu GL, Liu Z, Zhou CN, Wu M, Liu ZH. Overexpression of ETS2 in human esophageal squamous cell carcinoma. *World J Gastroenterol* 2003; **9**: 205-208

6 **Zhou J**, Ng AY, Tymms MJ, Jermiin LS, Seth AK, Thomas RS, Kola I. A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene* 1998; **17**: 2719-2732

7 **Baillat D**, Begue A, Stehelin D, Aumercier M. ETS-1 transcription factor binds cooperatively to the palindromic head to head ETS-binding sites of the stromelysin-1 promoter by counteracting autoinhibition. *J Biol Chem* 2002; **277**: 29386-29398

8 **Rutter JL**, Mitchell TI, Buttice G, Meyers J, Gusella JF, Ozelius LJ, Brinckerhoff CE. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. *Cancer Res* 1998; **58**: 5321-5325

9 **Nakada M**, Yamashita J, Okada Y, Sato H. Ets-1 positively regulates expression of urokinase-type plasminogen activator (uPA) and invasiveness of astrocytic tumors. *J Neuropathol Exp Neurol* 1999; **58**: 329-334

10 **Trojanowska M**. Ets factors and regulation of the extracellular matrix. *Oncogene* 2000; **19**: 6464-6471

11 **Liu Z**, Klominek J. Regulation of matrix metalloprotease activity in malignant mesothelioma cell lines by growth factors. *Thorax* 2003; **58**: 198-203

12 **Chung AS**, Yoon SO, Park SJ, Yun CH. Role of matrix metalloproteinases in tumor metastasis and angiogenesis. *J Biochem Mol Biol* 2003; **36**: 128-137

13 **Sasaki H** , Yukiue H, Moiriyama S, Kobayashi Y, Nakashima Y, Kaji M, Kiriyama M, Fukai I, Yamakawa Y, Fujii Y. Clinical significance of matrix metalloproteinase-1 and Ets-1 gene expression in patients with lung cancer. *J Surg Res* 2001; **101**: 242-247

14 **Span PN**, Manders P, Heuvel JJ, Thomas CM, Bosch RR, Beex LV, Sweep CG. Expression of the transcription factor Ets-1 is an independent t prognostic marker for relapse-free survival in breast cancer. *Oncogene* 2002; **21**: 8506-8509

15 **Ito T**, Nakayama T, Ito M, Naito S, Kanematsu T, Sekine I. Expression of the ets-1 proto-oncogene in human pancreatic carcinoma. *Mod Pathol* 1998; **11**: 209-215

16 **Nakayama T**, Ito M, Ohtsuru A, Naito S, Nakashima M, Sekine I. Expression of the ets-1 proto-oncogene inhuman thyroid tumor. *Mod Pahtol* 1999; **12**: 61-68

17 **Soni S**, Pande P, Shukla NK, Ralhan R. Coexpression of Ets-1 and p53 in oral carcinomas is associated with P-glycoprotein expression and poor prognosis. *J Cancer Res Clin Oncol* 2002; **128**: 336-342

18 **Pisani P**, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; **83**: 18-29

19 **Shimada K**, Ajani JA. Adjuvant therapy for gastric carcinoma patients in the past years: a review of western and oriental trials. *Cancer* 1999; **86**: 1657-1668

20 **Tahara E**. Molecular aspects of invasion and metastasis of stomach cancer. *Verh Dtsch Ges Pathol* 2000; **84**: 43-49

21 **Kola I**, Brookes S, Green AR, Garber R, Tymms M, Papas TS, Seth A. The Ets1 transcription factor is widely expressed during murine embryo development and is associated with mesodermal cells involved in morphogenetic processes such as organ formation. *Proc Natl Acad Sci U S A* 1993; **90**: 7588-7592
22 **Kallioniemi OP**, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum Mol Genet* 2001; **10**: 657-662
23 **Oikawa M**, Abe M, Kurosawa H, Hida W, Shirato K, Sato Y. Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-inducible factor-1. *Biochem Biophys Res Commun* 2001; **289**: 39-43
24 **Sato Y**, Teruyama K, Nakano T, Oda N, Abe M, Tanaka K, Iwasaka-Yagi C. Role of transcription factors in angiogenesis: Ets-1 promotes angiogenesis as well as endothelial apoptosis. *Ann N Y Acad Sci* 2001; **947**: 117-123
25 **Takai N**, Miyazaki T, Fujisawa K, Nasu K, Miyakawa I. Expression of c-Ets1 is associated with malignant potential in endometrial carcinoma. *Cancer* 2000; **89**: 2059-2067
26 **Behrens P**, Rothe M, Wellmann A, Krischler J, Wernert N. The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. *J Pathol* 2001; **194**: 43-50
27 **Ko JH**, Miyoshi E, Noda K, Ekuni A, Kang R, Ikeda Y, Taniguchi N. Regulation of the GnT-V promoter by transcription factor Ets-1 in various cancer cell lines. *J Biol Chem* 1999; **274**: 22941-22948
28 **Davidson B**, Reich R, Goldberg I, Gotlieb WH, Kopolovic J, Berner A, Ben Baruch G, Bryne M, Nesland JM. Ets-1 messenger RNA expression is a novel marker of poor survival in ovarian carcinoma. *Clin Cancer Res* 2001; **7**: 551-557
29 **Inoue H**, Matsuyama A, Mimori K, Ueo H, Mori M. Prognostic score of gastric cancer determined by cDNA microarray. *Clin Cancer Res* 2002; **8**: 3475-3479
30 **Chan D**, Wilson TJ, Xu D, Cowdery HE, Sanij E, Hertzog PJ, Kola I. Transformation induced by Ewing's sarcoma associated EWS/FLI-1 is suppressed by KRAB/FLI-1. *Br J Cancer* 2003; **88**: 137-145
31 **Shuey DJ**, McCallus DE, Giordano T. RNAi: gene-silencing in therapeutic intervention. *Drug Discov Today* 2002; **7**: 1040-1046

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2160-2163
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

# Expression of MTLC gene in gastric carcinoma

Guang-Bin Qiu, Li-Guo Gong, Dong-Mei Hao, Zhi-Hong Zhen, Kai-Lai Sun

**Guang-Bin Qiu, Li-Guo Gong, Dong-Mei Hao, Zhi-Hong Zhen, Kai-Lai Sun,** Department of Medical Genetics, China Medical University, Shenyang 110001, Liaoning Province, China
**Supported by** the National Natural Science Foundation of China, No.30171008; Funds of Educational Department, Liaoning Province, No. 20121034
**Correspondence to:** Kai-Lai Sun, Department of Medical Genetics, China Medical University, No.92 North 2nd Road, Heping District, Shenyang 110001, Liaoning Province, China. sunkailai@21cn.com
**Telephone:** +86-24-23256666-5325 **Fax:** +86-24-23265842
**Received:** 2003-03-12 **Accepted:** 2003-05-11

## Abstract

**AIM:** To investigate the expression of c-myc target from laryngeal cancer cells (MTLC) gene in gastric carcinoma (GC) tissues and the effect of MTLC over-expression on gastric carcinoma cell line BGC823.

**METHODS:** RT-PCR was performed to determine the expression of MTLC mRNA in GC and matched control tissues. BGC823 cells were transfected with an expression vector pcDNA3.1-MTLC by liposome and screened by G418. Growth of cells expressing MTLC was observed daily by manual counting. Apoptotic cells were determined by TdT-mediated dUTP nick-end labeling (TUNEL) assay.

**RESULTS:** The expression of MTLC mRNAs was down-regulated in 9(60%) of 15 cases of GC tissues. The growth rates of the BGC823 cells expressing MTLC were indistinguishable from that of control cells. A marked acceleration of apoptosis was observed in MTLC-expressing cells.

**CONCLUSION:** MTLC was down-regulated in the majority of GC tissues and could promote apoptosis of GC cell lines, which suggests that MTLC may play an important role in the carcinogenesis of gastric carcinoma.

Qiu GB, Gong LG, Hao DM, Zhen ZH, Sun KL. Expression of MTLC gene in gastric carcinoma. *World J Gastroenterol* 2003; 9(10): 2160-2163
http://www.wjgnet.com/1007-9327/9/2160.asp

## INTRODUCTION

Gastric carcinoma (GC) is one of the most common malignant tumors in the world[1,2]. Numerous data have shown that some genes such as p53, c-myc, bcl-2, COX-2 and PTEN[3-6] might be associated with the gastric carcinogenesis. However, the exact molecular mechanism underlying GC remains to be fully elucidated. Therefore, it is necessary to look for novel genes to obtain a thorough understanding about gastric carcinogenesis.

c-myc target from laryngeal cancer cells (MTLC) gene, a putative target of c-myc, was recently cloned in our laboratory (GenBank access number AF527367). MTLC was located in 6q25, a chromosome region involved in various kinds of cancers[7-11]. Previous studies have shown that its protein product expressed in nuclei and might take part in the regulation of cell cycle[12], suggesting that MTLC was potentially related to the carcinogenesis. In this study, we therefore performed RT-PCR and eukaryotic transfection to reveal the relationship between MTLC and GC.

## MATERIALS AND METHODS

### *Tissues and cell line*

All the gastric cancer and matched control tissues confirmed pathologically were obtained from the First Affiliated Hospital of China Medical University. Tumor tissues were dissected from the resected specimens. The normal tissue block was taken from the distal resection margin and was apart from cancer at least 1 cm. Gastric carcinoma cell line BGC823 was kept in our laboratory.

### *RT-PCR*

Total RNAs were extracted from cancer tissues by TRIZOL reagents (GibcoBRL, Grand Island, NY, USA), and were reverse-transcripted to the first strand of cDNA using reverse transcriptase system (Promega, Madison, WI, USA). MTLC cDNA was amplified by PCR under the following condition: first at 95 ℃for 1 min, 30 cycles at 95 ℃for 30 s, at 60 ℃for 1 min, at 72 ℃for 1.5min, and finally at 72 ℃for 10 min. PCR primers consisted of the sequences of forward: 5-ATGGATCCCTGCACTGGCTGATGAGTGTGTA-3 and reverse: 5-GTAAGCTTGAACAGTGCCTTCACCCTCG AGGT-3. β-actin gene was used as internal control.

### *Construction of MTLC expression vector*

MTLC segment amplified by PCR was ligated to pMD-18T vector (Takara, Dalian, China) by TA cloning. The recombinant was digested by *BamH I* and *EcoR I*, and then the target fragment was recollected and cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Both PCR product and the expression vector pcDNA3.1-MTLC were confirmed by sequencing to avoid mutation.

### *Transfection and screening of BGC823 cells*

BGC823 cells in logarithmic phase were seeded in 35 mm plates and cultured with DMEM containing 10 % serum overnight. Cells were transfected with 1 μg expression vector or empty parental vector by Lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) and subsequently screened by G418 at a final concentration of 5 g/L after cultured for 24 h.

### *Observation of cell growth*

Cells transfected by pcDNA3.1-MTLC or empty parental vector were plated in 35 mm plates at a concentration of $1\times10^5$ cells/plate with DMEM culture containing 10 % serum. Individual plates were trypsinized daily and the total number of viable cells per plate was determined by manual counting.

### *Detection of apoptosis*

DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) was used to determine the apoptosis of cells. $1\times10^5$ cells transfected by pcDNA3.1-MTLC or empty parental vector were seeded into a plate with a poly-L-lysine-coated slide on its center and grown for 24 h in DMEM culture containing 10 % serum. The cells were then maintained for additional

18 h in serum-free culture and then detected according to the protocol provided by the manufacturer. The samples were stained with propidium iodide (PI) to make a red background and then observed under fluorescence microscope.

## RESULTS

### *Expression of MTLC mRNA in GC tissues*

RT-PCR was performed in 15 paired tissues to reveal the expression levels of MTLC mRNA. The result of electrophoresis showed that the PCR product was a single band on agar gel (Figure 1). MTLC was down-regulated in cancer tissues in 9(60 %) of 15 cases after normalization by comparing the band intensities with software UVP Gelworks ID advanced version 2.5 (Figure 1).

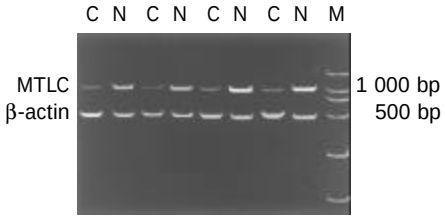



**Figure 1** RT-PCR products were electrophoresed on 1 % agarose gel containing ethidium bromide. The level of β-actin was used as internal control. M: DL2000 DNA marker; C: gastric cancer tissue; N: adjacent normal gastric tissue.

### *Effects of MTLC expression on cell growth*

One of the effects of c-myc on cells is to affect their growth properties. Therefore, we determined whether over-expression of MTLC could recapitulate this character. As seen in Figure 2, the growth rates of MTLC-expressing cells were indistinguishable from those of control cells transfected with the empty parental vector.

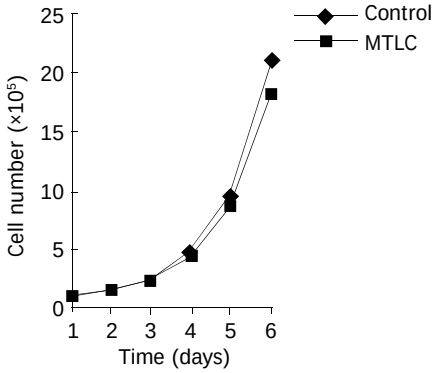



**Figure 2** Cells were determined by manual counting daily. The data was analyzed by Microsoft Excel.

### *Promotion of apoptosis by MTLC*

We studied the response of MTLC-expression BGC823 cell line to avoid its growth factors. Compared with the control

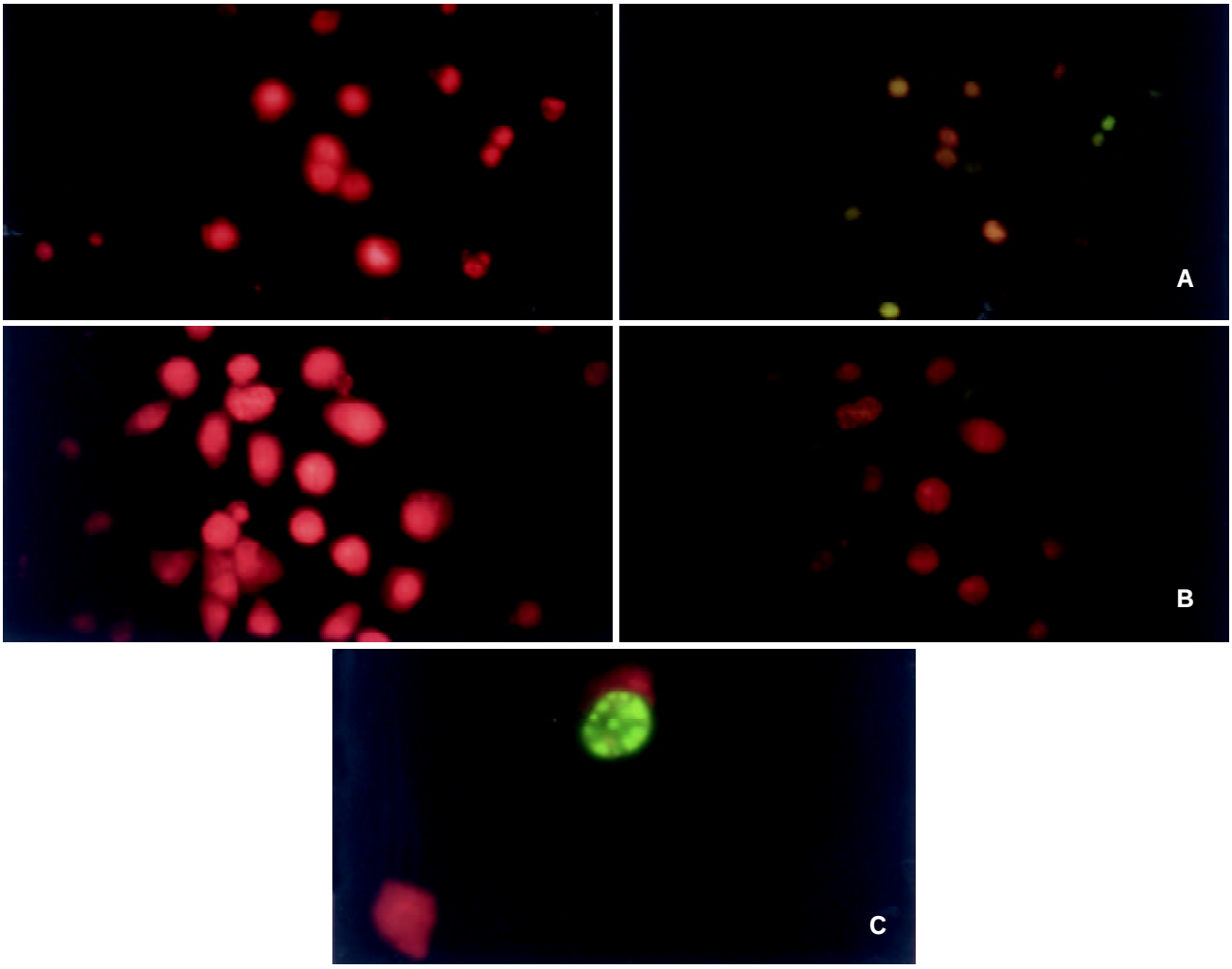



**Figure 3** Apoptosis of cells were detected by TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Green fluorescence of fluorescein-12-dUTP was detected in apoptotic cells, whereas red fluorescence of PI was observed in all cells. Both signals in a same field were photographed respectively. A: MTLC-expressing cells (×400); B: control cells (×400). C: a single apoptotic cell (×1000).

cells, MTLC-expression cells showed a marked acceleration of apoptosis (Figure 3).

**Table 1** Relative expressions of MTLC / β-actin in GC tissues and control tissues

| Case No. | GC | Control | Ratio[a] |
|---|---|---|---|
| 1 | 0.27 | 2.56 | 0.10 |
| 2 | 0.16 | 3.78 | 0.04 |
| 3 | 0.56 | 2.64 | 0.21 |
| 4 | 0.93 | 1.56 | 0.59 |
| 5 | 1.25 | 1.17 | 1.06 |
| 6 | 0.52 | 2.31 | 0.22 |
| 7 | 0.35 | 1.75 | 0.20 |
| 8 | 0.35 | 1.21 | 0.29 |
| 9 | 0.64 | 0.73 | 0.87 |
| 10 | 1.21 | 0.98 | 1.29 |
| 11 | 0.39 | 2.35 | 0.16 |
| 12 | 0.57 | 1.54 | 0.37 |
| 13 | 0.47 | 1.56 | 0.30 |
| 14 | 0.69 | 0.76 | 0.90 |
| 15 | 0.83 | 0.93 | 0.89 |

[a]The ratios less than 0.5 were defined as down-regulation.

## DISCUSSION

MTLC is a novel gene cloned in our laboratory recently and has no known function. Previous studies showed that it was located in 6q25, a chromosome region involved in a variety of human malignancies, including gastric cancer. Analysis of the 5' flanking sequence also demonstrated two E-boxes on the promoter region of MTLC, suggesting that MTLC may be a target of oncogene c-myc. C-myc, a helix-loop-helix leucine zipper transcription factor, can exert considerable control over transformation, differentiation, apoptosis, and cell cycle progression through a number of target genes, including CAD[13], ODC[14,15], LDH-A[16,17], cyclin E[18], MrDb[19], telomerase/hTERT[20-25], rcl[26], IRP2[27], cdc25A[28], and JPO1[29]. It was also shown that c-myc could contribute to gastric carcinogenesis[30-36], but the exact mechanism is still not clear.

In this study, therefore, we detected the expression of MTLC mRNA in gastric cancer tissues and the effects of MTLC over-expression in gastric carcinoma cell line BGC823 to reveal the relationship between MTLC and gastric cancer. Results of RT-PCR showed that MTLC was down-regulated in 60 % (9/15) cases of gastric cancer tissues, a considerable frequency approximating to other genes suppressed in gastric cancer[37-40], suggesting that MTLC may play an important role in carcinogenesis. Furthermore, we performed gene transfection to reveal the possible function of MTLC in gastric carcinogenesis. MTLC did not affect cell growth but remarkably promoted apoptosis in response to growth factor deprivation, although we could not yet explain how this occurred. Over-expression of some other c-myc targets such as p21[41-45] and GADD45[46-51] has been reported to exhibit similar effects through p53 pathway. However, the mechanism of MTLC promoting GC cells apoptosis needs to be further studied. It is also necessary to verify the down-regulation of MTLC in GC tissues by detecting more samples with various types or clinical stages.

## REFERENCES


1 **Parkin DM**. Global cancer statistics in the year 2000. *Lancet Oncol* 2001; **2:** 533-543

2 **Deng DJ.** Progress of gastric cancer etiology: N-nitrosamides 1999s. *World J Gastroenterol* 2000; **6:** 613-618

3 **Xu AG**, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins Bcl-2, p53 and C-myc in the development of gastric cancer. *World J Gastroenterol* 2001; **7:** 403-406

4 **Xue YW**, Zhang QF, Zhu ZB, Wang Q, Fu SB. Expression of cyclooxygenase-2 and clinicopathologic features in human gastric adenocarcinoma. *World J Gastroenterol* 2003; **9:** 250-253

5 **Guo XL**, Wang LE, Du SY, Fan CL, Li L, Wang P, Yuan Y. Association of cyclooxygenase-2 expression with Hp-cagA infection in gastric cancer. *World J Gastroenterol* 2003; **9:** 246-249

6 **Yang L**, Kuang LG, Zheng HC, Li JY, Wu DY, Zhang SM, Xin Y. PTEN encoding product: a marker for tumorigenesis and progression of gastric carcinoma. *World J Gastroenterol* 2003; **9:** 35-39

7 **Wang VW**, Bell DA, Berkowitz RS, Mok SC. Whole genome amplification and high-throughput allelotyping identified five distinct deletion regions on chromosomes 5 and 6 in microdissected early-stage ovarian tumors. *Cancer Res* 2001; **61:** 4169-4174

8 **Rodriguez C**, Causse A, Ursule E, Theillet C. At least five regions of imbalance on 6q in breast tumors, combining losses and gains. *Genes Chromosomes Cancer* 2000; **27:** 76-84

9 **Stilgenbauer S**, Bullinger L, Benner A, Wildenberger K, Bentz M, Dohner K, Ho AD, Lichter P, Dohner H. Incidence and clinical significance of 6q deletions in B cell chronic lymphocytic leukemia. *Leukemia* 1999; **13:** 1331-1334

10 **Gao H,** Wang Q, Wang B, Yan C, Wang S, Wang B, Zhu J, Huang C, Fu S. Genescan analysis of non-small cell lung cancer in the long arm of chromosome 6. *Zhonghua Yixue Yichuanxue Zazhi* 2002; **19:** 14-16

11 **Acevedo CM**, Henriquez M, Emmert-Buck MR, Chuaqui RF. Loss of heterozygosity on chromosome arms 3p and 6q in microdissected adenocarcinomas of the uterine cervix and adenocarcinoma in situ. *Cancer* 2002; **94:** 793-802

12 **Qiu G**, Xu Z, Huang D, Gong L, Li C, Sun X, Sun K. Cloning and characterization of MTLC, a novel gene in 6q25. *Zhonghua Yixue Yichuanxue Zazhi* 2003; **20:** 94-97

13 **Boyd** KE, Farnham PJ. Myc versus USF: discrimination at the cad gene is determined by core promoter elements. *Mol Cell Biol* 1997; **17:** 2529-2537

14 **Bello-Fernandez** C, Packham G, Cleveland JL. The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci U S A* 1993; **90:** 7804-7808

15 **Wu S**, Pena A, Korcz A, Soprano DR, Soprano KJ. Overexpression of Mxi1 inhibits the induction of the human ornithine decarboxylase gene by the Myc / Max protein complex. *Oncogene* 1996; **12:** 621-629

16 **Shim H**, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV. C-myc transactivation of LDH-A: Implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A* 1997; **94:** 6658-6663

17 **Hubank M**, Schatz DG. Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res* 1994; **22:** 5640-5648

18 **Perez-Roger** I, Solomon DL, Sewing A, Land H. Myc activation of cyclin E / Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes. *Oncogene* 1997; **14:** 2373-2381

19 **Grandori C**, Mac J, Siebelt F, Ayer DE, Eisenman RN. Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites *in vivo*. *EMBO J* 1996; **15:** 4344-4357

20 **Wang J**, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. *Genes Dev*1998; **12:** 1769-1774

21 **Greenberg RA**, O' Hagan RC, Deng H, Xiao Q, Hann SR, Adams RR, Lichtsteiner S, Chin L, Morin GB, DePinho RA. Telomerase reverse transcriptase gene is a direct target of c-Myc but is not functionally equivalent in cellular transformation. *Oncogene* 1999; **18:** 1219-1226

22 **Horikawa I**, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. *Cancer Res* 1999; **59:** 826-830

23 **Takakura M**, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res* 1999; **59:** 551-557

24 **Wick M**, Zubov D, Hagen G. Genomic organization and promoter characteriza- tion of the gene encoding the human

telomerase reverse transcriptase (hTERT). *Gene* 1999; **232:** 97-106
25 **Qiu GB**, He G, Gong LG, Zhao Z, Pan ZM, Tang YC, Sun KL. Cloning of hTERT cDNA fragment and application of anti-hTERT monoclonal antibody in mechanism of laryngeal carcinogenesis. *Yichuan Xuebao* 2003; **30:** 109-113
26 **Lewis BC**, Shim H, Li Q, Wu CS, Lee LA, Maity A, Dang CV. Identification of putative c-myc-responsive genes: characterization of rcl, a novel growth-related gene. *Mol Cell Biol* 1997; **17:** 4967-4978
27 **Wu KJ**, Polack A, Dalla-Favera R. Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-myc. *Science* 1999; **283:** 676-679
28 **Galaktionov K**, Chen X, Beach D. Cdc25 cell-cycle phosphatase as a target of c-myc. *Nature* 1996; **382:** 511-517
29 **Prescott JE**, Osthus RC, Lee LA, Lewis BC, Shim H, Barrett JF, Guo Q, Hawkins AL, Griffin CA, Dang CV. A novel c-myc-responsive gene, JPO1, participates in neoplastic transformation. *J Biol Chem* 2001; **276:** 48276-48284
30 **Yang Y**, Deng CS, Peng JZ, Wong BC, Lam SK, Xia HH. Effect of *Helicobacter pylori* on apoptosis and apoptosis related genes in gastric cancer cells. *Mol Pathol* 2003; **56:** 19-24
31 **Liu JR**, Chen BQ, Yang YM, Wang XL, Xue YB, Zheng YM, Liu RH. Effect of apoptosis on gastric adenocarcinoma cell line SGC-7901 induced by cis-9, trans-11-conjugated linoleic acid. *World J Gastroenterol* 2002; **8:** 999-1004
32 **Chen RC**, Su JH, Yang SM, Li J, Wang TJ, Zhou H. Effect of isoverbascoside, a phenylpropanoid glycoside antioxidant, on proliferation and differentiation of human gastric cancer cell. *Acta Pharmacol Sin* 2002; **23:** 997-1001
33 **Ishii HH**, Gobe GC, Pan W, Yoneyama J, Ebihara Y. Apoptosis and cell proliferation in the development of gastric carcinomas: associations with c-myc and p53 protein expression. *J Gastroenterol Hepatol* 2002; **17:** 966-972
34 **Kawanaka H**, Tomikawa M, Baatar D, Jones MK, Pai R, Szabo IL, Sugimachi K, Sarfeh IJ, Tarnawski AS. Despite activation of EGF-receptor-ERK signaling pathway, epithelial proliferation is impaired in portal hypertensive gastric mucosa: relevance of MKP-1, c-fos, c-myc, and cyclin D1 expression. *Life Sci* 2001; **69:** 3019-3033
35 **Hensel F**, Hermann R, Brandlein S, Krenn V, Schmausser B, Geis S, Muller-Hermelink HK, Vollmers HP. Regulation of the new coexpressed CD55 (decay-accelerating factor) receptor on stomach carcinoma cells involved in antibody SC-1-induced apoptosis. *Lab Invest* 2001; **81:** 1553-1563
36 **Ye YN**, Liu ES, Shin VY, Koo MW, Li Y, Wei EQ, Matsui H, Cho CH. A mechanistic study of proliferation induced by Angelica sinensis in a normal gastric epithelial cell line. *Biochem Pharmacol* 2001; **61:** 1439-1448
37 **Yoshikawa Y**, Mukai H, Hino F, Asada K, Kato I. Isolation of two novel genes, down-regulated in gastric cancer. *Jpn J Cancer Res* 2000; **91:** 459-463
38 **Liu DH**, Zhang XY, Fan DM, Huang YX, Zhang JS, Huang WQ, Zhang YQ, Huang QS, Ma WY, Chai YB, Jin M. Expression of vascular endothelial growth factor and its role in oncogenesis of human gastric carcinoma. *World J Gastroenterol* 2001; **7:** 500-505
39 **Rosivatz E**, Becker I, Specht K, Fricke E, Luber B, Busch R, Hofler H, Becker KF. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* 2002; **161:** 1881-1891
40 **Zhang J**, Wang Y, Shou C, Xu G, Chen X, Wu J, Xie Y, Li J, So S, Jiafu J. Detection of Mycoplasma hyorhinis in gastric cancer using bio-chip technology. *Zhonghua Yixue Zazhi* 2002; **82:** 961-965
41 **Bearss DJ**, Lee RJ, Troyer DA, Pestell RG, Windle JJ. Differential effects of p21(WAF1/CIP1) deficiency on MMTV-ras and MMTV-myc mammary tumor properties. *Cancer Res* 2002; **62:** 2077-2084
42 **Horiguchi-Yamada** J, Fukumi S, Saito S, Nakayama R, Iwase S, Yamada H. DNA topoisomerase II inhibitor, etoposide, induces p21WAF1/CIP1 through down-regulation of c-myc in K562 cells. *Anticancer Res* 2002; **22:** 3827-3832
43 **Seoane J**, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002; **419:** 729-734
44 **Bergsmedh A**, Szeles A, Spetz AL, Holmgren L. Loss of the p21 (Cip1/Waf1) cyclin kinase inhibitor results in propagation of horizontally transferred DNA. *Cancer Res* 2002; **62:** 575-579
45 **Wu YL**, Sun B, Zhang XJ, Wang SN, He HY, Qiao MM, Zhong J, Xu JY. Growth inhibition and apoptosis induction of Sulindac on Human gastric cancer cells. *World J Gastroenterol* 2001; **7:** 796-800
46 **Conzen SD**, Gottlob K, Kandel ES, Khanduri P, Wagner AJ, O'Leary M, Hay N. Induction of cell cycle progression and acceleration of apoptosis are two separable functions of c-myc: transrepression correlates with acceleration of apoptosis. *Mol Cell Biol* 2000; **20:** 6008-6018
47 **Wang A**, Gu J, Judson-Kremer K, Powell KL, Mistry H, Simhambhatla P, Aldaz CM, Gaddis S, MacLeod MC. Response of human mammary epithelial cells to DNA damage induced by BPDE: involvement of novel regulatory pathways. *Carcinogenesis* 2003; **24:** 225-234
48 **Chen Z**, Clark S, Birkeland M, Sung CM, Lago A, Liu R, Kirkpatrick R, Johanson K, Winkler JD, Hu E. Induction and superinduction of growth arrest and DNA damage gene 45 (GADD45) alpha and beta messenger RNAs by histone deacetylase inhibitors trichostatin A (TSA) and butyrate in SW620 human colon carcinoma cells. *Cancer Lett* 2002; **188:** 127-140
49 **Vairapandi M**, Balliet AG, Hoffman B, Liebermann DA. GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. *J Cell Physiol* 2002; **192:** 327-338
50 **Uberti D**, Carsana T, Bernardi E, Rodella L, Grigolato P, Lanni C, Racchi M, Govoni S, Memo M. Selective impairment of p53-mediated cell death in fibroblasts from sporadic Alzheimer's disease patients. *J Cell Sci* 2002; **115**(Pt 15): 3131-3138
51 **Okura T**, Nakamura M, Takata Y, Watanabe S, Kitami Y, Hiwada K. Troglitazone induces apoptosis via the p53 and Gadd45 pathway in vascular smooth muscle cells. *Eur J Pharmacol* 2000; **407:** 227-235

**Edited by** Ma JY

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2164-2168
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

# Identification of antigens by monoclonal antibody PD4 and its expression in *Escherichia coli*

Jin-Ying Ning, Guo-Xun Sun, Su Huang, Hong Ma, Ping An, Lin Meng, Shu-Mei Song, Jian Wu, Cheng-Chao Shou

**Jin-Ying Ning, Guo-Xun Sun, Su Huang, Hong Ma, Ping An, Lin Meng, Shu-Mei Song, Jian Wu, Cheng-Chao Shou,** Department of Biochemistry and Molecular Biology, School of Oncology and Beijing Institute for Cancer Research, Peking University, Beijing 100034, China
**Supported by** Key Project of National Natural Science Foundation of China, No.30130190, Beijing Natural Science Foundation, No.7012007, Oncology Key Program and Cancer Center of Peking University
**Correspondence to:** Dr. Cheng-Chao Shou, Department of Biochemistry and Molecular Biology, School of Oncology and Beijing Institute for Cancer Research, Peking University, Beijing 100034, China. cshou9_@hotmail.com
**Telephone:** +86-10-66160960 **Fax:** +86-10-66175832
**Received:** 2003-06-04 **Accepted:** 2003-07-24

## Abstract

**AIM:** To clone and express the antigen of monoclonal antibody (MAb) PD4 for further investigation of its function.

**METHODS:** MGC803 cDNA expression library was constructed and screened with PD4 as probes to clone the antigen. After failed in the library screening, immunoprecipitation and SDS-polyacrylamide gel electrophoresis were applied to purify the antigen for sequence analysis. The antigen coming from *Mycoplasma hyorhinis (M. hyorhinis)* was further confirmed with Western blot analysis by infecting *M. hyorhinis* -free HeLa cells and eliminating the *M. hyorhinis* from MGC803 cells. The full p37 gene was cloned by PCR and expressed successfully in *Escherichia coli* after site-directed mutations. Immunofluorescence assay was used to demonstrate if p37 protein could directly bind to gastric tumor cell AGS.

**RESULTS:** The cDNA library constructed with MGC803 cells was screened by MAb PD4 as probes. Unfortunately, the positive clones identified with MAb PD4 were also reacted with unrelated antibodies. Then, immunoprecipitation was performed and the purified antigen was identified to be a membrane protein of *Mycoplasma hyorhinis (M. hyorhinis*) by sequencing of N-terminal amino acid residues. The membrane protein was intensively verified with Western blot by eliminating *M. hyorhinis* from MGC803 cells and by infecting *M. hyorhinis*-free HeLa cells. The full p37 gene was cloned and expressed successfully in *Escherichia coli* after site-directed mutations. Immunofluorescence demonstrated that p37 protein could directly bind to gastric tumor cell AGS.

**CONCLUSION:** The antigen recognized by MAb PD4 is from *M. hyorhinis,* which suggests the actions involved in MAb PD4 is possibly mediated by p37 protein or *M. hyorhinis.* As p37 protein can bind directly to tumor cells, the pathogenic role of p37 involved in tumorigenesis justifies further investigation.

Ning JY, Sun GX, Huang S, Ma H, An P, Meng L, Song SM, Wu J, Shou CC. Identification of antigens by monoclonal antibody PD4 and its expression in *Escherichia coli*. *World J Gastroenterol* 2003; 9(10): 2164-2168
http://www.wjgnet.com/1007-9327/9/2164.asp

## INTRODUCTION

Gastric cancer is common in China[1-14]. For decades, a goal of cancer researchers is to be able to immunize patients with their own tumor tissues or tumor associated antigens after surgical operation to stimulate their immune response. The benefits of this aim include host immune surveillance to eliminate the metastatic cells and prevent its relapse. Many tumor associated antigens defined by monoclonal antibodies have been successfully applied clinically in detecting different tumors[15,16]. However, only a few of the genes for these markers have been cloned[17,18], primarily because the epitopes usually are not protein or not linear in amino acid sequence.

MAb PD4 has been derived from mouse immunized with human gastric cell line MGC803 and could specially react with some tumor cells[19]. Our previous studies showed that MAb PD4 could induce apoptosis of MGC803 cells[20], inhibit both the growth of ras transformed cell line Rat3-3 and the tumorigenesis in nude mice[21], suggesting that the antigen recognized by MAb PD4 could be associated with cancerous development. Obviously the critical step in investigating the molecular mechanisms involved in the antigen is to isolate its cDNA.

As it was shown previously that PD4 worked well in Western blot in which the recognized protein was around 40 kilo-Dalton in molecular weight[21]. First, we screened the cDNA library constructed with MGC803 cells by MAb PD4 as probes. Unfortunately, the positive clones identified with MAb PD4 were reacted with unrelated antibodies. We tried with other different tumor expression cDNA libraries and also did not get any specific clones. Then, immunoprecipitation was performed and the target molecule was identified to be a membrane protein of *M. hyorhinis* by N-termial amino acid residues sequencing. The membrane protein was intensively verified with Western blot by eliminating *M. hyorhinis* from MGC803 cells and by infecting *M. hyorhinis*-free Hela cells. Therefore, the actions involved in MAb PD4 were possibly mediated by p37 protein or *M. hyorhinis.*

p37 is a membrane protein of *M. hyorhinis* located on the outside of the cell membrane. It contains 1209 nucleotides and encodes 403 amino acid residues[22]. An analysis of the protein sequence has revealed that p37 has a 41 % similarity to a periplasmic binding-protein-dependent transport system found in Gram-negative bacteria[22]. Thus, p37 is thought to be part of a high affinity transport system from *M. hyorhinis*. Also, there are lines of evidence indicating the linkage between p37 or *M. hyorhinis* and cancer[23-25]. For example, antibodies against p37 could inhibit the invasive potential of FS9 cells and cause malignant cells to revert to a more normal behavior.

In this study, we identified the antigen recognized by MAb PD4, which was previously considered as an antibody against cancer. The full gene encoding the antigen was cloned and expressed successfully in *Escherichia coli (E. coli)* after site-directed mutation of the seven codes tryptophan TGA into universal codes tryptophan TGG and demonstrated that p37 could bind directly to tumor cell AGS. Considering the association between p37 and tumor development and invasion, this work provides a basis for further investigation of the pathogenic role of p37 involved in *M. hyorhinis* infection.

## MATERIALS AND METHODS

### *Cell culture and regents*

Human gastric cancer cell lines MGC803 and AGS, human ovarian cancer cell line HeLa, expression plasmid pGEX-4T-1, *E. coli* BL21(DE3) and MAb PD4 were all kept in our laboratory. Site-directed mutation kit was purchased from Promega Corp. Anti-glutathione-S-transferase (GST) mouse antibody, goat anti-mouse antibody conjugated with tetramethyl rhodaine isothiacy-anate (TRITC), 3,3'-diaminobenzidine (DAB), isopropylthiogalactoside (IPTG) were from Sigma. All primers for PCR and site-directed mutation were synthesized by Sangon Corp. (Shanghai, China). Various restriction endonucleases were products of New England BioLabs (NEB). RPMI1640 and F12K medium were from GIBCO BRL. RNA extraction kit was from Invitrogen Corp.

### *cDNA library construction, screening and clone identification*

mRNA purification and poly(A)+ mRNA enrichment were processed with a messenger RNA isolation kit (Strategene Corp.) from $5\times10^7$ MGC803 cells. A cDNA library was prepared and packaged with ZAP Express cDNA Gigapack III gold cloning kit (Strategene Corp.) according to the manufacture's instruction. The library quality was determined with its diversity and the average size of the inserted cDNA fragments. The non-amplified library was plated and transferred to nitrocellulose filters. Initial screening was performed with 1 $\mu g\cdot mL^{-1}$ MAb PD4 (diluted in phosphate-buffered saline containing 1 % bovine serum albumin). The filters were then washed in phosphate-buffered saline (PBS) and bound MAb PD4 was detected by alkaline phosphatase coupled to sheep anti-mouse antibodies followed by the mixture solution of nitro blue tetrazolium and bromochloroindolylphosphate. The positive bacteriophages were subcloned and the positive individual clones were checked with unrelated antibodies for its specificity.

### *Preparation of MGC803 cell membrane proteins*

$2\times10^8$ MGC803 cells were frozen and thawed repeatedly for 4-5 times in phosphate-buffered saline containing 1 $mmol\cdot L^{-1}$ PMSF, then centrifuged at 4 000×g for 30 min at 4 ℃. The supernatant was collected, centrifuged at 100 000×g for 1 h at 4 ℃. The precipitated membrane debris was suspended with 1-2 ml lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 % NP-40, 5 % sodium deoxycholate, 1 mM PMSF, 2 $\mu g\cdot mL^{-1}$ aprotinin), shaken for 30 min at 4 ℃, then centrifuged at 100 000×g for 1 h at 4 ℃. The supernatant was analyzed by Western blot with MAb PD4.

### *Purification and identification of the antigen protein*

The lysates from MGC803 cells were immunoprecipitated for overnight at 4 ℃ by MAb PD4 coupled with protein A-sepharose 4B beads (Sigma). The normal mouse IgG was used as control. The immunoprecipitated beads were washed three times with lysis buffer and subjected to 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel-separated proteins were transferred onto PVDF membranes. A piece cut off from the membrane was used for Western blot with MAb PD4 and the left transferred membrane was stained with ponceau S. After the identification, the corresponding band with the Western blot was cut off and the sequence of N-terminal amino acid residues was performed in Life Science Center of Peking University.

### *Further identification of the antigen from mycoplasma infected cells*

When *M. hyorhinis*-free HeLa cells grew to appropriate confluence, the conditional medium from cultured MGC803 cells, which reacted with MAb PD4, was added into the cultured HeLa cells system. After 72 h incubation, total proteins were extracted from HeLa cells with lysis buffer (1 % Triton X-100/PBS, 1 $mmol\cdot L^{-1}$ PMSF) and Western blot was performed with MAb PD4 as first antibody. Moreover, cultured MGC803 cells were treated with BM cyclin antibiotics for 3 weeks (Roche, Mannheim, Germany) which were recognized as the most effective against mycoplasma infection[26]. Then the total proteins of MGC803 cells were extracted, followed by Western blot as above.

### *Cloning, site-directed mutations and sequencing of p37 gene*

3 mL supernatant from cultured MGC803 cells infected with *M. hyorhinis* was collected, centrifuged at 12 000×g for 5 min, rinsed once with PBS, dissolved in 30 μl ddH2O, then boiled for 5 min, centrifuged for 5 min at 10 000×g. 2 μl supernatant was used as the template for PCR to amplify p37 gene. Upstream primer 5'-aatcggatcc gaggtagcttttatgctc-3' (including *Bam*H I site) and downstream primer 5'-aaagaattctcatttaatggcttttt c-3' (including *Eco*R I site) were synthesized by Sangon Co. (Shanghai. China). The PCR program was consisted of 30 cycles at 94 ℃ for 1 min, at 48 ℃ for 1 min, at 72 ℃ for 90 s, and at 72 ℃ for 10 min for the final extension. The PCR product was inserted into a pBluescript vector after digestion with endonucleases *Bam*HI and *Eco*R I. The seven tryptophan TGA codes were mutated into universal codes tryptophan TGG using mutation vector pALTER-1 according to the instructions of the site-directed mutation kit (Promega) and verified by DNA sequencing. The 5' phosphated mutated primers were as follows: (1) 5'-p(A GGTCAATG**G**G ATAAAAGTA)-3', (2)5'-p(GCAAGTTG **G**ACTGATGA AAATC A TAAGTG**G**AATGGTAATG)-3', (3)5'-p (GGAATGATTTG**G**ATAAAAGGTAAT G )-3', (4)5'-p (AAAAAGCTTG**G**AATGATA AAGATTG**G**AATAC ATTTAGAAA TTTT)-3', (5)5'-p(GG T TCTTTTGCTTG**G**A CACATAAC A)-3'. The bold letters were changed from A.

### *Expression, purification and identification of GST-P37 fusion protein*

The recombinant vector pGEX-4T-p37 was constructed through inserting the mutated p37 into plasmid pGEX-4T-1, then it was introduced into *E. coli* BL21(DE3). The transformed bacteria were induced with 0.1 mM IPTG to express the fusion protein at 30 ℃ overnight. Then the induced bacteria were collected and lysed by ultrasonocation. After centrifugation, the supernatant was incubated with glutathione-sepharose-4B and binding protein was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 15 mM reduced glutathione). The GST-p37 protein in elution buffer was tested by SDS-PAGE and identified by Western blot after concentrated with sucrose and dialyzed with PBS.

### *Binding assay with immunofluorescence microscopy*

AGS cells were seeded into 6-well culture plates in F-12K medium and crept on cover slides for 24 h. Then the cells were washed twice by PBS. The GST-p37 and GST proteins (10 $\mu g\cdot mL^{-1}$) dissolved in serum-free F-12K medium were added respectively. After incubation for 1 h, cells were gently washed 3 times by PBS and fixed for 15 min with freshly prepared 4 % paraformaldehyde. Then the cells were washed twice by PBT buffer (PBS with 0.1 % bovine serum albumin and 0.01 % Tween 20) and blocked with 10 % normal goat serum for 1 h at 37 ℃. Anti-GST antibodies were added as the first antibody and incubated for 1 h at 37 ℃. After the cells were washed twice for 5 min each by PBT buffer, TRITC-conjugated goat anti-mouse IgG antibodies were used as secondary antibody to detect bound GST-p37 and GST

proteins. The cells were washed twice with PBT buffer and briefly rinsed with water before mounted onto slides for observation under fluorescence microscopy.

## RESULTS

### *cDNA library construction, screening and identification*

We obtained 2 μg poly(A)+ enriched mRNA for synthesizing the cDNA library. Double-stranded cDNA was generated by means of "nick-translation". *Eco*R I adapters were ligated to the double-stranded cDNA, which was then digested with *Xho*I and size selected. Only the cDNA molecules larger than 500 bp were collected and ligated into ZAP expression vectors. The diversity of the primary library was $1.2\times10^6$ pfu. The recombinant rate was 9/9, and the average size of cDNA inserts was about 1.5 kb. A total of $10^6$ non-amplified library clones were screened with MAb PD4 and the positive clones were confirmed with unrelated antibodies as controls. Anyhow no specific positive clone reacted with MAb PD4 was obtained.

### *Purification of the antigen recognized by MAb PD4*

First, the total membrane protein from MGC803 was analyzed by Western blot with MAb PD4, and a specific 40 kilo-Dalton band could be found (Figure 1A). Then immunoprecipitation was performed and the protein complex was subjected to 12 % SDS-PAGE. The gel-separated proteins were analyzed with Western blot, and the target band was cut off from the membrane (Figure 1B). The sequence of N-terminal amino acid residues revealed that 16 amino acid residues (CSNTGVVKQEDVSVSQ) were completely identical with protein p37 from *M. hyorhinis*, which suggested the antigen recognized by MAb PD4 was from mycoplasma, not from tumor cells, and the PD4 was a MAb against *M. hyorhinis.*

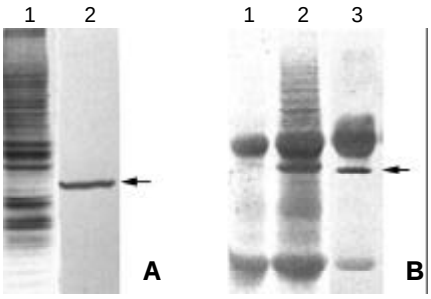



**Figure 1** Purification of the antigen by MAb PD4. (A) 1. SDS-PAGE and Coomassie blue staining of the membrane proteins extracted from MGC803 cells; 2. Western blot analysis of the membrane proteins extracted from MGC803 with MAb PD4, the arrow indicating the target protein of MAb PD4. (B) 1. SDS-PAGE analysis of immunoprecipited protein complex bound with normal mouse IgG. (B) 2. SDS-PAGE analysis of immunoprecipited protein complex bound with MAb PD4. (B) 3. Western blot analysis of immunoprecipited protein complex bound with MAb PD4, arrow indicating the target protein of MAb PD4.

### *Further identification of the antigen with mycoplasma infected cells*

If the antigen was really derived from *M. hyorhinis*, it should be transferred along with *M. hyorhinis* infection and also would disappear when the infected mycoplasma was cleared away from host cells. HeLa cells, which were mycoplasma-free and not reactive with PD4, were treated with conditional medium from *M. hyorhinis* infected cell MGC803. After 72 h treatment, the totally extracted cell protein could react with PD4 (Figure 2A). Meanwhile, the reacting band with PD4 disappeared after MGC803 cells were treated by BM cyclin antibiotics. This result indicated that the antigen of MAb PD4 was a protein from *M. hyorhinis.*

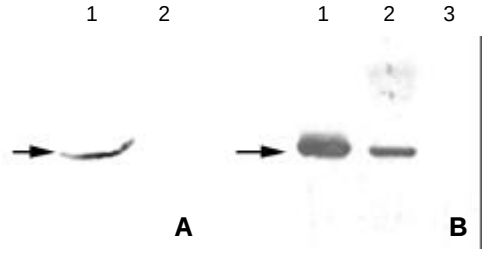



**Figure 2** Further identification of the antigen from mycoplasma infection. (A) 1. Western blot analysis of the total protein from Hela cells with MAb PD4, which was treated with cultured MGC803 medium. (A) 2. Western blot analysis of the total protein from untreated HeLa cells with MAb PD4. (B) Western blot analysis of the total proteins from MGC803 cells treated with BM cyclin antibiotics differently. Lane 1: untreated, lane 2: treated for two weeks, and lane 3: treated for three weeks. As indicated by the arrow, the band reacted with MAb PD4 disappeared gradually following the treatment.

### *Cloning, expression and purification of GST-P37 protein*

The results above suggested that the effect of MAb PD4 on tumor cells was mediated by p37 or *M. hyorhinis.* To investigate the molecular mechanisms of the effect, the gene p37 was cloned and mutated by site-directed mutagenesis successfully. After checked by DNA sequencing, the full length p37 was expressed as fusion protein GST-p37 and it could react with PD4 specifically by Western blot (Figure 3).

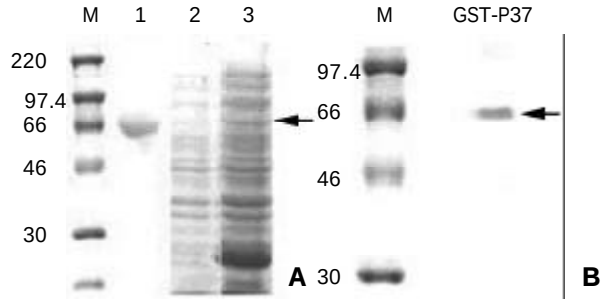



**Figure 3** Analysis of GST-p37 with SDS-PAGE and Western blot. (A) Coomassie blue staining of the SDS-PAGE gel. M protein standards. lane 1: protein of BSA, lane 2: total proteins from un-induced bacteria, lane 3: total proteins from IPTG induced bacteria, the arrow indicating GST-p37 band. (B) Western blot analysis of purified protein with MAb PD4, the arrow indicating GST-p37 band.

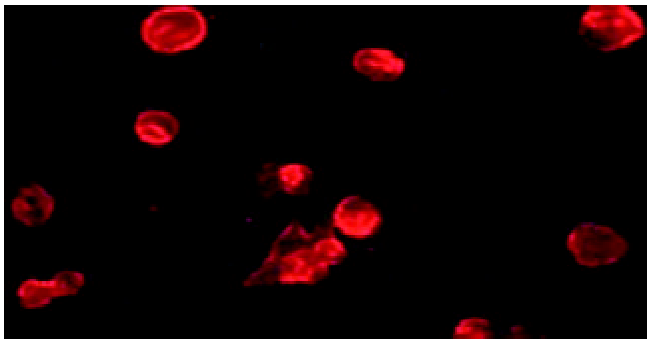


**Figure 4** p37 binding AGS cells assay. Observation of the immunofluorecence under microscopy, which showed GST-p37 protein binding to AGS cells, whereas GST protein has no such binding ability (data not shown).

### *Binding assay*

Because p37 is a dominant protein component of *M. hyorhinis* located on the outside of the cell membrane, we hypothesized that p37 was capable of mediating *M. hyorhinis* binding to host cells. In current study, we first demonstrated that p37 could directly bind to gastric cell line AGS (Figure 4), which provided the basis for further related investigation of p37.

## DISCUSSION

Our study indicated that the antigen recognized by MAb PD4, which was derived from mice immunized with gastric tumor cell line MGC803, was a protein from *M. hyorhinis.* This result suggests that the actions of MAb PD4, which we showed before, are mediated possibly by p37 protein or *M. hyorhinis*. Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication that can cause a wide variety of diseases in animals. Some mycoplasmas cause respiratory or urogenital diseases in humans[27,28], but others chronically colonize on respiratory and urogenital tracts without apparent clinical significance. There have been some discrete reports about the correlation between mycoplasma infection and cancer since last century. In 1960s, two studies reported that infection of mycoplasma (*M. orale* and one unspeciated) caused chromosomal changes[29]. Other researches in the 1980s reported that an arthropod spiroplasma could rapidly transform mouse and monkey cells[30]. However, investigating the possible association between mycoplasma infection and carcinogenesis did not become more active until Tsai *et al*. reported that continuous infection of *M. penetrans* or *M. fermentans* could lead to multiple stage of malignant transformation of murine embryonic C3H cells, accompanied by abnormal karyotypes and some oncogene upregulation[30, 31].

Also, there are documents indicating the linkage between p37 protein or *M. hyorhinis* and cancer. One is that elevated tumor invasion, as suggested by a leukocyte adherence inhibition (LAI) response, correlates with the presence of *M. hyorhinis* in patients with lung cancer, colon cancer, and breast cancer[32]. Furthermore, two independent research groups showed that p37 on the surface of FS9 mouse fibrosarcoma cells correlated with a highly invasive phenotype, as measured in an *in vitro* cellular invasion assay[22,23]. Antibodies against p37 inhibited the invasive potential of FS9 cells in the assay *in vitro*[23]. Using Abercrombie' s confronted explant assay, it was shown that antibodies against p37 caused malignant cells to revert to a more normal behavior[24]. More recently, p37 antibodies were reported to reduce the lung metastasis of colon cancer in nude mouse models[25]. These results, taken together, suggest that *M. hyorhinis* infection causes enhanced tumor invasion and correlates with tumorigenesis, and that p37 is an essential component of this phenomenon.

In order to further investigate the pathogenic role of p37 in *M. hyorhinis* infection, we cloned the full p37 gene and expressed it successfully in *E. coli*. Eighty percent of the expressed GST-p37 fusion protein was soluble at the inducing condition (IPTG 0.1 mM, 30 ℃ overnight). Western blot analysis indicated the expressed p37 protein was intact. As p37 is a dominant protein component of *M. hyorhinis* located on the outside of the cell membrane, we hypothesize that p37 may be capable of mediating *M. hyorhinis* binding to host cells. In the current study, we first demonstrated that p37 could directly bind to gastric cell line AGS, which provided the basis for further related investigation of p37.

*M. hyorhinis* contamination is very common in routine cell culture and mycoplasma may be more prone to stimulate immune response when mice are immunized with tumor cells infected with mycoplasma. Thus, the obtained monoclonal antibodies are possibly raised to mycoplasma, especially when the infected tumor cells are used as both immunogen and target cells for screening positive clones. This should draw more attention during preparing monoclonal antibodies. Interestingly, when we recently performed immunohistochemistry with PD4 to detect *M. hyorhinis* infection in paraffin embedded carcinoma tissues, the results indicated that positive rates in gastric carcinoma, esophageal cancer, colon carcinoma and lung cancer were around 50 %, but they were less than 25 % in other gastric diseases, such as chronic superficial gastritis, gastric ulcer and intestinal metaplasia[33]. Meanwhile, we have also isolated *M. hyorhinis* successfully from human gastric cancer tissues by direct culture (data not published). These results strongly suggest an association between mycoplasma infection and tumorigenesis. Undoubtedly, the molecular mechanism of p37 action on tumor cells needs to be intensively investigated.

## REFERENCES


1 **Xue FB**, Xu YY, Wan Y, Pan BR, Ren J, Fan DM. Association of *H pylori* infection with gastric carcinoma: a Meta analysis. *World J Gastroenterol* 2001; **7**: 801-804

2 **Wu YL**, Sun B, Zhang XJ, Wang SN, He HY, Qiao MM, Zhong J, Xu JY. Growth inhibition and apoptosis induction of Sulindac on Human gastric cancer cells. *World J Gastroenterol* 2001; **7**: 796-800

3 **Cai L**, Yu SZ, Zhan ZF. Cytochrome P450 2E1 genetic polymorphism and gastric cancer in Changle, Fujian Province. *World J Gastroenterol* 2001; **7**: 792-795

4 **Xu CT**, Huang LT, Pan BR. Current gene therapy for stomach carcinoma. *World J Gastroenterol* 2001; **7**: 752-759

5 **Cai L**, Yu SZ, Zhang ZF. Glutathione S-transferases M1, T1 genotypes and the risk of gastric cancer: A case-control study. *World J Gastroenterol* 2001; **7**: 506-509

6 **Niu WX**, Qin XY, Liu H, Wang CP. Clinicopathological analysis of patients with gastric cancer in 1200 cases. *World J Gastroenterol* 2001; **7**: 281-284

7 **Miehlke S**, Kirsch C, Dragosics B, Gschwantler M, Oberhuber G, Antos D, Dite P, Luter J, Labenz J, Leodolter A, Malfertheiner P, Neubauer A, Ehninger G, Stolte M, Bayerdorffer E. *Helicobacter pylori* and gastric cancer: current status of the Austrian Czech German gastric cancer prevention trial (PRISMA Study). *World J Gastroenterol* 2001; **7**: 243-247

8 **Li XY**, Wei PK. Diagnosis of stomach cancer by serum tumor markers. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 568-570

9 **Gao HJ**, Yu LZ, Bai JF, Peng YS, Sun G, Zhao HL, Miu K, Lü XZ, Zhang XY, Zhao ZQ. Multiple genetic alterations and behavior of cellular biology in gastric cancer and other gastric mucosal lesions: *H pylori* infection, histological types and staging. *World J Gastroenterol* 2000; **6**: 848-854

10 **Cai L**, Yu SZ, Ye WM, Yi YN. Fish sauce and gastric cancer: an ecological study in Fujian Province, China. *World J Gastroenterol* 2000; **6**: 671-675

11 **Cai L**, Yu SZ, Zhang ZF. *Helicobacter pylori* infection and risk of gastric cancer in Changle County, Fujian Province, China. *World J Gastroenterol* 2000; **6**: 374-376

12 **Feng DY,** Chen RX, Peng Y, Zheng H, Yan YH. Effect of HCV NS-3 protein on P53 protein expression in hepatocarcinogenesis. *World J Gastroenterol* 1999; **5**: 45-46

13 **Qiao GB**, Han CL, Jiang RC, Sun CS, Wang Y, Wang YJ. Overexpression of P53 and its risk factors in esophageal cancer in urban areas of Xi'an. *World J Gastroenterol* 1998; **4**: 57-60

14 **Deng ZL**, Ma Y. Aflatoxin sufferer and *p53* gene mutation in hepatocellular carcinoma. *World J Gastroenterol* 1998; **4**: 28-29

15 **Devine PL**, McGuckin MA, Ward BG. Circulating mucins as tumor markers in ovarian cancer. *Anticancer Res* 1992; **12**: 709-718

16 **Kawa S**, Tokoo M, Hasebe O, Hayashi K, Imai H, Oguchi H, Kiyosawa K, Furuta S, Homma T. Comparative study of CA242 and CA19-9 for the diagnosis of pancreatic cancer. *Br J Cancer* 1994; **70**: 481-486

17 **Grimm T**, Johnson JP. A modified screening method for pcDNA-1 expression libraries which is applicable to both surface and intracellular antigens. Cloning of a colon carcinoma antigen. *J*

*Immunol Methods* 1995; **186**: 305-312

18 **Merlo GR**, Siddiqui J, Cropp CS, Liscia DS, Lidereau R, Callahan R, Kufe DW. Frequent alteration of the DF3 tumor-associated antigen gene in primary human breast carcinomas. *Cancer Res* 1989; **49**: 6966-6971

19 **Dong ZW**, Wei SM, Mu ZY. Monoclonal antibodies against human gastric cancer. *Zhonghua Zhongliu Zazhi* 1989; **1**: 1-6

20 **Xiao H**, Shou CC, Dong ZW. Induction of apoptosis in human gastric carcinoma cell line MGC803. *Zhonghua Zhongliu Zazhi* 1998; **1**: 91-95

21 **Yin WN**, Dong ZW, Deng GR. Study of the inhibitory effect of monoclonal antibody PD4 to Ha-ras transfected cell line Rat3-3. *Zhonghua Zhongliu Zazhi* 1991; **13**: 82-86

22 **Dudler R**, Schmidhauser C, Parish RW, Wettenhall RE, Schmidt TA. A mycoplasma high-affinity transport system and the *in vitro* invasiveness of mouse sarcoma cells. *The EMBO Journal* 1988; **7**: 3963-3970

23 **Steinemann C**, Fenner M, Binz H, Parish RW. Invasive behavior of mouse sarcoma cells is inhibited by blocking a 37,000-dalton plasma membrane glycoprotein with Fab Fragments. *Proc Natl Acad Sci U S A* 1984; **81**: 3747 -3750

24 **Schmidhauser C**, Dudler R, Schmidt T, Parish RW. A mycoplasmal protein influences tumour cell invasiveness and contact inhibition *in vitro*. *J Cell Sci* 1990; **95**: 499-506

25 **Ushio S**, Iwaki K, Taniai M, Ohta T, Fukuda S, Sugimura K, Kurimoto M. Metastasis -promoting activity of a novel molecule, Ag 243-5, derived from mycoplasma, and the complete nucleotide sequence. *Microbiol Immunol* 1995; **39**: 393-400

26 **Uphoff CC,** Drexler HG. Comparative antibiotic eradication of mycoplasma infections from continuous cell lines. *In Vitro Cell Dev Biol Anim* 2002; **38**: 86-89

27 **Loo VG**, Richardson S, Quinn P. Isolation of Mycoplasma pneumoniae from pleural fluid. *Diagn Microbiol Infect Dis* 1991; **14**: 443-445

28 **Taylor-Robinson D**. Genital mycoplasma infections. *Clin Lab Med* 1989; **9**: 501-523

29 **Paton GR**, Jacobs JP, Perkins FT. Chromosome changes in human diploid-cell cultures infected with Mycoplasma. *Nature* 1965; **207**: 43-45

30 **Tsai S**, Wear DJ, Shih JW, Lo SC. Mycoplasmas and oncogenesis: persistent infection and multistage malignant transformation. *Proc Natl Acad Sci U S A* 1995; **92**: 10197-10201

31 **Feng SH**, Tsai S, Rodriguez J, Lo SC. Mycoplasmal infections prevent apoptosis and induce malignant transformation of interleukin-3-dependent 32D Hematopoietic cells. *Molecular Cellular Biology* 1999; **19**: 7995-8002

32 **Ilantzis C**, Thomson DM, Michaelidou A, Benchimol S, Stanners CP. Identification of a human cancer related organ-specific neoantigen. *Microbiol Immunol* 1993; **37**: 119-128

33 **Huang S**, Li JY, Wu J, Meng L, Shou CC. Mycoplasma infections and different human carcinomas. *World J Gastroenterol* 2001; **7**: 266-269

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2169-2173
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Different approaches to caudate lobectomy with "curettage and aspiration" technique using a special instrument PMOD: A Report of 76 cases

Shu-You Peng, Jiang-Tao Li, Yi-Ping Mou, Ying-Bin Liu, Yu-Lian Wu, He-Qing Fang, Li-Ping Cao, Li Chen, Xiu-Jun Cai, Cheng-Hong Peng

**Shu-You Peng, Jiang-Tao Li, Ying-Bin Liu, Yu-Lian Wu, He-Qing Fang, Li-Ping Cao, Li Chen, Xiu-Jun Cai, Cheng-Hong Peng,** Department of Surgery, 2nd Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
**Yi-Ping Mou,** Department of Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
**Correspondence to:** Shu-You Peng, Department of Surgery, 2nd Affiliated Hospital, School of Medicine, Zhejiang University. No 88 Jie Fang Road, Hangzhou 310009, Zhejiang Province, China sypeng@mail.hz.zj.cn
**Telephone:** +86-571-87783766 **Fax:** +86-571-87022776
**Received:** 2003-08-06 **Accepted:** 2003-09-17

## Abstract

**AIM:** To study different approaches to caudate lobectomy with "curettage and aspiration" technique using Peng's multifunctional operative dissector (PMOD). The surgical procedure of isolated complete caudate lobectomy was specially discussed.

**METHODS:** In 76 cases of various types of caudate lobectomy, three approaches were used including left side approach, right side approach, and anterior approach. Among the 76 cases, isolated complete caudate lobectomy was carried out in 6 cases with transhepatic anterior approach. The surgical procedure consisted of mobilization of the total liver, ligation and separation of the short hepatic veins, splitting the liver parenchyma through the Cantlie's plane, ligation and division of the caudate portal triads from the hilum, dissection of the root of major hepatic veins, detachment of the caudate lobe from liver parenchyma.

**RESULTS:** The mean operative time was 285±51 min, the mean blood loss was 1 600 ml. No severe complications were observed. Among the 6 cases receiving isolated complete caudate lobectomy with transhepatic anterior approach, one case died 17 months after operation due to disease recurrence and liver failure, the other 5 cases have been alive without recurrence, with one longest survival of 49 months.

**CONCLUSION:** The choice of approach is essential to the success of caudate lobectomy. As PMOD and "curettage and aspiration" technique can delineate intrahepatic or extrahepatic vessels clearly, caudate lobe resection has become safer, easier and faster.

Peng SY, Li JT, Mou YP, Liu YB, Wu YL, Fang HQ, Cao LP, Chen L, Cai XJ, Peng CH. Different approaches to caudate lobectomy with "curettage and aspiration" technique using a special instrument PMOD: A Report of 76 cases. *World J Gastroenterol* 2003; 9(10): 2169-2173
http://www.wjgnet.com/1007-9327/9/2169.asp

## INTRODUCTION

The caudate lobe of the liver is difficult to resect because it lies deep beneath the confluence of main hepatic veins and between porta hepatis and inferior vena cava[1]. Isolated or combined caudate lobectomy is the treatment of choice for a mass originating in caudate lobe or for hepatobiliary cancer invading the hepatic hilum. Among various types of caudate lobectomy, isolated complete resection of caudate lobe is technically the most difficult one. Anterior approach is considered to be a safe, potentially curative option for isolated resection of the entire caudate lobe, especially in the presence of cirrhosis, as we can spare innocent hepatic parenchyma using this approach[2,3]. With the use of a specially designed instrument-Peng's multifunctional operative dissector (PMOD), we developed a new surgical technique called "curettage and aspiration" technique with which transection of the liver parenchyma can be carried out in a nearly bloodless field[4]. Precise anatomy of caudate lobe can be achieved with this technique and instrument. Therefore isolated complete resection of caudate lobe has become easier and faster as the liver is split into two halves by anterior approach. From 1994 to June 2003, we performed 76 cases of various types of hepatectomy with caudate lobectomy, which included 6 isolated complete caudate lobectomies by anterior transhepatic approach. This review studied different approaches to caudate lobectomy especially the anterior transhepatic approach for isolated complete caudate lobectomy with "curettage and aspiration" technique using PMOD.

## MATERIALS AND METHODS

### *Patients*

Seventy-six cases were enrolled in the study. Various types of hepatectomy with caudate lobectomy were performed in 51 cases, isolated caudate lobectomy was performed in 25 cases. Forty-five cases had hepatocellular carcinoma (HCC) originating from or invading caudate lobe, 7 cases had benign tumors and stone of caudate lobe, 17 cases had cholangiocarcinoma, 7 cases had metastatic tumor in the liver (6 cases of colonic carcinoma, 1 case of adrenal carcinoma). These 6 patients received isolated complete caudate lobectomy by anterior approach. Among them, four were male and 2 female, their age ranged from 32 to 65 years (mean 52 years); five cases had HCC accompanied by cirrhosis, and one had hemoangioma.

### *Surgical procedures*

In the majority of cases, a reversed L-shaped skin incision from xiphoid to the tip of the twelfth right rib was used, giving an excellent exposure, which was of vital importance for caudate lobectomy. The whole abdominal cavity was explored to rule out intra-abdominal metastasis.

The choice of approach is essential to the success of caudate lobectomy. Approaches are dependent largely on the size, location of the lesion and the severity of cirrhosis. In this series, four approaches were used for various types of

caudate lobectomy. Left side approach was suitable for small tumors situated in Spiegelian lobe or when caudate lobe was to be resected combined with the left liver. Left lateral segmentectomy, left hemi-hepatectomy or left trisegmentectomy was carried out before caudate lobe was exposed and resected. Right side approach was more suitable for tumor located in the caudate process or when the caudate lobe was resected together with the right liver, mostly right hemi-hepatectomy. The combined approach was a combination of the left side and right side approach. The caudate lobe might be approached mainly from the right or left side although dissection from both sides was necessary in many cases. Anterior transhepatic approach was suitable for cases when isolated complete resection of caudate lobe was indicated and innocent liver parenchyma should not be resected due to cirrhosis of the liver (Figure 1). The characteristics of this approach were that the liver was split through the interlobar plane into two halves, so as to fully expose the caudate lobe.

The anterior transhepatic approach included 7 steps. (1) The falciform ligament was separated up to the front of suprahepatic IVC, then the incision was turned to the right and left. The coronary ligaments, triangular ligaments and hepato-renal ligament were separated, respectively. The adrenal gland was detached from the liver, and hepatogastric ligament was completely separated. (2) The short hepatic veins (SHV) were dissected and ligated caudal cranially, three to five thick short hepatic veins were separated in this process (Figure 2). (3) Tapes were used to encircle the suprahepatic and infrahepatic IVC, respectively (Figure 3). (4) The interlobar plane was split and the anterior surface of the paracaval portion and the hilar plate were explored. (5) The ascending caudate portal triads were ligated and separated (Figure 4). (6) The caudate lobe was separated from the major hepatic veins (MHV) (Figure 5). (7) The caudate lobe was detached from the neighboring liver parenchyma. No large branches here needed to be ligated, the small vessels encountered could be cauterized with PMOD. Thus isolated complete caudate lobe was resected and two halves of the liver were sutured (Figure 6).

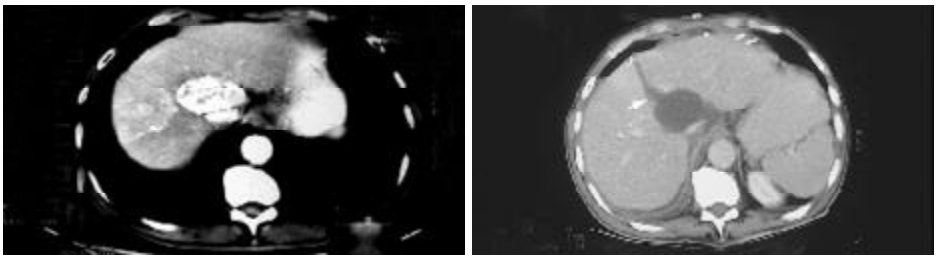


**Figure 1** Pre-operative CT scan shows the tumor originating in caudate lobe, post-operative CT scan shows splitting line(arrow).

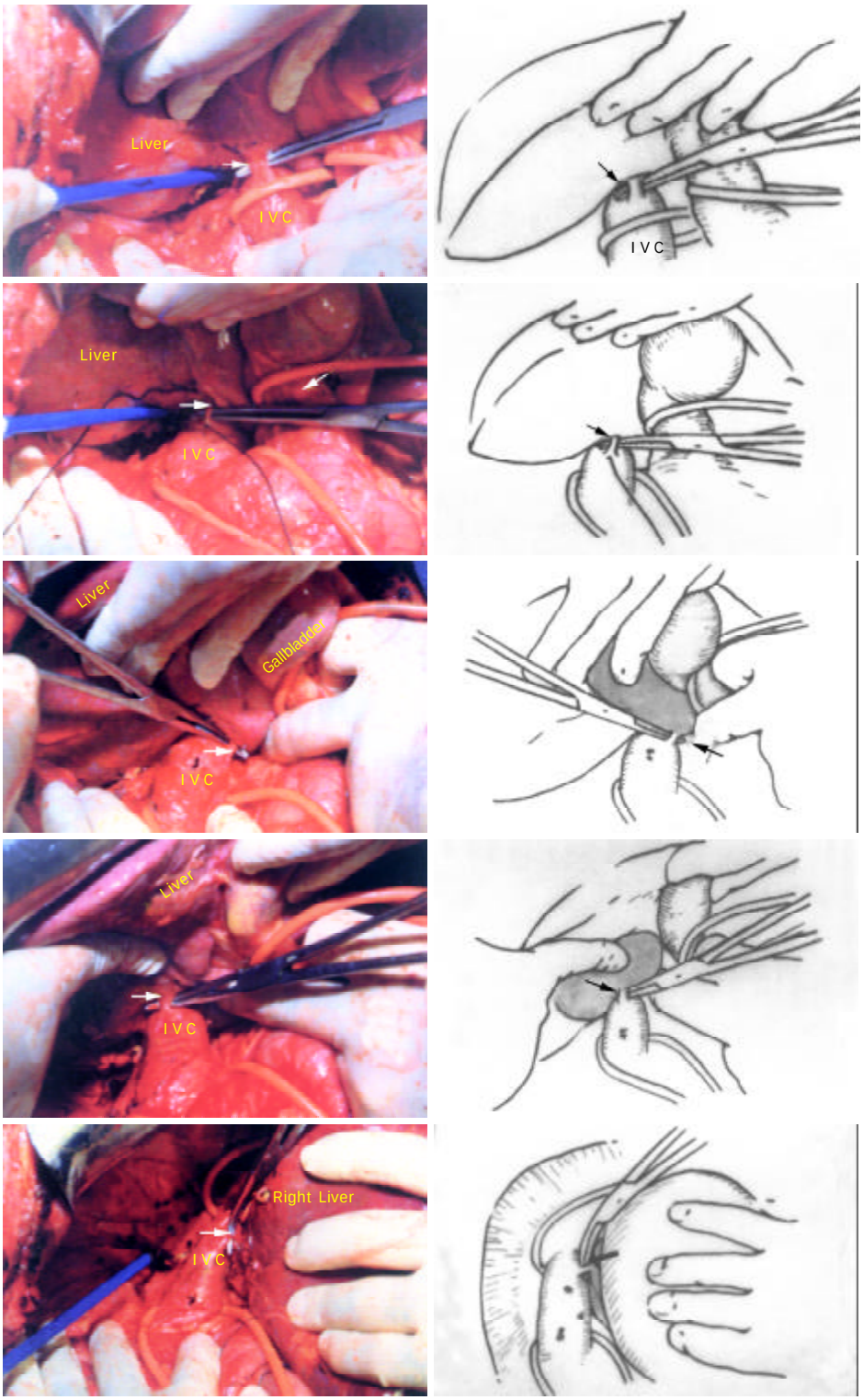



**Figure 2** Five short hepatic veins ligated from caudal direction to cranial direction respestively (Arrow).

In this series, the mobilization of caudate lobe was from left side first in most of the cases. Bartlett *et al*. reported that the ligamentous attachment should be separated and the tip of caudate lobe was mobilized before separation of the caudate veins[5].

The liver parenchyma was transected by means of "curettage and aspiration" technique using PMOD with intermittent inflow occlusion at the hepatoduodenal ligament (Pringle's maneuver), the time limit was 10 min each time with reperfusion for 2 min. Total vascular exclusion was seldom necessary except that when the tumor involved IVC or major hepatic veins. PMOD is a special instrument, it has the functions of dissection, coagulation and aspiration separately or synchronously. As a result, the surgical field was nearly bloodless and the intrahepatic duct structures could be identified, isolated and treated individually.

## RESULTS

Isolated or combined caudate lobectomy was successfully performed in 76 patients (Table 1). Isolated caudate lobectomy was performed in 25 cases. Of these 25 cases, isolated complete caudate lobectomy was performed in 19 cases, 6 cases (31.6 %,6/19) underwent complete resection by anterior transhepatic approach (Table 2). In these 6 cases, the size of tumor was from 3 cm to 8 cm, the mean operating time was 285±51 min, and the total occlusion time ranged from 50 to 110 min, the operative blood loss ranged from 500-3 000 ml (mean 1 600 ml).

No intraoperative death and signs of liver failure occurred in all the 6 cases, serum aspartate transaminase level recovered to normal range within one week postoperatively. One case had ascites because of liver cirrhosis, one case had right pleural effusion and was cured after aspiration two times. One case had bile leakage and was cured after two weeks' conservative treatment. Abdominal drains were placed in all the patients and removed within one week except the case with bile leakage.

All the 6 cases received a long-term follow-up. One case died in the 17th month postoperatively due to disease recurrence and liver failure, the other 5 cases have been alive without recurrence, with the longest survival of 49 months in 1 case.

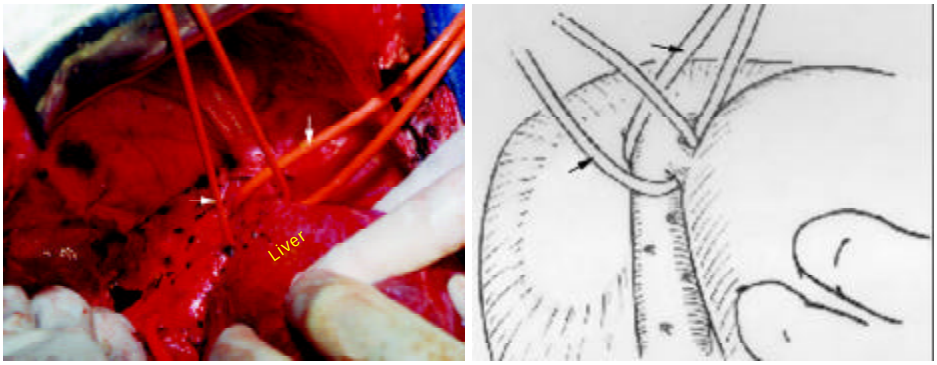



**Figure 3** The suprahepatic inferior vena cava and right hepatic vein (RHV) dissected and encircled with tapes (thick arrow: RHV, thin arrow: suprahepatic IVC).

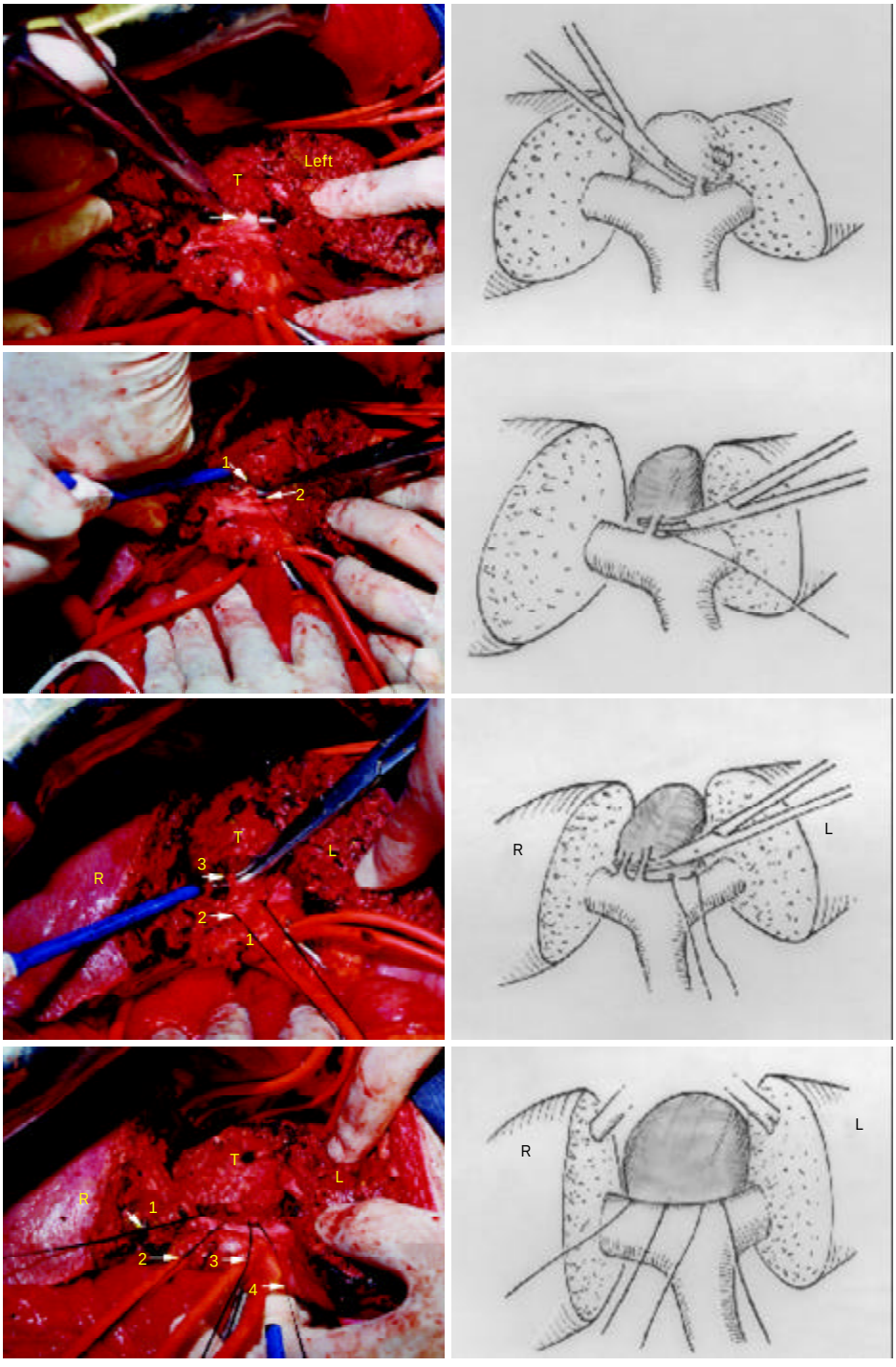



**Figure 4** Four groups of portal triads to the caudate lobe(Arrow) were divided, the tumor was detached from the hilum.

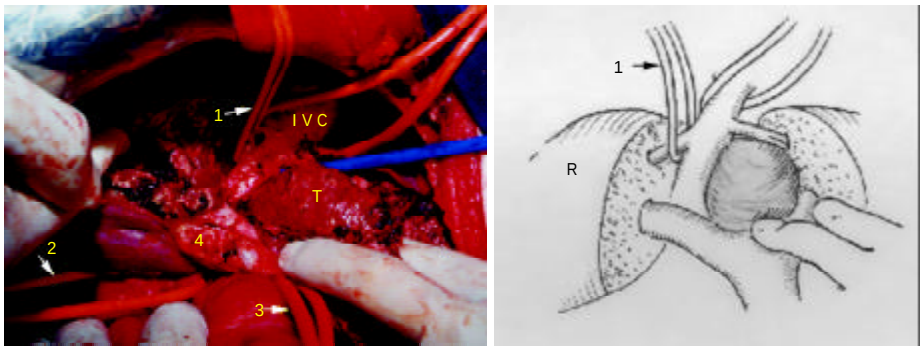



**Figure 5** The tumor still attaches to MHV (1: tape across RHV, 2: tape across IVC, 3: tape across pedicle, 4: the portion of bifurcation, T: tumor).

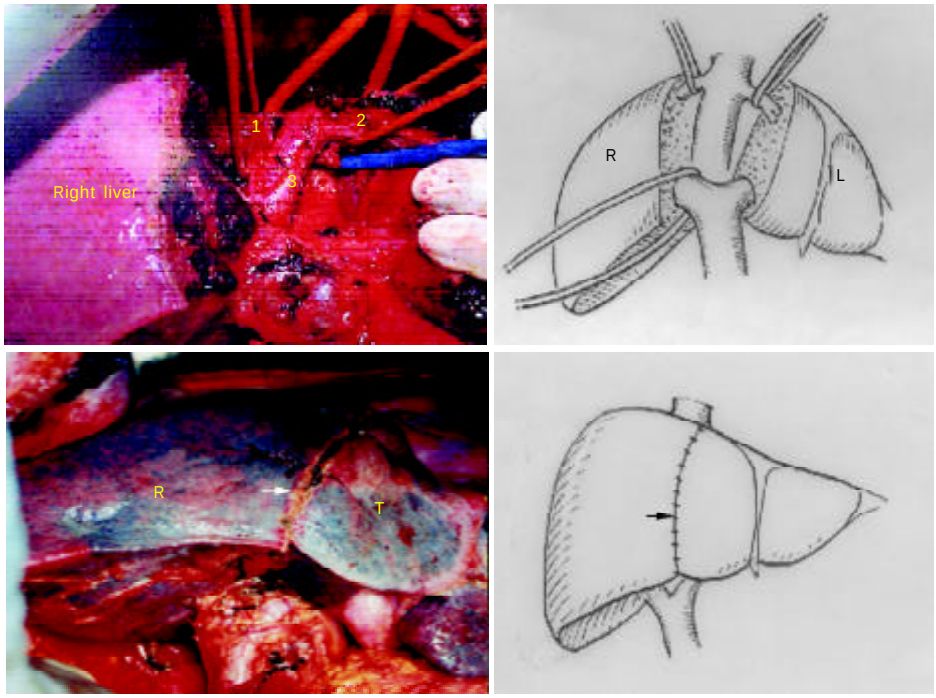



**Figure 6** Completely resected tumor. Two halves of the liver were sutured. (1: RHV, 2: common trunk of MHV and LHV, 3:IVC, R: right liver, L: left liver, Arrow: interlobar plane, T: tumor).

**Table 1** Resection procedures

| Operation | No. of patients |
|---|---|
| Complete caudate lobectomy(49cases) | |
| Isolated | 19 (total) |
| Anterior transhepatic approach | 6 |
| Other approaches | 13 |
| Combined | 30 (total) |
| Right hepatectomy+S1(C) | 13 |
| Left hepatectomy+S1(C) | 14 |
| Extend left hepatectomy+S1(C) | 2 |
| VI segmentectomy+S1(C)+T colon | 1 |
| Partial caudate lobectomy(27 cases) | |
| Isolated | 6 (total) |
| Combined | 21 (total) |
| Left hepatectomy+S1(P) | 5 |
| Right hepatectomy+S1(P) | 8 |
| Right hepatectomy+S1(P)+adrenal gland | 1 |
| V segmentectomy +S1(P) | 1 |
| VI segmentectomy +S1(P) | 4 |
| VII segmentectomy +S1(P) | 1 |
| V+VI segmentectomy +S1(P) | 1 |
| Total | 76 |

S1: caudate lobe, T Colon: transverse colon, P: partial, C:complete.

## DISCUSSION

The caudate lobe is a single anatomic segment that is defined by the presence of portal venous and hepatic arterial branches, which supply the lobe, drain biliary ducts and hepatic veins[6]. Three parts make up the entire caudate lobe, namely, the usual caudate lobe (Spiegelian lobe), the caudate process, and the paracaval portion[7]. Partial or complete caudate lobectomy with major hepatectomy is often necessary for extirpation of the tumor. But in China, most liver cancers occur in cirrhotic liver. The poor liver function of our cases prevented us from performing a combined or preparatory resection of major segment or other liver segments for tumors situated only in the caudate lobe.

Caudate lobectomy is classified by complete and partial resection, it is also classified by isolated and combined resection. Therefore caudate lobectomy is generally composed of four types: isolated complete resection, combined complete resection, isolated partial resection and combined partial resection. To select an appropriate surgical approach is essential for resection of caudate lobe. In our experience, right side approach is suitable for the tumor confined in caudate process or for cases when hepatectomy is indicated for cancer involvement. Left side approach is suitable for the combined resection of the left liver overlying the caudate lobe or for tumor confined in Spiegelian lobe. Isolated complete caudate lobectomy without resection

of innocent parenchyma is sometimes performed by splitting the parenchyma through interlobar plane as anterior approach. In addition, central segmentectomy is also classified as anterior approach, anterior segmentectomy together with caudate lobectomy has been considered as an appropriate treatment for hilar cholangiocarcinoma without infiltration of the posterior hepatic branch[8,9].

Some of the isolated caudate lobectomies could be performed through both left side and right side approaches when the tumor was small, but when the tumor was large or IVC and/or major hepatic veins were compressed by the tumor, the above methods might not be appropriate due to the possibility of laceration of major hepatic veins. Under such circumstances, anterior transhepatic approach is the best choice for isolated complete caudate lobectomy. In 1992, Yamamoto *et al*. described a patient with cirrhosis and a 3×3 cm HCC in the paracaval portion of the caudate lobe for whom they performed isolated caudate lobectomy by splitting the cirrhotic liver into two halves. The anterior transhepatic approach could provide a safe strategic alternative for isolated complete caudate lobectomy[10]. The separation of hepatic parenchyma overlying the caudate lobe could expose the major hepatic veins and the hilar plate to direct view, facilitating control of venous bleeding and interruption of the ascending paracaval portal branches along the hilar plate. Usually it would be associated with a significant amount of blood loss. In this series, we used PMOD to transect the liver parenchyma by means of "curettage and aspiration" technique, all of the intrahepatic ducts could be identified and isolated before it was transected. Asahara *et al*. reported that the minor operative time and blood loss were 355 min and 1 100 ml[2]. Because PMOD could facilitate the transection of cirrhotic liver, the minor operation time and blood loss were 234 min and 500 ml respectively. In our series with "curettage and aspiration" technique, not only the blood loss was decreased obviously, but also no atrophy of the medial segment was observed on postoperative computed tomography. So the benefit exceeded the risk of splitting the medium hepatic plane. For selective caudate lobectomy, the most dangerous part is the parenchyma division of its anterosuperior portion, where the roots of major hepatic veins are running next to the line of dissection. Without an outflow occlusion, any inadvertently deep cleavage could result in sudden and massive hemorrhage from a hepatic vein. Although total vascular exclusion could limit blood loss from the hepatic venous system and decrease the risk of air embolism. Such a maneuver would result in hemodynamic instability in up to 40 %[11]. In our series, Tapes were used to encircle the suprahepatic and infrahepatic IVC for precautions. The author usually isolated the common trunk and the right hepatic vein to pre-place the tapes for controlling the three major hepatic veins. In case massive hemorrhage occurred from a hepatic vein, we could control the hepatic vein to substitute the total vascular exclusion. In addition, during hepatic transection, usually dissection was carried out along the section plane, but when large vessels were shown, curettage was proceeded in parenchyma in parallel with the vessels in deep cleavage plane by altering direction, so sudden and massive hemorrhage occurred rarely, and we could treat vessels in direct view even if there was hemorrhage. In fact, we did not use tapes in all the 6 cases.

The anterior transhepatic approach for isolated complete caudate lobectomy is a curative procedure for primary or metastatic hepatobiliary neoplasm originating from caudate lobe, especially in the presence of cirrhosis when the tumor is large and involves IVC and/or the major hepatic veins. As PMOD and "curettage and aspiration" technique can delineate intrahepatic or extra hepatic vessels clearly, caudate lobe resection has become safer, easier and faster.

## ACKNOWLEDGMENT

We are deeply grateful to Professor Fang Zheng (Department of Anatomy, School of Medicine, Zhejiang University) for producing the figures of this paper.

## REFERENCES

1 **Lucandri G**, Stipa F, Sapienza P, Ziparo V, Stipa S. Resection of the caudate lobe for hepatocellular carcinoma. *Eur J Surg* 1998; **164**: 395-398
2 **Asahara T**, Dohi K, Hino H, Nakahara H, Katayama K, Itamoto T, Ono E, Moriwaki K, Yuge O, Nakanishi T, Kitamoto M. Isolated caudate lobectomy by anterior approach for hepatocellular carcinoma originating in the paracaval portion of the caudate lobe. *J Hepatobiliary Pancreat Surg* 1998; **5**: 416-421
3 **Yamamoto J**, Kosuge T, Shimada K, Yamasaki S, Takayama T, Makuuchi M. Anterior transhepatic approach for isolated resection of the caudate lobe of the liver. *World J Surg* 1999; **23**: 97-101
4 **Peng S**, Mou Y, Peng C, Cai X, Jiang X, Li J. Resection of caudate lobe of liver: report of 26 cases. *Zhonghua Waike Zazhi* 1999; **37**: 12-13
5 **Bartlett D**, Fong Y, Blumgart LH. Complete resection of the caudate lobe of the liver: technique and results. *Br J Surg* 1996; **83**: 1076-1081
6 **Blumgart LH**, Fong Y. Surgery of the liver and biliary tract. 3rd ed. *Health Science Asia Elsevier Science* 2000: 1639
7 **Takayama T**, Makuuchi M. Segmental liver resections, present and future-caudate lobe resection for liver tumors. *Hepatogastroenterology* 1998; **45**: 20-23
8 **Shimada H**, Izumi T, Note M, Seki H, Nakagawara G. Anterior segmentectomy with caudate lobectomy for hilar cholangiocarcinoma. *Hepatogastroenterology* 1993; **40**: 61-64
9 **Nagino M**, Nimura Y, Kamiya J, Kanai M, Uesaka K, Hayakawa N, Yamamoto H, Kondo S, Nishio H. Segmental liver resections for hilar cholangiocarcinoma. *Hepatogastroenterology* 1998; **45**: 7-13
10 **Yamamoto J**, Takayama T, Kosuge T, Yoshida J, Shimada K, Yamasaki S, Hasegawa H. An isolated caudate lobectomy by the transhepatic approach for hepatocellular carcinoma in cirrhotic liver. *Surgery* 1992; **111**: 699-702
11 **Yanaga K**, Matsumata T, Hayashi H, Shimada M, Urata K, Sugimachi K. Isolated hepatic caudate lobectomy. *Surgery* 1994; **115**: 757-761

**Edited by** Wang XL and Zhu LH

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2174-2177
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Inhibitory effects of antisense RNA of HAb18G/CD147 on invasion of hepatocellular carcinoma cells *in vitro*

Yu Li, Peng Shang, Ai-Rong Qian, Li Wang, Yong Yang, Zhi-Nan Chen

**Yu Li, Peng Shang, Ai-Rong Qian, Li Wang, Yong Yang, Zhi-Nan Chen,** Department of Cell Biology, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China
**Supported by** National Natural Science Foundation of China, No. 39989002
**Correspondence to:** Dr. Zhi-Nan Chen, Department of Cell Biology, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China. chcerc2 @fmmu.edu.cn
**Telephone:** +86-29-3374547 **Fax:** +86-29-3293906
**Received:** 2003-05-11 **Accepted:** 2003-06-07

## Abstract

**AIM:** To study the inhibitory effects of antisense RNA of HAb18G/CD147 on invasion of hepatocellular carcinoma (HCC) cells *in vitro*.

**METHODS:** Antisense RNA of HAb18G/CD147 vector PCI-asHAb18G was constructed by reversely inserting HAb18G/CD147 cDNA to eukaryotic expression vector PCI-neo. The HCC cell line HHCC was transfected by PCI-asHAb18G via cation liposome. Expression of HAb18G/CD147 of transfected cells selected by G418 (geneticin) was observed by immunohistochemical SP staining and FACS (fluorescence activated cell sorting). Gelatin zymography was used to determine the effect of PCI-asHAb18G on reducing secretions of MMP-2 and MMP-9 of the transfected cells. Boyden chamber was employed to test the invasion of HCC cells *in vitro*.

**RESULTS:** The construction of antisense RNA vector PCI-asHAb18G was verified correct by partial nucleotide sequencing and restricted endonuclease digestion. The expression of HAb18G/CD147 in transfected HHCC was inhibited by PCI-asHAb18G. Secretions of MMP-2 and MMP-9 of transfected HHCC were reduced and the invasion of transfected HHCC was inhibited compared to HHCC, respectively.

**CONCLUSION:** Invasion of HCC cells can be inhibited by antisense RNA of HAb18G/CD147. HAb18G/CD147 may be used as a potential target of drugs for anti-invasion and metastasis of HCC.

Li Y, Shang P, Qian AR, Wang L, Yang Y, Chen ZN. Inhibitory effects of antisense RNA of HAb18G/CD147 on invasion of hepatocellular carcinoma cells *in vitro*. *World J Gastroenterol* 2003; 9(10): 2174-2177
http://www.wjgnet.com/1007-9327/9/2174.asp

## INTRODUCTION

Invasion and metastasis are malignant characteristies of HCC and the main mortal reason for patients[1,2]. In the invasive and metastatic process of malignant tumor, molecules existing in extracellular matrix (ECM) and receptors or ligands existing on the surfaces of tumor cells play critical roles[3,4]. HAb18G is such a molecule obtained by screening cDNA library with HAb18 specific monoclonal antibody (mAb) against HCC[5-7]. The gene sequence of HAb18G is homologous to that of CD147. CD147, also named extracelluar matrix metalloproteinase inducer (EMMPRIN), is originated from human lung cancer cell line LX-1, firstly found in human HCC tissue in our laboratory. Previous studies demonstrated that EMMPRIN, a member of the immunoglobulin superfamily, concentrated on the surfaces of most tumor cells, promoted invasion of tumor cells by stimulating stromal cells to produce elevated levels of several matrix metalloproteinase (MMPs)[8,9] which play very important roles in several aspects of tumor progression, including growth, invasion, metastasis, and angiogenesis[10-12]. To study the relationship between HAb18G/CD147 and metastasis of HCC, we constructed a vector of antisense RNA of HAb18G/CD147 and investigated its inhibitory effects on invasion of HCC cells *in vitro*.

## MATERIALS AND METHODS

### *Plasmid, bacteria strain and cell line*

Plasmid pBluescript ks(+/-)/HAb18G including full length cDNA of HAb18G/CD147 was constructed by our laboratory. Eukaryotic expressing vector PCI-neo was purchased from Promega. *E.Coli* strain JM109 was purchased from Huamei Co, Ltd. Human HCC cell line HHCC was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. Human embryo dermal fibroblast cell line (fb) was kindly presented by Dr. Han (Department of Plastic Surgery, Fourth Military Medical University).

### *Construction and identification of antisense vector*

Full length cDNA fragment of HAb18G/CD147 was obtained from pBluescript ks(+/-)/HAb18G by cutting with *Xba*I and *Xho*I and inserted reversely into eukaryotic expressing vector PCI-neo by cutting with the same restriction endonucleases. The antisense vector of HAb18G/CD147, named as PCI-asHAb18G, was transformed into *E.coli* stain JM109 and identified by restricted endonuclease digestion and DNA sequences analysis by an automatic fluorescence sequenator using $T_3$ sequencing primer.

### *Cloning of cell transfected with antisense RNA vector*

Empty vector PCI-neo and antisense vector PCI-asHAb18G were transfected into human HCC cell line HHCC via cation liposome Lipfectinamine$^{2000}$ (Gibco) respectively. The two kinds of transfected cells were named as HHCC/neo and HHCC/asHAb18G respectively. All transfection procedures were performed according to the product manual. After two days of transfection, cells were selected by culture medium containing G418 (400 mg/L) for 4 weeks. The single clone was picked out by using limiting dilution method and cultured constantly in the culture medium containing G418 (100 mg/L).

### *Immunohistochemical staining of transfected cells*

Transfected cells were plated on glass slides overnight, fixed with cold acetone, and stained with SP (streptomycin avidin-peroxidase) according to the manufacturer' s instructions.

Briefly, mAb HAb18 was used as primary antibody and the goat anti-mouse mAb coupled with biotin as secondary antibody followed by indirect immunohistochemical staining with the mixture of streptomycin-avidin peroxidase and its substrate DAB. HHCC/neo served as the control.

### *FACS analysis*

HHCC/asHAb18G suspension was prepared by adding primary antibody and secondary antibody coupled with fluorescein into $10^8$ cells per liter. Then the cells were fixed and analyzed by flow cytometer.

### *Gelatin zymography*

Five experimental groups of cells were HHCC, fb, HHCC/neo+fb, HHCC/asHb18G +fb and HHCC+fb, which were plated into 100 mL culture flasks respectively. The ratio of HCC cells/fb cells was 1:1. After cultured in completed DMEM medium for 24 hours, all the groups of cells were washed three times with serum free DMEM and cultured for 2-3 days in DMEM with 20 mL/L bovine serum. Subsequently, the supernatants were collected and centrifuged to remove the cell debris. Proteins were precipitated with 800 g/L saturated $(NH_4)_2SO_4$. Precipitations were dissolved in 10 mmol/L Tris-HCl, pH 7.5 and dialyzed. Dialyzed samples were determined with SDS-PAGE that was modified in four points. Gelatin (Sigma) was added into the separating gel with 1.0 g/L, concentration of the stacking gel was 5 %, samples were not boiled and sample buffers did not contain DTT. After electrophoresis, the gel was washed with 0.1 mol/L NaCl and incubated for 24 hours at 37 ℃. Finally, the gel was dyed and decolorized.

### *Reconstituted basement membrane invasion detection*

Matrigel (main component was type IV collagen, purchased from Cell-biology Department of Pecking University) was added onto the inner surface of Boyden chambers (Millipore) to form the reconstituted basement membrane. Three groups of cells were HHCC+fb, HHCC/asHAb18G+fb, HHCC/neo+fb (the ratio of HCC cells/fb cells was 1:1), which were added on the reconstituted basement membrane respectively. Then the chambers were put in the 24-well plates and cultured overnight. Cells infiltrated through the reconstituted basement membrane and appeared on the outer surfaces of the membrane were stained with HE. The numbers of the cells were counted under high-power microscope.

## RESULTS

### *Construction and identification of PCI-asHAb18G*

Two fragments were obtained by digesting HAb18G/CD147 antisense vector with *Xho*I and *Xba*I , one was human HAb18G/CD147 cDNA fragment about 1.7 kb and the other was 5.5 kb fragment. Two fragments of 1.1 kb and 6.1 kb were also obtained with *Sma*I digestion (both MCS of vector and 1.1 kb site of HAb18G/CD147 cDNA respectively have a *Sma*I site). The construction of the vector was verified correct by endonuclease digestion (Figure 1) and nucleotide sequencing. The vector was named as PCI-asHAb18G.

### *Immunohistochemical staining and FACS*

HHCC/as HAb18G was negative, HHCC/neo and HHCC groups were positive (Figures 2-4). Average value of the fluorescence intensities of HHCC/asHAb18G cells was 5, and that of HHCC/neo cells was 500 (Figure 5).

### *Gelatin zymography and cell invasion*

The secretions of MMP-9 and MMP-2 in the transfected cells HHCC/asHAb18G co-cultured with fb were inhibited compared to those in the HHCC/neo and intact HHCC. The cells of HHCC/asHAb18G infiltrated through the reconstituted basement membrane were less than those of HHCC/neo and intact HHCC (Figure 7).

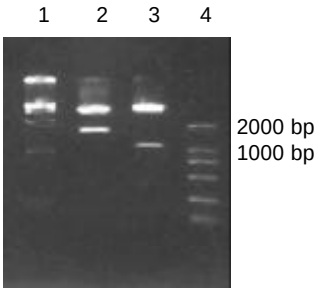



**Figure 1** Restricted endonuclease analysis of recombinant plasmid PCI-asHAb18G. 1: DNA marker DL15000, 2: PCI-asHAb18G/*Xho*I+*Xba*I, 3: PCI-asHAb18G/*Sma*I, 4: DNA marker DL2000.

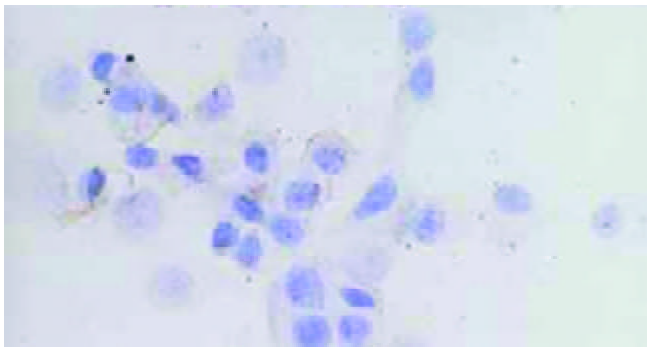


**Figure 2** Negative staining of HAb18G/CD147 on membrane of HHCC/asHAb18G SP ×400.

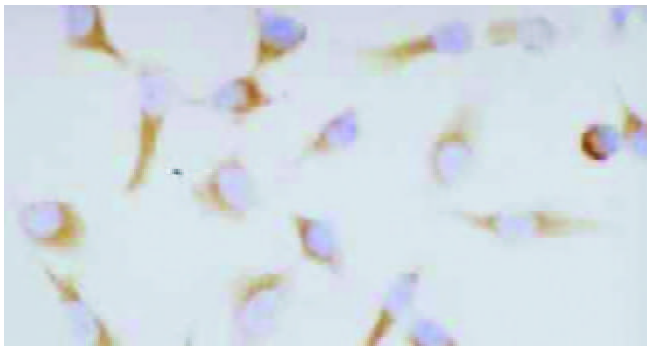


**Figure 3** Positive staining of HAb18G/CD147 on membrane of HHCC/neo SP ×400.

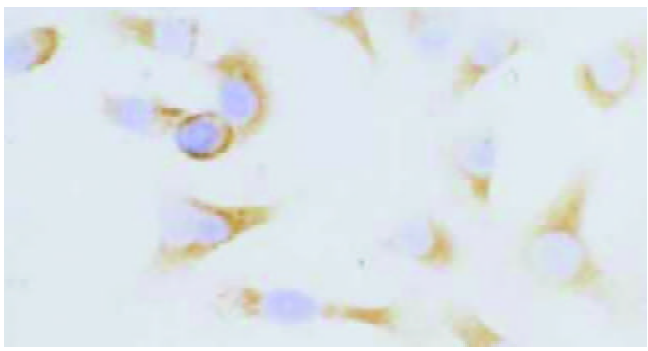


**Figure 4** Positive staining of HAb18G/CD147 on membrane of HHCC SP ×400.

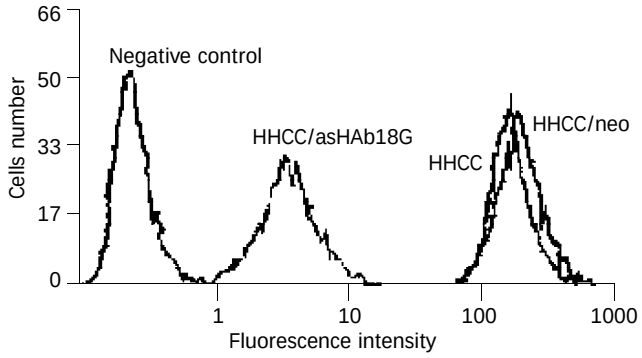



**Figure 5** FACS analysis of expressions of HAb18G / CD147 in four kinds of cells.

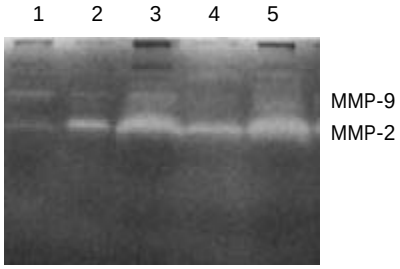



**Figure 6** Gelatin zymography of secretions of MMP-2 and MMP-9 in five groups of cells. 1: fb, 2: HHCC, 3: HHCC / neo+fb, 4: HHCC / asHAb18G+fb, 5: HHCC+fb.

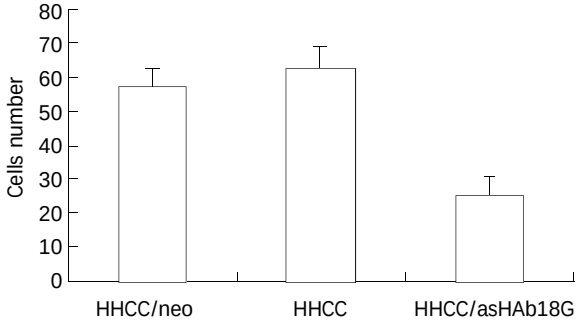



**Figure 7** Inhibitory effects of antisense RNA on invasion of HHCC / asHAb18G.

## DISCUSSION

Matrix metalloproteinases (MMPs) play an important part in tumor progression and tumor cell survival, with a positive correlation between MMP expression and the invasive and metastatic potential of malignant tumors, including colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian, and gastric carcinomas. CD147 is a heavily glycosylated transmembrane glycoprotein containing two immunoglobulin superfamily domains, which induces MMPs production in the adjacent stromal cells. Some results implied that a CD147 counter-receptor might existed on the fb cell surface, but such a counter- receptor has not been identified[13]. Other studies[14] showed that CD147 also acted in an autocrine fashion to increase productions of MMPs and invasiveness in tumor cells themselves. CD147 enriched in HCC tissue was first reported by our laboratory and might be a potential target for anti-invasion and metastasis therapies. Our previous studies showed that HAb18G/CD147 was highly expressed in HCC tissues and lowly expressed in normal tissues. HAb18G/CD147, not only participated adhesion of cell-cell or cell-matrix but also enhanced metastatic potentials of human hepatoma cells by disrupting the regulation of store-operated Ca(2+) entry by NO/cGMP[15,16].

The principle of antisense technology is the sequence-specific binding of an antisense oligonucleotide to target mRNA, resulting in the prevention of gene translation. The specificity of hybridisation makes antisense treatment an attractive strategy selectively modulating the expression of genes involved in the pathogenesis of diseases. In 1998, the first antisense drug (fomivirsen) was approved by the US Food and Drugs Administration (FDA) for the treatment of cytomegalovirus-induced retinitis in patients with AIDS[17]. Now, several antisense oligonucleotides have been under clinical trials, including oligonucleotides targeting the mRNA of Bcl-2[18,19], protein-kinase-C alpha[20,21], RAF kinase[22,23], H-ras[24,25], C-myb [26], DNA methyltransferase[27] and RI-alpha regulatory subunit of protein kinase A (PKA)[28,29]. Antisense oligonucleotides are well tolerated and might have therapeutic activities.

Our experimental findings showed that the expression of HAb18G/CD147 on HCC cells transfected by PCI-asHAb18G was decreased by analysis of immunohistochemical staining and FACS. Gelatin zymography demonstrated that secretions of MMP-2 and MMP-9 of HHCC/asHAb18G were lower than those of HHCC/neo and HHCC. Matrigel invasion assay indicated that invasion of HHCC/asHAb18G cells was inhibited significantly. All of the results confirm that antisense RNA targeting HAb18G/CD147 mRNA interfered with translation and expression of HAb18G/CD147, weakened the productions of MMPs, and inhibited the invasion of HCC cells through reconstituted basement membrne *in vitro*.

We suggest that HAb18G/CD147 be used as a novel target for anti-hepatoma metastatic therapy. Intervention in HAb18G/CD147 function by agents, such as antisense RNA, may have potential therapeutic value in the prevention of hepatoma invasion and metastasis.

## REFERENCES

1 **Li Y**, Tang ZY, Ye SL, Liu YK, Chen J, Xue Q, Chen J, Gao DM, Bao WH. Establishment of cell clones with different metastatic potential from the metastatic hepatocellular carcinoma cell line MHCC97. *World J Gastroenterol* 2001; **7**: 630-636

2 **Xiao CZ**, Dai YM, Yu HY, Wang JJ, Ni CR. Relationship between expression of CD44v6 and nm23-H1 and tumor invasion and metastasis in hepatocellular carcinoma. *World J Gastroenterol* 1998; **4**: 412-414

3 **Liu LX**, Jiang HC, Liu ZH, Zhou J, Zhang WH, Zhu AL, Wang XQ, Wu M. Integrin gene expression profiles of human hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 631-637

4 **Su JM**, Gui L, Zhou YP, Zha XL. Expression of focal adhesion kinase and alpha5 and beta1 integrins in carcinomas and its clinical significance. *World J Gastroenterol* 2002; **8**: 613-618

5 **Lou C**, Chen ZN, Bian HJ, Li J, Zhou SB. Pharmacokinetics of radioimmunotherapeutic agent of direct labeling mAb 188Re-HAb18. *World J Gastroenterol* 2002; **8**: 69-73

6 **Yang LJ**, Sui YF, Chen ZN. Preparation and activity of conjugate of monoclonal antibody HAb18 against hepatoma F(ab')(2) fragment and staphylococcal enterotoxin A. *World J Gastroenterol* 2001; **7**: 216-221

7 **Bian HJ**, Chen ZN, Deng JL. Direct technetium-99m labeling of anti-hepatoma monoclonal antibody fragment: a radioimmunoconjugate for hepatocellular carcinoma imaging. *World J Gastroenterol* 2000; **6**: 348-352

8 **Kanekura T**, Chen X, Kanzaki T. Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. *Int J Cancer* 2002; **99**: 520-528

9 **Bordador LC**, Li X, Toole B, Chen B, Regezi J, Zardi L, Hu Y. Ramos DM. Expression of emmprin by oral squamous cell carcinoma. *Int J Cancer* 2000; **85**: 347-352

10 **Hou L**, Li Y, Jia YH, Wang B, Xin Y, Ling MY, Lü S. Molecular mechanism about lymphogenous metastasis of hepatocarcinoma

cells in mice. *World J Gastroenterol* 2001; **7**: 532-536

11 **Roeb E**, Schleinkofer K, Kernebeck T, Potsch S, Jansen B, Behrmann I, Matern S, Grotzinger J. The matrix metalloproteinase 9 (mmp-9) hemopexin domain is a novel gelatin binding domain and acts as an antagonist. *J Biol Chem* 2002; **277**: 50326-50332

12 **Wang TN,** Albo D, Tuszynski GP. Fibroblasts promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase-9 production. *Surgery* 2002; **132**: 220-225

13 **Guo HM**, Li RS, Zucker S, Bryan P. Toole EMMPRIN (CD147), an Inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. *Cancer Research* 2000; **60**: 888-891

14 Sun JX, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer Research* 2001; **61**: 2276-2281

15 **Jiang JL**, Yu MK, Chen ZN, Chan HC. cGMP-regulated store-operated calcium entry in human hepatoma cells. *Cell Biol Int* 2001; **25**: 993-995

16 **Jiang JL**, Zhou Q, Yu MK, Ho LS, Chen ZN, Chan HC. The involvement of HAb18G/CD147 in regulation of store-operated calcium entry and metastasis of human hepatoma cells. *J Biol Chem* 2001; **276**: 46870-46877

17 **Vitravene Study Group.** A randomized controlled clinical trial of intravitreous fomivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with AIDS. *Am J Ophthalmol* 2002; **133**: 467-474

18 **Waters JS**, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, Cotter FE. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2000; **18**: 1812-1823

19 **Marcucci G,** Byrd JC, Dai G, Klisovic MI, Kourlas PJ, Young DC, Cataland SR, Fisher DB, Lucas D, Chan KK, Porcu P, Lin ZP, Farag SF, Frankel SR, Zwiebel JA, Kraut EH, Balcerzak SP, Bloomfield CD, Grever MR, Caligiuri MA. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. *Blood* 2003; **101**: 425-432

20 **Tolcher AW**, Reyno L, Venner PM, Ernst SD, Moore M, Geary RS, Chi K, Hall S, Walsh W, Dorr A, Eisenhauer E. A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clin Cancer Res* 2002; **8**: 2530-2535

21 **Cripps MC**, Figueredo AT, Oza AM, Taylor MJ, Fields AL, Holmlund JT, McIntosh LW, Geary RS, Eisenhauer EA. Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada clinical trials group study. *Clin Cancer Res* 2002; **8**: 2188-2192

22 **McPhillips F**, Mullen P, Monia BP, Ritchie AA, Dorr FA, Smyth JF, Langdon SP. Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian *cancer*. *Br J Cancer* 2001; **85**: 1753-1758

23 **Coudert B,** Anthoney A, Fiedler W, Droz JP, Dieras V, Borner M, Smyth JF, Morant R, de Vries MJ, Roelvink M, Fumoleau P. European organization for research and treatment of cancer (EORTC). Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer. *Eur J Cancer* 2001; **37**: 2194-2198

24 **Adjei AA,** Dy GK, Erlichman C, Reid JM, Sloan JA, Pitot HC, Alberts SR, Goldberg RM, Hanson LJ, Atherton PJ, Watanabe T, Geary RS, Holmlund J, Dorr FA. Phase I trial of ISIS 2503, an antisense inhibitor of H-ras, in combination with gemcitabine in patients with advanced cancer. *Clin Cancer Res* 2003; **9**: 115-123

25 **Cunningham CC**, Holmlund JT, Geary RS, Kwoh TJ, Dorr A, Johnston JF, Monia B, Nemunaitis J. A phase I trial of H-ras antisense oligonucleotide ISIS 2503 administered as a continuous intravenous infusion in patients with advanced carcinoma. *Cancer* 2001; **92**: 1265-1271

26 **Luger SM**, O'Brien SG, Ratajczak J, Ratajczak MZ, Mick R, Stadtmauer EA, Nowell PC, Goldman JM, Gewirtz AM. Oligodeoxynucleotide-mediated inhibition of c-*myb* gene expression in autografted bone marrow: a pilot study. *Blood* 2002; **99**: 1150-1158

27 **Goffin J**, Eisenhauer E. DNA methyltransferase inhibitors-state of the art. *Ann Oncol* 2002; **13**: 1699-1716

28 **Wang H**, Hang J, Shi Z, Li M, Yu D, Kandimalla ER, Agrawal S, Zhang R. Antisense oligonucleotide targeted to RIalpha subunit of cAMP-dependent protein kinase (GEM231) enhances therapeutic effectiveness of cancer chemotherapeutic agent irinotecan in nude mice bearing human cancer xenografts: *in vivo* synergistic activity, pharmacokinetics and host toxicity. *Int J Oncol* 2002; **21**: 73-80

29 **Agrawal S**, Kandimalla ER, Yu D, Ball R, Lombardi G, Lucas T, Dexter DL, Hollister BA, Chen SF. GEM 231, a second-generation antisense agent complementary to protein kinase A RIalpha subunit, potentiates antitumor activity of irinotecan in human colon, pancreas, prostate and lung cancer xenografts. *Int J Oncol* 2002; **21**: 65-72

**Edited by** Zhu LH

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2178-2181
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Role of serum total sialic acid in differentiating cholangiocarcinoma from hepatocellular carcinoma

Prachya Kongtawelert, Pisit Tangkijvanich, Siriwan Ong-Chai, Yong Poovorawan

**Prachya Kongtawelert, Siriwan Ong-Chai,** Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200 Thailand
**Pisit Tangkijvanich,** Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330 Thailand
**Yong Poovorawan,** Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330 Thailand
**Correspondence to:** Dr. Yong Poovorawan, Viral Hepatitis Research Unit, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, 10330 Thailand. yong.p@chula.ac.th
**Telephone:** +662-256-4909 **Fax:** +662-256-4929
**Received:** 2003-06-04 **Accepted:** 2003-08-02

## Abstract

**AIM:** This study was designed to evaluate the clinical application of serum total sialic acid (TSA) in the diagnosis of cholangiocarcinoma (CCA).

**METHODS:** Serum TSA was determined by periodate-resorcinol microassay in 69 patients with CCA, 59 patients with hepatocellular carcinoma (HCC), 37 patients with cirrhosis, 61 patients with chronic hepatitis and 50 healthy blood donors.

**RESULTS:** The mean serum TSA concentration in CCA (2.41±0.70 mmol/L) was significantly higher than those of HCC, cirrhosis, chronic hepatitis and healthy blood donors (1.41±0.37 mmol/L, 1.13±0.31 mmol/L, 1.16±0.26 mmol/L, and 1.10±0.14 mmol/L, respectively; $P<0.001$). Based on ROC curve analysis, a cut-off point of 1.75 mmol/L discriminated between CCA and HCC with a sensitivity, specificity and accuracy of 82.6 %, 83.1 %, and 82.8 %, respectively.

**CONCLUSION:** Based on our results, serum TSA would be a useful marker for the differential diagnosis of CCA from HCC.

Kongtawelert P, Tangkijvanich P, Ong-Chai S, Poovorawan Y. Role of serum total sialic acid in differentiating cholangiocarcinoma from hepatocellular carcinoma. *World J Gastroenterol* 2003; 9(10): 2178-2181
http://www.wjgnet.com/1007-9327/9/2178.asp

## INTRODUCTION

Cholangiocarcinoma (CCA) constitutes a common primary liver cancer in Southeast Asia where the liver fluke, *Opisthorchis viverrini*, is endemic[1]. Most patients with CCA are diagnosed at advanced stages, therefore, treatment of the cancer is usually palliative and the prognosis is poor[2]. Currently, there is no ' gold standard' tumor marker for the diagnosis of CCA. This is particularly remarkable for early detection of the tumor itself, for screening of the high-risk groups, and for differentiating CCA from hepatocellular carcinoma (HCC), another primary liver cancer which is common in Southeast Asia and frequently associated with chronic hepatitis B or C[3]. Among the available serum tumor markers, the most commonly used is a high-molecular-weight glycolipid, carbohydrate antigen 19-9 (CA 19-9). CA 19-9, however, is not a sensitive or specific tumor marker for CCA. As a single diagnostic test, CA 19-9 increases in approximately 65 % of liver fluke-associated CCA[4]. Elevated concentrations of this marker have also been observed in patients with a variety of gastrointestinal cancers, as well as benign cholestasis and acute cholangitis[5]. As a result, a more sensitive and specific serum marker for the diagnosis of CCA is considered necessary.

Sialic acid, a class of important ketoses that contain nine carbon atoms, is an acetylated derivative of neuraminic acid (2-keto-5-amino-3, 5-dideoxy-D-nonulosonic acid)[6]. The unique structural features of this molecule, which includes a negative charge owing to a carboxyl group, enable it to play an important role in cellular functions, such as cell-to-cell recognition and transformation to malignancy[7]. Elevated levels of serum total sialic acid (TSA) have been reported in patients diagnosed with various cancers such as lymphoma, malignant melanoma, lung cancer and gastrointestinal cancers[8,9]. Recently, it has been shown that most patients with CCA have an elevated concentration of serum TSA, and determination of this marker yields high diagnostic values that differentiate between CCA and benign hepatobiliary diseases[10]. However, the diagnostic role of the serum marker in discriminating CCA from HCC has never been verified.

Therefore, the aim of this study was to use a simple technique (microassay) to determine the clinical application of serum TSA in the diagnosis of CCA by comparison with HCC and other chronic liver diseases including chronic hepatitis and cirrhosis.

## MATERIALS AND METHODS

### *Subjects*

Sera for the measurement of TSA levels were obtained from 5 groups of subjects who were attending King Chulalongkorn Memorial Hospital and Udonthani Hospital from January 1998 to July 1999.

Group 1 consisted of 50 adult healthy blood donors as control subjects.

Group 2 consisted of 61 patients with chronic hepatitis which was diagnosed based on histopathology.

Group 3 consisted of 37 patients with cirrhosis. The diagnosis of cirrhosis was based on histopathology and/or clinical features such as the presence of ascites, or esophageal varices.

Group 4 consisted of 59 patients with HCC. The diagnosis of HCC was based on histopathology and/or imaging techniques combined with serum alpha-fetoprotein levels above 400 ng/ml.

Group 5 comprised 69 patients with CCA. All patients in this group were residents of Thailand's northeastern provinces where *O. viverrini* was endemic. The peripheral type CCA was diagnosed based on liver tumor features detected by ultrasound/CT scan and confirmed by histology. Criteria for

diagnosis of the hilar type included findings of primary mass at the hilum and the evidence of bile duct dilatation on ultrasound/ CT scan and confirmed by characteristic features on cholangiography or histopathology.

All subjects were informed about the objective of the study, and subsequently provided their consent. Blood was obtained during investigation at the initial presentation, sera were separated by centrifugation and stored at -70 ℃ at Viral Hepatitis Research Unit until tested for TSA concentrations.

### *Measurement of serum TSA by periodate-resorcinol microassay*

Serum TSA determination was performed as previously described[6], with some modifications. Briefly, 40 μL of samples or pure standard sialic acid solution (2-10 μg/well) was added to the wells of a 96-well microtiter plate. Then, 50 μL of 1.3 mM periodic acid (prepared from stock 0.32 M), was added to each well and mixed by shaking the plate for 5 minutes on a microplate shaker at room temperature. The plate was placed (floated) in an icebox for 60 minutes, then 100 μL of 0.6 g/dL of resorcinol reagent (prepared from stock 6 g/dL) was added and mixed by shaking as described above. The plate was covered with a glass and heated at 80 ℃ for 60 minutes in water bath, then it was removed and placed on the shaker for about 2 minutes mixing as well as cooling the contents down to room temperature. Then, 100 μL of 95 % tert-butyl alcohol was added to each well and mixed once again as described above. The absorbance at 620 nm was measured immediately by a microtiter plate reader.

The precision of the test was determined by analysis of an intra- and inter-assay coefficient variation (CV). The method described herein demonstrated an intra- and inter-assay CV of 0.79 % and 4.68 %, respectively. Furthermore, the recovery percentage of this assay was 94.25 %.

### *Statistical analysis*

The data were expressed as mean values ± standard deviation. Statistical significance in the mean values was evaluated by the Student' s *t* test. Receiver-operating characteristic (ROC) curves were constructed to establish the diagnostic cut-off level of serum TSA in discriminating CCA from other groups. Sensitivity, specificity, positive and negative predictive values and diagnostic accuracy were calculated in accordance with standard methods. *P* value below 0.05 was considered statistically significant.

## RESULTS

Comparison of the characteristics of the subjects in each group (Table 1) showed that the mean age of patients with CCA (60.2±12.6 years) was significantly higher than those with HCC and cirrhosis (53.0±12.0 and 50.0±13.3 years, respectively; $P$=0.001), chronic hepatitis and blood donors (42.0±13.0 and 34.6±10.3 years, respectively; $P$<0.001). There was no significant difference in the sex distribution in each group. Mean total bilirubin level was significantly higher in patients with CCA compared with those with HCC, cirrhosis and chronic hepatitis ($P$<0.05), whereas mean serum alkaline phosphatase was significantly higher in patients with CCA compared to those with cirrhosis and chronic hepatitis ($P$<0.05).

The distributions of serum TSA levels in each group are shown in Figure 1. The mean serum TSA concentration in patients with CCA (2.41±0.70 mmol/L) was significantly higher than that in those with HCC, cirrhosis, chronic hepatitis and blood donors (1.41±0.37 mmol/L, 1.13±0.31 mmol/L, 1.16±0.26 mmol/L, and 1.10±0.14 mmol/L, respectively; $P$<0.001). The mean concentration of serum TSA in patients with HCC was also significantly higher than that in those with cirrhosis, chronic hepatitis and blood donors ($P$=0.001). However, there was no significant difference in mean serum level of TSA among patients with cirrhosis, chronic hepatitis and blood donors.

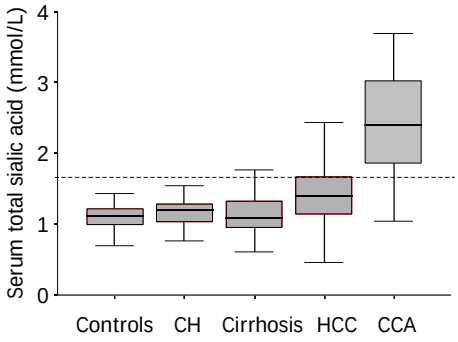



**Figure 1** Serum total sialic acid (TSA) concentrations in each group. Cut-off value of serum TSA as a tumor marker was 1.75 mmol/L.

In order to discriminate CCA from HCC with an optimal accuracy, an analysis of the ROC curve was performed. As shown in Figure 2A, the area under the curve of CCA and HCC was 0.885 [95 % confidence interval (CI) 0.828-0.0.942]. This result indicated that approximately 89 % of randomly selected patients from the positive group (CCA) would have a higher TSA value than a patient randomly selected from the HCC group. Similarly, the area under the ROC curve of CCA and the other three groups (cirrhosis, chronic hepatitis and healthy controls) was 0.964 (95 % CI 0.938-0.0.989) (Figure 2B), indicating that approximately 96 % of patients with CCA would have a higher level of serum TSA than patients with cirrhosis, chronic hepatitis and healthy controls.

Based on the ROC curve analysis, a cut-off point of serum TSA concentration considered as the highest accuracy for diagnosing CCA was 1.75 mmol/L. At this concentration, the sensitivity, specificity and accuracy for differentiating CCA from HCC were 82.6 %, 83.1 %, and 82.8 %, respectively.

**Table 1** Clinical characteristics of the subjects in this study

| Group | No | Age (yr) | Sex (M/F) | TB (mg/dl) | ALT (IU/L) | Alb (g/l) | AP (IU/L) |
|---|---|---|---|---|---|---|---|
| Controls | 50 | 34.6±10.3[a] | 29/21 | - | - | - | - |
| CH | 61 | 42.0±13.0[a] | 46/15 | 1.6±0.9[a] | 98.1±72.9 | 3.8±0.3 | 201.5±106.2[a] |
| Cirrhosis | 37 | 50.0±13.3[a] | 26/11 | 2.5±1.3[a] | 65.0±49.6 | 3.3±0.5 | 290.2±212.5[a] |
| HCC | 59 | 53.0±12.0[a] | 50/9 | 3.0±2.1[a] | 79.9±46.1 | 3.4±0.3 | 612.9±393.7 |
| CCA | 69 | 60.2±12.6 | 46/23 | 7.5±7.6 | 71.4±68.7 | 3.4±0.5 | 894.3±850.2 |

Controls=healthy blood donors, CH=chronic hepatitis, HCC=hepatocellular carcinoma, CCA=cholangiocarcinoma. TB=total bilirubin, ALT=alanine aminotransferase, Alb=albumin, AP=alkaline phosphatase. Data were expressed as mean±standard deviation. Statistical analysis compared to CCA: [a]$P$<0.05.

Likewise, when compared to patients with cirrhosis, chronic hepatitis and healthy blood donors, the same cut-off level of serum TSA exhibited a sensitivity, specificity and accuracy for diagnosing CCA of 82.6 %, 86.0 %, and 84.8 %, respectively.

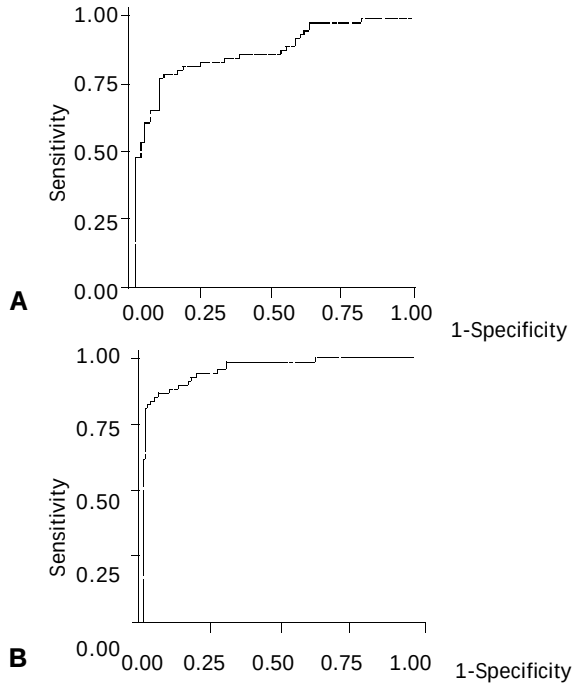



**Figure 2** ROC curves of serum TSA in distinguishing CCA from other groups. A: CCA and HCC (the area under the curve was 0.885), B: CCA and cirrhosis, chronic hepatitis, blood donors (the area under the curve is 0.964).

## DISCUSSION

Liver fluke-associated CCA is typically associated with its insidious onset of clinical manifestations, which frequently escapes detection until late stages. The clinical features of CCA appeared to differ according to the location of the tumor along the intrahepatic duct, whether in the hilum or periphery[11, 12]. The hilar type tends to present with progressive jaundice or ascending cholangitis due to tumor obstructtion in the confluence of the hepatic ducts, while the peripheral type usually presents with weight loss and progressive right upper quadrant pain caused by the expanding tumors. In areas where both liver flukes and chronic viral hepatitis are prevalent, it remains an essential problem to differentiate CCA from HCC. Although HCC is usually associated with cirrhosis, while conversely, CCA develops in a non-cirrhotic liver, the occurrence of CCA in cirrhosis has been increasingly recognized[13]. Moreover, approximately 10 % of patients with HCC presented with obstructive jaundice, a clinical feature that might imitate the hilar type of CCA[14]. Accordingly, a sensitive and specific serum marker of CCA would be considered as a valuable adjunct to non-invasive imaging for the diagnosis of the cancer.

Serum TSA has been reported as a diagnostic maker for patients with various cancers such as lymphoma, malignant melanoma, lung cancer and gastrointestinal cancers[8,9]. A variety of methods for the detection and estimation of free and glycosidically-bound sialic acids have been performed. These techniques could be broadly classified as colorimetric, fluorometric, enzymatic methods, as well as the highly sensitive high performance liquid chromatographic (HPLC) method[7]. Among these, the most widely used technique is the colorimetric method, which includes the Ehrlich method, the periodate-thiobabituric acid method, and the periodic-resorcinol assay. In this report, we employed a newly developed periodate-resorcinol micorassay that has several advantages over the conventional methods. These include use of smaller samples (5 μL), a larger number of samples simultaneously analyzed, and a greater speed in measuring absorbance by a microtiter plate spectrophotometer. Moreover, this method enables the direct transfer of data to a computer, or even an adaptation to an automated system.

In this study, we demonstrated that increased serum TSA concentration yielded a high sensitivity, specificity and accuracy for the diagnosis of CCA. Our data showed that the mean serum TSA level in patients with CCA was significantly increased compared to those with cirrhosis, chronic hepatitis and healthy controls ($P<0.001$). Notably, the mean concentration of serum TSA in patients with CCA was substantially higher than that in those with HCC ($P<0.001$). Based on the ROC curve analysis, a cut-off point of 1.75 mmol/L provided a satisfactorily high sensitivity, specificity and accuracy of above 80 % for the differential diagnosis of CCA from HCC. The data of the present study are in agreement with those previously reported which described an increase in serum TSA in patients with CCA[10,15]. Thus, serum TSA would be a useful marker, particularly in conjunction with radiological studies such as ultrasonography or CT scan, for the detection of CCA in patients presenting with clinical features of liver mass and jaundice.

The mechanisms underlying the substantial difference in levels of TSA between patients with CCA and HCC are unclear. One possible explanation is that, in certain cancers, increased activity of sialytransferase might lead to spontaneous shedding of aberrant sialic acid-containing cell surface glycoconjugates into the circulation[16]. Alternatively, since TSA has been well described as being associated with the acute-phase protein response[17], increased activity of serum or tissue sialidase in combination with inflammatory response could considerably elevate serum TSA levels in patients with CCA. Also, the difference between the ages of the CCA and HCC groups might be responsible for this discrepancy, since serum TSA concentration slightly increased with advancing age[18]. Nonetheless, the age-dependent difference between groups might not be sufficient to interfere with the discrimination of serum TSA values in our study. To confirm this observation, the group of age-matched patients with HCC should also be examined for serum TSA levels.

In conclusion, we have demonstrated that serum TSA concentrations, which were determined by a simple microassay, are significantly higher in patients with CCA than in those with HCC, cirrhosis, chronic hepatitis and blood donors. At a cut-off point of 1.75 mmol/L, this serum marker can yield a satisfactory accuracy for the differential diagnosis of CCA from HCC. Accordingly, the measurement of serum TSA level might be useful for the diagnosis of CCA, particularly in the regions where liver flukes are common.

## ACKNOWLEDGEMENT

We would like to thank Venerable Dr. Mettanando Bhikkhu of Wat Nakprok, Bangkok, for editing the manuscript. This work was supported by grants from the National Research Council, Bangkok, the Center of Excellent Fund, Center of Excellence Viral Hepatitis Research Unit, Faculty of Medicine, Chulalongkorn University, the Thailand Research Fund, Senior Research Scholar (YP) and Junior Research Scholar (PK).

## REFERENCES

1 **Parkin DM**, Srivatanakul P, Khlat M, Chenvidhya D, Chotiwan P, Insiripong S, L'Abbe KA, Wild CP. Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int J Cancer* 1991; **48**: 323-328

2 **Watanapa P**. Cholangiocarcinoma in patients with opisthorchiasis. *Br J Surg* 1996; **83**: 1062-1064
3 **Tangkijvanich P**, Hirsch P, Theamboonlers A, Nuchprayoon I, Poovorawan Y. Association of hepatitis viruses with hepatocellular carcinoma in Thailand. *J Gastroenterol* 1999; **34**: 227-233
4 **Pungpak S**, Akai PS, Longenecker BM, Ho M, Befus AD, Bunnag D. Tumour markers in the detection of opisthorchiasis-associated cholangiocarcinoma. *Trans R Soc Trop Med Hyg* 1991; **85**: 277-279
5 **Duffy MJ**. CA 19-9 as a marker for gastrointestinal cancers: a review. *Ann Clin Biochem* 1998; **35**( Pt 3): 364-370
6 **Bhavanandan VP**, Sheykhnazari M. Adaptation of the periodate-resorcinol method for determination of sialic acids to a microassay using microtiter plate reader. *Anal Biochem* 1993; **213**: 438-440
7 **Narayanan S**. Sialic acid as a tumor marker. *Ann Clin Lab Sci* 1994; **24**: 376-384
8 **Kokoglu E**, Sonmez H, Uslu E, Uslu I. Sialic acid levels in various types of cancer. *Cancer Biochem Biophys* 1992; **13**: 57-64
9 **Polivkova J**, Vosmikova K, Horak L. Utilization of determining lipid-bound sialic acid for the diagnosis and further prognosis of cancer. *Neoplasma* 1992; **39**: 233-236
10 **Wongkham S**, Boonla C, Kongkham S, Wongkham C, Bhudhisawasdi V, Sripa B. Serum total sialic acid in cholangiocarcinoma patients: an ROC curve analysis. *Clin Biochem* 2001; **34**: 537-541
11 **Uttaravichien T,** Buddhisawasdi V. Experience of non-jaundiced cholangiocarcinoma. *Hepatogastroenterology* 1990; **37**: 608-611
12 **Kullavanijaya P**, Tangkijvanich P, Poovorawan Y. Current status of infection-related gastrointestinal and hepatobiliary diseases in Thailand. *Southeast Asian J Trop Med Public Health* 1999; **30**: 96-105
13 **Hui CK**, Yuen MF, Tso WK, Ng IO, Chan AO, Lai CL. Cholangiocarcinoma in liver cirrhosis. *J Gastroenterol Hepatol* 2003; **18**: 337-341
14 **Qin LX**, Tang ZY. Hepatocellular carcinoma with obstructive jaundice: diagnosis, treatment and prognosis. *World J Gastroenterol* 2003; **9**: 385-391
15 **Wongkham S**, Bhudhisawasdi V, Chau-in S, Boonla C, Muisuk K, Kongkham S, Wongkham C, Boonsiri P, Thuwajit P. Clinical significance of serum total sialic acid in cholangiocarcinoma. *Clin Chim Acta* 2003; **327**: 139-147
16 **Singhal A**, Hakomori S. Molecular changes in carbohydrate antigens associated with cancer. *Bioessays* 1990; **12**: 223-230
17 **Crook MA**, Treloar A, Haq M, Tutt P. Serum total sialic acid and acute phase proteins in elderly subjects. *Eur J Clin Chem Clin Biochem* 1994; **32**: 745-747
18 **Stefenelli N**, Klotz H, Engel A, Bauer P. Serum sialic acid in malignant tumors, bacterial infections, and chronic liver diseases. *J Cancer Res Clin Oncol* 1985; **109**: 55-59

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2182-2185
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Prevention of hepatocellular carcinoma in mice by IL-2 and B7-1 genes co-transfected liver cancer cell vaccines

Ning-Ling Ge, Sheng-Long Ye, Ning Zheng, Rui-Xia Sun, Yin-Kun Liu, Zhao-You Tang

**Ning-Ling Ge, Sheng-Long Ye, Ning Zheng, Rui-Xia Sun, Yin-Kun Liu, Zhao-You Tang,** Liver Cancer Institute of Zhongshan Hospital Affiliated to Fudan University, Shanghai 200032, China
**Supported by** the National Key Technologies Research and Development during the 9th Five-Year Plan period, Program of China, No. 96-906-01-20
**Correspondence to:** Dr. Sheng-Long Ye, Liver Cancer Institute of Zhongshan Hospital Affiliated to Fudan University, Shanghai 200032, China. slye@shmu.edu.cn
**Telephone:** +86-21-64041990 Ext 2150 **Fax:** +86-21-64037181
**Received:** 2003-05-12 **Accepted:** 2003-06-02

## Abstract

**AIM:** To study the immunoprotective effect of liver cancer vaccine with co-transfected IL-2 and B7-1 genes on hepatocarcinogenesis in mice.

**METHODS:** The murine liver cancer cell line Hepal-6 was transfected with IL-2 and/or B7-1 gene via recombinant adenoviral vectors and the liver cancer vaccines were prepared. C57BL/6 mice were immunized with these vaccines and challenged with the parental Hepal-6 cells afterwards. The immunoprotection was investigated and the reactive T cell line was assayed.

**RESULTS:** The immunoprotection of the tumor vaccine was demonstrated. The effect of IL-2 and B7-1 genes co-transfected Hepal-6 liver cancer vaccine (Hep6-IL2/B7 vaccine) on the onset of tumor formation was the strongest. When attacked with wild Hepal-6 cells, the median survival period of the mice immunized with Hep6-IL2/B7 vaccine was the longest (68 days, $\chi^2$=7.70-11.69, $P$<0.05) and the implanted tumor was the smallest ($z$ =3.20-44.10, $P$<0.05). The effect of single IL-2 or B7-1 gene-transfected vaccine was next to the IL2/B7 gene co-transfected group, and the mean survival periods were 59 and 54 days, respectively. The mean survival periods of wild or enhanced green fluorescence protein gene modified vaccine immunized group were 51 and 48 days, respectively. The mice in control group all died within 38 days and the implanted tumor was the largest ($z$=3.20-40.21, $P$<0.05). The cellular immunofunction test and cytotoxicity study showed that the natural killer (NK) cell, lymphokine activated killer (LAK) cell and cytotoxic T lymphocyte (CTL) activities were significantly increased in mice immunized with the Hep6-IL2/B7 vaccine, (29.5±2.5 %, 65.0±2.9 %, 83.1±1.5 % respectively, compared with other groups, $P$<0.05).

**CONCLUSION:** The Hep6-IL2/B7 liver cancer vaccines can induce the mice to produce activated and specific CTL against the parental tumor cells, and demonstrate stronger effect on the hepatocarcinogenesis than single gene modified or the regular tumor vaccine. Therefore, the vaccines may become a novel potential therapy for recurrence and metastasis of HCC.

Ge NL, Ye SL, Zheng N, Sun RX, Liu YK, Tang ZY. Prevention of hepatocellular carcinoma in mice by IL-2 and B7-1 genes co-transfected liver cancer cell vaccines. *World J Gastroenterol* 2003; 9(10): 2182-2185
http://www.wjgnet.com/1007-9327/9/2182.asp

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of liver in humans and the rate of incidence and mortality is very high in East Asia and China[1]. Though many approaches, such as surgical resection, transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), radiotherapy and liver transplantation were developed to treat it, and the effective and survival rates were increased, a large number of patients died from recurrence and metastasis[2-6].

Some studies showed immunogene modified tumor vaccines could induce effective specific active T cell immune response to prevent tumor recurrence and metastasis[7-10]. Also some reports demonstrated that specific active immunotherapy based on specific antitumor T cell immunity could be a potential modality for further improving the survival of HCC patients on preventing tumor recurrence and metastasis[11-14]. But HCC is poorly immunogenic. Poor or no immunogenicity and lack of costimulating molecules on the surface of tumor cells are some of the causes of most tumors including HCC[15].

The successful induction of an anti-tumor immune response has been reported in a number of tumor models including HCC animal models by using B7-1 (CD80) transfected tumor cells as a vaccine[16]. The rationale of B7-1 based immunotherapy is that T cells require both an antigen-specific signal delivered through the T-cell receptor and a co-stimulatory signal to be fully activated and proliferated, and secrete cytokines or generate cytotoxic T lymphocyte (CTL) to cytolyse tumor cells[17-19].

Interleukin 2 (IL-2) is a growth factor that stimulates the proliferation of cytotoxic T cells, helper T cells, natural killer (NK) cells, and lymphokine activated killer (LAK) cells, all of which can participate in the anti-tumor response[20-22]. Many HCC animal models demonstrated that local secretion of IL-2 abrogated the tumorigenicity of cytokine-producing tumor cells and inducing a long-lasting protective immune response against a subsequent tumor graft[23,24].

In this study, we compared the effect and immunological mechanism of mouse Hepal-6 liver cancer cell vaccine modified by IL-2 and/or B7-1 genes on protecting the C57BL/6 mice from challenge of wild parental Hepal-6 cells. The results provide some experimental evidences that gene modified tumor vaccine can prevent recurrence and metastasis of liver cancer.

## MATERIALS AND METHODS

### *Cell lines*

For the experiments, three tumor cell lines were used. Hepal-6 was a liver cancer cell line derived from C57BL/6 mice generously provided by Dr. Li-Xin Wei from Eastern Hepato-Biliary Surgery Hospital, Second Military Medical University,

Shanghai. P815 (NK cell resistant) and Yac-1 (NK cell sensitive) were leukemia cell lines derived from C57BL/6 mice and generous gifts from Prof. Xue-Tao Cao from Institute of Immunology, Second Military Medical University, Shanghai.

### Mice

Female C57BL/6 mice, 6-8 weeks old (15-19 g), were purchased from the Animal Center of Chinese Academy of Sciences and housed in a specific-pathogen-free animal facility in Shanghai Medical University.

### Replication-deficient recombinant adenoviral vectors

Recombinant adenoviruses carrying human IL-2 and B7-1 genes (AdVhIL-2 and AdVhB7-1) were obtained from Prof. Xue-Tao Cao. The control AdVEGFP (recombinant adenovirus carrying enhanced green fluorescence protein) was gifted from Dr. Gambotto from Pittsburgh University, USA.

### Reagents

CD3-FITC, CD4-PE, CD8-PE and CD25-PE, monoclonal antibodies (mAbs) specific for murine CD3, CD4, CD8 and CD25 respectively,used for flow cytometry were purchased from Pharmingen Co. (USA). MTS kits used for cytolytic assay were purchased from Promega Co. (USA). CD80-FITC which was mAbs specific for human CD80 was purchased from Pharmingen Co. (USA). Human IL-2 ELISA Kits used to detect the active part p70 of IL-2 in solution were purchased from DIACLONE Co. (USA).

### Preparation of gene modified Hepal-6 tumor cell vaccines

Hepal-6 cells were transfected with various recombinant adenoviruses according to the five protocols listed below, namely 200 pfu/cell AdhIL-2, 200 pfu/cell AdhB7-1, co-transfection of 200 pfu/cell AdhIL-2 and 200 pfu/cell AdhB7-1, 200 pfu/cell AdEGFP, and wild Hepal-6 cell not transfected with any gene. All of the modified Hepal-6 cells in the five groups were inactivated with mitomycin-C (80 ug/ml) to prepare tumor vaccines. These tumor vaccines were named as Hep6-IL2, Hep6-B7, Hep6-IL2B7, Hep6-EGFP and Hep6 tumor cell vaccines, respectively.

### Immunization of C57BL/6 mice with various gene modified Hepal-6 tumor cell vaccines

The mice were divided into six groups with 6 mice in each. Five groups were injected $5\times10^6$ cell vaccines/mouse subcutaneously in the left scapula of those above 5 tumor cell vaccines, respectively. The sixth group was control group and the mice were injected culture medium. All the mice were injected twice a week.

### Immunoprotection of tumor cell vaccines to mice

On the 7th day after the final immunization, the mice were injected $8\times10^6$/mouse of wild Hepal-6 cell subcutanously in right scapula. The mice were scored for tumor growth twice a week according to the tumor volume, and calculated as $V=L*W^2/2$ (L: length, W: width)[25]. The survival time of the mice was investigated.

### Preparation of lymphocytes in spleens from mice immunized with gene modified Hepal-6 tumor cell vaccines

The mice in each group were killed by dislocating cervical vertebra on the 7th day after the final immunization, and the spleens were cut aseptically and minced into suspension of single splenocytes. Then the suspensions were centrifuged at 1 000 rpm for 5 min and RBCs in the splenocytes were lysed with sterile distilled water for 10 sec to get lymphocytes, and then the debris was filtered. The filtered cells were centrifuged, and the lymphocytes were harvested and maintained in 1 640 medium (GIBCO BRL, USA) containing 10 % FCS (HYCLONE, USA).

### Immunostaining and FACS analysis

The freshly prepared spleocytes were analyzed by direct immunofluorescence staining with mAbs CD3-FITC, CD4-PE, CD8-PE and CD25-PE. Flow cytometry analysis was then performed with a FACScan (Becton Dickson Co.) in the Department of FACS, the Chinese Academy of Sciences.

### Induction of the cytolytic activity of LAK cells and CTL from the lymphocytes

The harvested lymphocytes ($2\times10^6$ cell/ml) were cultured in 1 640 containing 10 % FCS with rhIL-2 at 1 000 U/ml at 37 ℃ in a 5 % $CO_2$ incubator for 5 days and then cocultured with mitomycin Ctreated Hepal-6 cells (20:1 of responder to tumor cell ratio ) in 1 640 containing 10 % FCS with rhIL-2 at 50 U/ml at 37 ℃ in a 5 % $CO_2$ incubator for 5-7 days.

### In vitro cytolytic assay of NK, LAK cells, and CTL

MTS assay was employed to test the cytolytic activity of NK cell, LAK cell and CTL from stimulated lymphocytes. Target cells were Yac-1, P815 and Hepal-6 respectively and incubated with various lymphocytes at 37 ℃ in a 5 % $CO_2$ incubator for 2-4 hours at 100:1 of effector/target ratio. Assays were performed in triplicate wells. Cytolytic activity was calculated according to the formula provided by the MTS kits: cytolytic activity (%) =[(ODe-ODb)+(ODt-ODb)-(ODa- ODb)]×100 %/(ODt- ODb).

### Statistical analysis

The data were analyzed with SAS 6.12 and STATA 6.0 software, and expressed as $\bar{x}\pm s$. The cytolytic activity of NK cell, LAK cell and CTL was determined by *t* test. The growth curves of tumor volume were analyzed with a generalized regression model and the mice survival period was analyzed with Log-Rank test.

## RESULTS

### In vivo immuno-protection by various tumor cell vaccines

After the immunized mice were challenged with wild Hepal-6 cells, the change of the tumor volume was assessed as is shown in Figure 1. The tumors grew progressively in Hep6-EGFP and Hep6 tumor vaccine immunized mice. The tumors in Hep6-IL2 and Hep6-B7 tumor vaccine immunized mice grew more slowly than those in Hep6-EGFP, Hep6 tumor vaccine immunized mice and the control. Compared with the above 5 groups of mice, the tumors in Hep6-IL2B7 tumor vaccine immunized mice grew most slowly and the tumor volume was the smallest ($z$=3.20-44.10, $P$<0.05) and about 50 % of the tumor disappeared 68 days after attacked by the wild Hepal-6 cell.

The survival periods of each group of mice were compared. The median survival time of Hep6, Hep6-EGFP, Hep6-B7 and Hep6-IL2 tumor vaccine immunized mice was 51, 48, 54 and 59 days, respectively and significantly longer than that of the control group ($\chi^2$=10.61-23.81, $P$<0.05). The median survival period of the control mice was the shortest and all the mice died within 41 days. The median survival period of the Hep6-IL2B7 tumor vaccine immunized mice was significantly longer than that of other groups ($\chi^2$=7.70-11.69, $P$<0.05) and all the mice in this group were alive for more than 68 days after attacked by the wild Hepal-6 cell.

### Expression of B7-1 and IL-2 genes transduced Hepal-6 tumor vaccine

Flow cytometry analysis confirmed that the transfected tumor

vaccines Hep6-IL2B7 and Hep6-B7 expressed a higher level of CD80 (55.47 % and 59.11 %, respectively) than Hep6-IL2, Hep6 and Hep6-EGFP tumor vaccines (12.48 %, 11.87 %, and 7.93 %, respectively).

In the supernant of Hep6-B7, Hep6 and Hep6-EGFP tumor vaccines, no IL-2 was detected, while a high level IL-2 was detected in the supernant of Hep6-IL2 and Hep6-IL2B7 tumor vaccines (1 885 pg and 460 pg per $3*10^5$ cells, respectively).

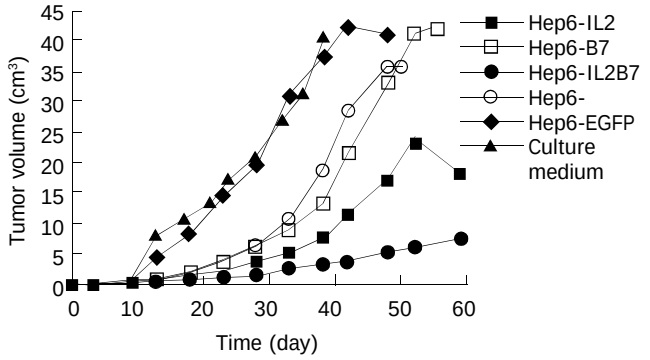



**Figure 1** Change of tumor volumes after cancer vaccine immunization.

### *Flow cytometry analysis*

The CD4/CD8 ratio in lymphocytes of the Hep6-IL2B7 tumor vaccine immunized mice was the lowest (0.91) among all the study groups and the CD25 positive rate was the highest (32.85 %). The CD4/CD8 ratios in lymphocytes of the Hep6-B7, Hep6-IL2 and Hep6 tumor vaccine immunized mice and normal mice were 1.02, 1.17, 1.24 and 1.31, respectively, while the CD25 positive rates were 28.62 %, 26.13 %, 19.32 % and 3.59 %, respectively.

### *Cytolytic assay of NK cell, LAK cell, CTL of spleen lymphocytes*

The results are shown in Table 1. The cytolytic activities of the NK cells in each group of tumor vaccine immunized mice were all significantly higher than those in the normal mice ($t$=11.3-15.5, $P$<0.05) and it was the highest in the Hep6-IL2B7 group (29.5±2.5 %, $t$=10.4-15.5, $P$<0.05). The cytolytic activities of the LAK cells in Hep6-IL2 and Hep6-IL2B7 tumor vaccine immunized mice were significantly higher than those in the normal mice (60.9±1.7 %, 65.0±2.9 %, $t$=10.6-40.2, $P$<0.05). The cytolytic activities of the CTL induced by Hep6-IL2B7 tumor vaccine were significantly higher than those in other groups (83.1±1.5 %, $t$=13.7-53.3, $P$<0.05).

**Table 1** The NK cell, LAK cell and CTL activities of each group mice against different target cells ($\bar{x}$±$s$ %)

| Effector cell | Target cell | | |
|---|---|---|---|
| | Yac-1 | p815 | Hepal-6 |
| Normal group | 19.0±1.9 | 48.4±1.9[c] | 29.9±1.3[e] |
| Hep6-IL2 cancer vaccine immunized group | 32.5±2.7[a] | 60.9±1.7 | 64.4±12.3[e] |
| Hep6-B7 cancer vaccine immunized group | 17.0±1.7 | 47.7±1.2[c] | 71.2±1.5[e] |
| Hep6-IL2B7 cancer vaccine immunized group | 29.5±2.5[a] | 65.0±2.9 | 83.1±1.5 |
| Hep6 cancer vaccine immunized group | 18.5±1.4 | 42.5±1.3[c] | 52.8±1.2[e] |

NK cell activities: [a]$P$<0.05 *vs* normal, Hep6-B7 and Hep6 cancer vaccine immunized group; LAK cell activities: [c]$P$<0.05 *vs* Hep6-IL2 and Hep6-IL2B7 cancer vaccine immunized group; CTL activities: [e]$P$<0.05 *vs* Hep6-IL2B7 cancer vaccine immunized group.

## DISCUSSION

Hepatocellular carcinoma was poor immunogenicity partly because of poor antigen expression and lack of co-stimulatory molecules[26]. Though successful induction of anti-tumor immunity by means of B7-1 gene[11,14,16] or cytokine (IL-2, IL-4, IL-12, GM-CSF, IFN, and so on) gene[9,12-14,27] transfection has been reported in some HCC animal models, prevention of tumor recurrence and metastasis still needs to be improved.

Some animal experiments showed that IL-2 gene-modified tumor vaccine constantly secreted a relatively high level of IL-2 in local areas, induced local and systemical specific antitumor immuno-reactions and immuno-memory T cells in the body of the animals, prevented the challenge of the next parental tumor and decreased the recurrence rate[28-30]. Reports showed the locally secreted high level of IL-2 generated much less side effects than a systemically administered high dose of IL-2[31].

Double signal systems were needed in the process of inducing effective specific active antitumor T cell immune response[18]. B7-1 was an important co-stimulating molecule for tumor antigen presentation, and could provide the second signal system for T cell immune[11,32,33]. Tumors could escape from the immune surveillance of host when they were lack of it[17]. B7-1 gene-modified tumor vaccine could break the immuno-tolerance of the tumor to the host and induce effective specific antitumor immunology[32,34].

Some reports showed a single immunogene modified tumor vaccine could not induce very effective immune response because double signal systems were needed in the process[18,19] and multi-gene modified tumor vaccine could improve the immunogenicity of tumor vaccines in different ways and promote T cell immunity[18,19,35-39]. So in our experiment, we investigated the immunoprotection of single IL-2 or B7-1 gene or both IL-2 and B7-1 genes immunized mice HCC cell line Hepal-6 tumor vaccines.

In our experiment, the results showed Hepal-6 liver cancer cell line expressed a low level of B7-1 molecule and no IL-2 was secreted, and the tumorigenicity was 100 %. The vaccine prepared from wild Hepal-6 cells showed very weak protection against wild tumor cell attacking. The tumor vaccine modified with IL-2 or B7-1 gene induced a stronger immunoprotective effect than the wild vaccines, and could significantly improve the survival rate and median survival time. More excitingly, the immunoprotection induced by IL-2 and B7-1 genes co-transfected tumor cell vaccine was even better than that of either of the genes. These two genes improved the immunogenicity of the liver cancer cell vaccine synergically and induced the mice to generate stronger anti-tumor immunoreactions. The immunological analysis of spleno-lymphocytes from the mice showed that IL-2 and B7-1 genes co-transfected liver cancer cell vaccine induced lymphocytes to express a significantly higher CD25 positive level and a lower CD4/CD8 ratio. This result suggested the T cells were activated. The cytotoxic analysis showed after immunization with this vaccine, specific CTLs were induced in the mice.

The results in this mouse liver cancer model demonstrated that after immunization with IL-2 and B7-1 genes co-transfected liver cancer cell vaccine, more effective systemic anti-tumor cell immunoreactions could be induce in mice than that with either of the gene modified vaccines, and the induced immuno-memory reaction could protect against the challenge of the wild parental Hepal-6 cell. The results suggest that the co-transfected gene tumor vaccine can prevent recurrence and metastasis of liver cancer.

## ACKNOWLEDGMENTS

We are grateful to Drs. Yan Zhao and Wei-Hua Bao for their advice and help in the study.

## REFERENCES


1 **Pisani P,** Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; **83**: 18-29
2 **Tang ZY**. Hepatocellular carcinoma-cause, treatment and metastasis. *World J Gastroenterol* 2001; **7**: 445-454
3 **Hanazaki K,** Kajikawa S, Shimozawa N, Mihara M, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J. Survival and recurrence after hepatic resection of 386 consecutive patients with hepatocellular carcinoma. *J Am Coll Surg* 2000; **191**: 381-388
4 **Takayama T,** Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomized trial. *Lancet* 2000; **356**: 802-807
5 **Huang YH,** Wu JC, Lui WY, Chau GY, Tsay SH, Chiang JH, King KL, Huo TI, Chang FY, Lee SD. Prospective case-controlled trial of adjuvant chemotherapy after resection of hepatocellular carcinoma. *World J Surg* 2000; **24**: 551-555
6 **Poon RTP,** Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* 2000; **232**: 10-24
7 **Natsume A,** Mizuno M, Ryuke Y, Yoshida J. Antitumor effect and cellular immunity activation by murine interferon-beta gene transfer against intracerebral glioma in mouse. *Gene Ther* 1999; **6**: 1626-1633
8 **Parmiani G,** Rodolfo M, Melani C. Immunological gene therapy with ex vivo gene-modified tumor cells: a critique and a reappraisal. *Hum Gene Ther* 2000; **11**: 1269-1275
9 **Arienti F**, Belli F, Napolitano F, Sule-Suso J, Mazzocchi A, Gallino GF, Cattelan A, Santantonio C, Rivoltini L, Melani C, Colombo MP, Cascinelli N, Maio M, Parmiani G, Sanantonio C. Vaccination of melanoma patients with interleukin 4 gene-transduced allogeneic melanoma cells. *Hum Gene Ther* 1999; **10**: 2907-2916
10 **Del Vecchio M**, Parmiani G. Cancer vaccination. *Forum* 1999; **9**: 239-256
11 **Li Z,** Sui Y, Jiang Y, Lei Z, Shang J, Zheng Y. Reconstruction of SEA-B7.1 double signals on human hepatocellular carcinoma cells and analysis of its immunological effect. *Biochem Biophys Res Commun* 2001; **288**: 454-461
12 **Yamashita YI,** Shimada M, Hasegawa H, Minagawa R, Rikimaru T, Hamatsu T, Tanaka S, Shirabe K, Miyazaki JI, Sugimachi K. Electroporation-mediated interleukin-12 gene therapy for hepatocellular carcinoma in the mice model. *Cancer Res* 2001; **61**: 1005-1012
13 **Barajas M,** Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, Sangro B, Melero I, Qian C, Prieto J. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology* 2001; **33**: 52-61
14 **Shi M,** Wang FS, Wu ZZ. Synergetic anticancer effect of combined quercetin and recombinant adenoviral vector expressing human wild-type p53, GM-CSF and B7-1 genes on hepatocellular carcinoma cells in vitro. *World J Gastroenterol* 2003; **9**: 73-78
15 **Dan Q**, Sanchez R, Delgado C, Wepsic HT, Morgan K, Chen Y, Jeffes EW, Lowell CA, Morgan TR, Jadus MR. Non-immunogenic murine hepatocellular carcinoma Hepa1-6 cells expressing the membrane form of macrophage colony stimulating factor are rejected *in vivo* and lead to CD8+ T-cell immunity against the parental tumor. *Mol Ther* 2001; **4**: 427-437
16 **Tatsumi T**, Takehara T, Kanto T, Kuzushita N, Ito A, Kasahara A, Sasaki Y, Hori M, Hayashi N. B7-1 (CD80)-gene transfer combined with interleukin-12 administration elicits protective and therapeutic immunity against mouse hepatocellular carcinoma. *Hepatology* 1999; **30:** 422-429
17 **Chen L**, McGowan P, Ashe S. Tumor immunogenicity determines the effect of co-stimulation by B7 on T-cell-mediated tumor immunity. *J Exp Med* 1994; **179**: 523-532
18 **Paul DB,** Barth RF, Yang W, Shen GH, Kim J, Triozzi PL. B7.1 expression by the weakly immunogenic F98 rat glioma does not enhance immunogenicity. *Gene Ther* 2000; **7**: 993-999
19 **Buggins AG,** Lea N, Gaken J, Darling D, Farzaneh F, Mufti GJ, Hirst WJ. Effect of costimulation and the microenvironment on antigen presentation by leukemic cells. *Blood* 1999; **94**: 3479-3490
20 **Heike Y,** Takahashi M, Ohira T, Naruse I, Hama S, Ohe Y, Kasai T, Fukumoto H, Olsen KJ, Podack EE, Saijo N. Genetic immunotherapy by intrapleural, intraperitoneal and subcutaneous injection of IL-2 gene-modified Lewis lung carcinoma cells. *Int J Cancer* 1997; **73**: 844-849
21 **Atkins MB**. Interleukin-2: clinical applications. *Semin Oncol* 2002; **29**: 12-17
22 **Herberman RB**. Cancer immunotherapy with natural killer cells. *Semin Oncol* 2002; **29**: 27-30
23 **Guarini A**, Riera L, Cignetti A, Montacchini L, Massaia M, Foa R. Transfer of the interleukin-2 gene into human cancer cells induces specific antitumor recognition and restores the expression of CD3 / T-cell receptor associated signal transduction molecules. *Blood* 1997; **89**: 212-218
24 **Kim JH**, Gong SJ, Yoo NC, Lee H, Shin DH, Uhm HD, Jeong SJ, Cho JY, Rha SY, Kim YS, Chung HC, Roh JK, Min JS, Kim BS. Effects of interleukin-2 transduction on the human hepatoma cell lines using retroviral vector. *Oncol Rep* 1999; **6**: 49-54
25 **Vanhaesebroeck B**, Mareel M, Van Roy F, Grooten J, Fiers W. Expression of the tumor necrosis factor gene in tumor cells correlates with reduced tumorigenicity and reduced invasiveness *in vivo*. *Cancer Res* 1991; **51**: 2229-2238
26 **Tatsumi T**, Takehara T, Katayama K, Mochizuki K, Yamamoto M, Kanto T, Sasaki Y, Kasahara A, Hayashi N. Expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on human hepatocellular carcinoma. *Hepatology* 1997; **5**: 1108-1114
27 **Qian SB,** Chen SS. Transduction of human hepatocellular carcinoma cells with human alpha-interferon gene via retroviral vector. *World J Gastroenterol* 1998; **4**: 210-213
28 **Hajkova R,** Indrova M, Jandlova T, Bubenik J, Reinis M. Interleukin 2 gene therapy of surgical minimal residual tumor disease: characterization of cytolytic effector cells from tumor progressore and regressors. *Folia Biol Praha* 1999; **45**: 227-231
29 **Mizuno H,** Yanoma S, Nishimura G, Hattori S, Ito T, Okudera K, Tsukuda M. Therapeutic efficiency of IL-2 gene transduced tumor vaccine for head and neck carcinoma. *Cancer Lett* 2000; **152**: 175-185
30 **Palmer K,** Moore J, Everard M, Harris JD, Rodgers S, Rees RC, Murray AK, Mascari R, Kirkwood J, Riches PG, Fisher C, Thomas JM, Harries M, Johnston SR, Collins MK, Gore ME. Gene therapy with autologous, interleukin 2-secreting tumor cells in patients with malignant melanoma. *Hum Gene Ther* 1999; **10**: 1261-1268
31 **Tagawa M.** Cytokine therapy for cancer. *Curr Pharm Des* 2000; **6**: 681-699
32 **Takahashi T,** Hirano N, Takahashi T, Chiba S, Yazaki Y, Hirai H. Immunogene therapy against mouse leukemia using B7 molecules. *Cancer Gene Ther* 2000; **7**: 144-150
33 **Yang G,** Mizuno MT, Hellstrom KE, Chen L. B7-negative versus B7-positive P815 tumor: differential requirements for priming of an antitumor immune response in lymph nodes. *J Immuno* 1997; **158**: 851-858
34 **Antonia SJ**, Extermann M, Flavell RA. Immunologic nonresponsiveness to tumors. *Crit Rev Oncog* 1998; **9**: 35-41
35 **Barnard AL,** Farzaneh F, Gaken J, Darling D. Local versus systemic interlukin-2: tumor formation by wild-type and B7-1-positive murine melanoma cells. *Cancer Gene Ther* 2000; **7**: 207-214
36 **Fujiwara H**, Yamauchi N, Sato Y, Sasaki K, Takahashi M, Okamoto T, Sato T, Iyama S, Koshita Y, Hirayama M, Yamagishi H, Niitsu Y. Synergistic suppressive effect of double transfection of tumor necrosis factor-alpha and interleukin 12 genes on tumorigenicity of Meth-A cells. *Jpn J Cancer Res* 2000; **91**: 1296-1302
37 **Hurwitz AA**, Townsend SE, Yu TF, Wallin JA, Allison JP. Enhancement of the anti-tumor immune response using a combination of interferon-gamma and B7 expression in an experimental mammary carcinoma. *Int J Cancer* 1998; **77**: 107-113
38 **Mazzocchi A,** Melani C, Rivoltini L, Castelli C, Del Vecchio M, Lombardo C, Colombo MP, Parmiani G. Simultaneous transduction of B7-1 and IL-2 genes into human melanoma cells to be used as vaccine: enhancement of stimulatory activity for autologous and allogeneic lymphocytes. *Cancer Immunol Immunother* 2001; **50**: 199-211
39 **Jang YJ,** Nam SY, Kim MS, Seong RH, Park YS, Chung YH, Chung HY. Simultaneous expression of allogenic class II MHC and B7.1 (CD80) molecules in A20 B-lymphoma cell line enhances tumor immunogenicity. *Mol Cells* 2002; **13**: 130-136


**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2186-2193
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Oxidative DNA damage in peripheral leukocytes and its association with expression and polymorphisms of $hOGG_1$: A study of adolescents in a high risk region for hepatocellular carcinoma in China

Tao Peng, Han-Ming Shen, Zhi-Ming Liu, Lu-Nan Yan, Min-Hao Peng, Le-Qun Li, Ren-Xiang Liang, Zong-Liang Wei, Barry Halliwell, Choon Nam Ong

**Tao Peng, Zhi-Ming Liu, Min-Hao Peng, Le-Qun Li,** Department of Hepatobiliary Sugery, First Affiliated Hospital of Guangxi Medical University, Nanning, 530021, Guangxi Zhuang Autonomous Region, China
**Han-Ming Shen, Choon Nam Ong,** Department of Community, Occupational and Family Medicine, National University of Singapore, Singapore
**Lu-Nan Yan,** Department of General Surgery, First Affiliated Hospital of West China University of Medical Sciences, Chengdu, 610041, Sichuan Zhuang Autonomous Region, China
**Ren-Xiang Liang, Zong-Liang Wei,** Fusui Cancer Research Institute, Fusui County, 532100, Guangxi Province, China
**Barry Halliwell,** Department of Biochemistry, National University of Singapore, Singapore
**Supported by** the Guangxi Natural Sciences Grant, No.GKZ9912028 and No.GKJ0236030, Guangxi Educational Committee Grant, No. GZBH 2000-272, Guangxi Health Ministry Medicine Grant, No. Z2001087 and Singapore Science Grant, No.R-186-000-044-213
**Correspondence to:** Dr. Tao Peng, Department of Hepatobiliary Surgery, First Affiliated Hospital of Guangxi Medical University, Nanning, 530021, Guangxi Zhuang Autonomous Region, China. pengpang@hotmail.com
**Telephone:** +86-771-5352400
**Received:** 2003-07-12 **Accepted:** 2003-07-24

## Abstract

**AIM:** To study the oxidative DNA damage to adolescents of hepatocellular carcinoma (HCC) families in Guangxi Zhuang Autonomous Region, China.

**METHODS:** Peripheral leukocytes' DNA 7, 8-dihydro-8-oxoguanine (8-oxoG) and repair enzyme $hOGG_1$ were quantified by flow-cytometry. $hOGG_1$-Cys326Ser single nucleotide polymorphism (SNP) was distinguished by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) assay.

**RESULTS:** There was a positive correlation between 8-oxoG and repair enzyme $hOGG_1$ expression ($P<0.001$). HCC children ($n=21$) in Fusui county had a higher level of $hOGG_1$ ($P<0.01$) and a lower level of 8-oxoG ($P<0.05$) than the controls ($n=63$) in Nanning city. Children in Nanning exposed to passive-smoking had a higher $hOGG_1$ expression ($P<0.05$) than the non-exposers. 8-oxoG and $hOGG_1$ were negatively correlated with body mass index, while $hOGG_1$ was positively correlated with age. There was a peak of 8-oxoG level nearby the 12 year point. Individuals with the $hOGG_1$ 326Ser allele had a significantly marginal higher concentration of leukocyte 8-oxoG level than $hOGG_1$ 326Cys allele.

**CONCLUSION:** This is the first report using flow-cytometry to simultaneously quantify both the DNA oxidative damage and its repairing enzyme hOGG1. The results provide new insights towards a better understanding of the mechanisms of oxidative stress in a population highly susceptible to hepatocarcinogenesis.

Peng T, Shen HM, Liu ZM, Yan LN, Peng MH, Li LQ, Liang RX, Wei ZL, Halliwell B, Ong CN. Oxidative DNA damage in peripheral leukocytes and its association with expression and polymorphisms of $hOGG_1$: A study of adolescents in a high risk region for hepatocellular carcinoma in China. *World J Gastroenterol* 2003; 9(10): 2186-2193
http://www.wjgnet.com/1007-9327/9/2186.asp

## INTRODUCTION

Reactive oxygen species (ROS) possess a high reactivity of a variety of biological molecules, among which, DNA is one of the most important targets[2]. Oxidative DNA damage, caused by either endogenous or exogenous source of ROS, has been linked to aging, chronic degenerative diseases, inflammatory diseases and cancers[3-6]. Among various types of DNA base modifications induced by ROS attack, 7,8-dihydro-8-oxoguanine (8-oxoG) has been the most widely studied and is considered as a key biomarker of oxidative DNA damage[7]. Leaving unrepaired, 8-oxoG is highly mutagenic because of its propensity to mispair with adenine during DNA replication, ultimately yielding GC to TA transversion[8].

To minimize 8-oxoG accumulation within genomes, this lesion is subjected to DNA repair primarily through the base excision repair pathway[9]. A key component of this pathway in eukaryotes is $OGG_1$, a DNA glycosylase/β-lyase that recognizes 8-oxoG opposite cytosine[10]. Inactivation of the $OGG_1$ gene generates a mutator phenotype characterized by GC-TA transversions in yeast[10]. Analysis of the human $OGG_1$ gene ($hOGG_1$) and its transcripts in normal and tumoral tissues has revealed alternative splicing, polymorphisms and somatic mutations[11]. The repair effectiveness of $OGG_1$ may be modulated by gene polymorphisms. A Cys326Ser substitution in exon 7 has been the most extensively studied. The Cys326 isoform is postulated to exhibit reduced 8-oxoG repair activity[12], increase susceptibility to squamous cell carcinoma of lung cancer[13,14], otolaryngeal cancer[15] and esophageal cancer[16], nevertheless, controversy still remains[17-21].

Dietary aflatoxin exposure[22,23] and hepatitis infection[24,25] are two well known risk factors in liver carcinogenesis, which involves ROS generation and oxidative DNA damage. A synergistic effect of aflatoxin $B_1$ ($AFB_1$) and hepatitis virus B (HBV) may be involved in the hepatocellular carcinoma (HCC) formation, and may be responsible for the predominance of one hotspot GC→TA transversion in the p53 gene of affected individuals[22,23].

HCC is the third most common cause of cancer death in China[26], and the main killer in a south-western province, Guangxi Zhuang Autonomous Region[27]. The age-standardized mortality of HCC for males and females in this province was 32.5/100 000 and 8.5/100 000, respectively[1], accounting for 50 % and 25 % of all the cancer deaths in this region in men and women, respectively[1]. Thus far, dietary $AFB_1$ exposure[28] and HBV infection[1] are the well documented risk factors

for the extraordinarily high prevalence of HCC in this area. Our earlier data[29,30] together with that of Stern *et al*[31] have highlighted a frequency of 36-73 % of p53-249 codon mutation in HCCs in this region, which is consistent with the notion that, the p53-249 hot-spot mutation is a fingerprint of $AFB_1$ contamination, and possibly synergistic with HBV infection in hepatocarcinogenesis[22].

In the present study, on the assumption that environmental carcinogens may impose oxidative stress on the residents living in Guangxi Zhuang Autonomous Region, we examined the level of 8-oxoG and $hOGG_1$ expression in leukocytes of a random sample of adolescents aged 4-18 in an area of Guangatxi exposed to a high level of aflatoxin and high risk of HCC, using a newly developed flow cytometry method. Furthermore, we examined the relationship between DNA damage and genetic polymorphisms of oxidative damage repair gene $hOGG_1$.

## MATERIALS AND METHODS

### *Study subjects*

This collaborative study by the Guangxi University and Fusui Cancer Institute was part of a community-based health survey in the Nanning region of Guangxi Zhuang Autonomous Region, conducted during April to June 2001. Based on the local cancer registry from 1974 to 1999, the HCC incidence rate in this region ranged from 32 to 97 per 100 000 for males and 4.27 to 17.32 per 100 000 for females, respectively. The aims of the study were explained in detail prior to the survey. From a total of 472 informed residents, 162 adults and 123 adolescents (60.4 % response rate) participated in on a voluntary basis.

After written consents were obtained from all the individuals or children' s parent/guidance. Ten-milliliter venous blood was collected with heparin as anticoagulant. Buffy-coat and plasma separated soon after collection, and kept in liquid-nitrogen during transportation, and stored at -80 ℃ till analysis. Body weight, height, age, gender, occupation, alcohol and smoking habit and family history of hepatitis infections were also recorded using a structured questionnaire approved by the Guangxi Medical University. Plasma of all subjects was screened to differentiate HBV, HCV, HDV, HEV and HGV infections. 25.6 % (73/285) subjects were positive of HV infection (63/HBV(+), 1/HBV&HCV(+), 1/HCV(+), 4/HBV&HDV(+), 2/HEV(+), 2/HBV&HGV(+)). These cases were excluded, in order to rule out the possibility of influence from hepatitis viruse infection[24,25]. Furthermore, subjects with known exposure to known environmental or occupational hazards (such as cigarette smoking, alcohol or pesticides), as well as those who were currently under medication or known to have a chronic illness were also excluded. Fifty-six boys and 28 of girls aged from 4 to 18 (mean±SD, 11.45±3.0 years) born and grown up in this region met the above mentioned criteria, and were selected for this investigation.

### *Determination of DNA 8-oxoguanine and $hOGG_1$ in leukocytes*

Prior to the investigation, extensive experiments were conducted to optimize the antibody/probe titers and the amount of cells to be used for flow cytometry. It was found that reproducible data could be achieved with 40 μl-buffy coat (around $0.8\text{-}1.1\times10^6$ WBC). The buffy-coat was first transferred from -80 ℃ to 4 ℃, gently thawed for 4 h, and then shifted to room temperature till completely thawed. Forty micro liters buffy-coat were counted for leukocytes using a hemocytometer, and another 40 μl buffy-coat was transferred into an Eppendorf tube containing 1 ml of PBS with 1 % paraformaldehyde, stood on ice for 30 min. After washed twice with PBS, Cells were then fixed in ice-cool 70 % ethanol and kept at -20 ℃ till staining.

8-oxoG was stained by a Biotrin OxyDNA Assay Kit (Fluorescein isothiocyanate, FITC-conjugated probe, Biotrin Int. Ltd., Dublin, Ireland.) according to the manufacturer' s protocol with some minor modifications[32,33]. For $hOGG_1$ staining, first antibody (1st Ab, goat-anti-$hOGG_1$) was obtained from Santa Cruz Biotech, Inc (California, U.S.A.) (goat polyclonal antibody, against a peptide mapping at the amino terminus of $OGG_1$ of human origin, reacts with all $OGG_1$ splice variants of human origin). The second antibody (2nd Ab, R-phycoerythrin, PE-conjugated rabbit anti-goat IgG) was purchased from Sigma-Aldrich Inc., (St. Louis, MO, USA). Before staining, the cells were treated by Biotrin Blocking Solution at 37 ℃ for 60 min. After washed with Biotrin Washing Solution, the cells were first incubated with 1st Ab (goat-anti-$hOGG_1$, 1:100 in 3 % FBS/PBS), followed by a mixture of Biotrin 8-oxoG probe (FITC-conjugate, 1:10 dilution) and 2nd Ab (rabbit-anti-goat, PE conjugate, 1:400 dilution) in the Biotrin Washing Solution at 37 ℃ in the dark for 60 min per step. The cells were then re-suspended and quantified by a flow cytometer (Coulter Epics Elite Flow Cytometer, Coulter Corporation, Miami, USA), at 488 nm excitation and 525 nm (FITC), 578 nm (PE) emission, respectively. Blood from a healthy adult volunteer was included and served as an internal control. Data were analyzed using the WinMDI2.8 software (http: //facs.scripps.edu/software.html). 8-oxoG and $hOGG_1$ levels were determined as percentage of positive-staining-cells (oxoGP, $hOGG_1P$) and mean of relative fluorescence intensity (RFI for oxoGI, $hOGG_1I$) of 10 000 cells counted by the flow cytometer[34]. To eliminate possible batch-to-batch variations, all the samples were analyzed at the same batch by the same cytometer.

### *Genotyping of $hOGG_1$ Cys326Ser single nucleotide polymorphism (SNP)*

PCR-SSCP analysis of $hOGG_1$ Cys326Ser SNP was modified from a technique described by Kohno *et al*[12]. DNA extracted from leukocytes using phenol-chloroform method was amplified by PCR. The primers used were 5' -actgt-cacta-gtctc-accag-3' (forward) and 5' -tgaat-tcgga-aggtg-cttgg-ggaat-3' (reverse) (Research Biolabs Pte., Ltd., Singapore). PCR polymerase and dNTP were from Finnzymes (ESPOO, Finland). Twenty-μl PCR reaction mixture contained 2 μl 10×PCR-buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.5 μM of each primer, 1 U Finnzyme polymerase and -100 ng of sample DNA. PCR reaction on a thermocycler (Biometra TGradient, Göttingen, Germany) began with pre-incubation at 94 ℃ for 5 min, followed by 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 54 ℃ for 30 s, and elongation at 72 ℃ for 30 s. PCR products were diluted with 4 volumes of loading buffer (0.5×TBE, 0.05 % bromophenol blue, 0.05 % xylene cyanol, 20 mM methylmercury hydroxide), heat-denatured at 85 ℃ for 5 min and rapidly cooled on ice before loading. Twenty microliters of each sample were separated on 10 % non-denaturing polyacrylamide gels with or without 5 % glycerol. Electrophoresis was conducted at 200 V constant for 5 h at 4-5 ℃. Silver staining followed Forsberg *et al*[35]. Genotype of each band-pattern was confirmed by a subsequent sequencing in a commercial laboratory (Research Biolabs Pte., Ltd, Singapore) using BigDye™ Terminator kits on a ABI PRISM® 377-96 DNA Sequencer. A blank was inserted into each batch PCR to monitor PCR contamination, and for each sample, at least one independent repeat of PCR-SSCP assay was done.

### *Statistical analysis*

All analyses were performed using the SPSS 10.0 program (Chicago, USA). Two cases were excluded from flow cytometry assays because of inadequate leukocytes. The frequency distributions of 8-oxoGP, 8-oxoGI, $hOGG_1P$ and $hOGG_1I$ skewed to the right while body mass index (BMI)

skewed to the left. In order to obtain acceptable fit to the normal distribution and stabilize the variance, 8-oxoGI, $hOGG_1I$ and BMI were log-transformed while 8-oxoGP and $hOGG_1P$ were transformed by the formula: log ((100+X)/(100-X)). Non-parameter Mann-Whitney test and one-way ANOVA were used to examine the influence of gender and $hOGG_1$ genotype on the four biomarkers, respectively. A partial correlation was calculated to examine the closeness of relationship between continuous variables (biomarkers, age and BMI). Multiple linear regressions were conducted to examine the influence of risk factors on the four biomarkers, respectively. A two-tailed *P* value <0.05 was considered significant.

## RESULTS

### *Levels of 8-oxoG and $hOGG_1$*

Figures 1a to 1d show the images of leukocytes stained for 8-oxoG and $hOGG_1$ under laser confocal microscope. The mean levels of the four biomarker parameters in male and female children are summarized in Table 1. The positive-staining cell percentage of 8-oxoG and $hOGG_1$ ranged between 45-99 % and 44-98 %, respectively. It was noted that the majority (>72 %) of cases had over 90 % positive-staining leukocytes for both 8-oxoG and $hOGG_1$. The data suggested that between male and female children, there was no significant difference in both 8-oxoG and $hOGG_1$ expressed either as percentage or fluorescent intensity.

### *Association between 8-oxoG, $hOGG_1$, age and BMI*

Table 2 reveals that there were significant correlations between 8-oxoG level and $hOGG_1$ expression even after adjustment of age and BMI. Furthermore, a positive correlation was seen between age and $hOGG_1$ expression, either measured as percentage or intensity. In contrast, a negative correlation was noted between BMI and 8-oxoGP, and between BMI and $hOGG_1P$. These associations remained significant even when BMI or age was adjusted. These data suggested that age and BMI could independently influence the levels of oxidative damage and $hOGG_1$ gene expression.

### *Association between biomarkers and $hOGG_1$ Cys326Ser SNP*

The median levels and 95 % confidence interval (95 % CI) of the four biomarker parameters of their respective $hOGG_1$ Cys326Ser SNP genotype are presented in Table 3. Children with $hOGG_1$ Cys326Ser heterozygote were found to have a higher level of 8-oxoG than those with Cys/Cys homozygote, although statistical significance was observed only in 8-oxoG intensity. Also, comparison of subgroup Cys/Cys *vs.* (Cys/Ser + Ser/Ser) revealed similar results (data not shown).

### *Multivariate analysis*

To explore possible interactions of risk factors, a linear regression was then conducted for the four biomarkers, respectively (Table 4). Based on the multiple regression analysis against 8 variables, $hOGG_1$ expression level increased with aging, leaner children had a lower level of 8-oxoG, and $hOGG_1$ 326Ser allele had an increasing effect on 8-oxoG level while a reducing effect on $hOGG_1$ expression. However, the 8 factors listed in Table 4 explained only less than 50 % of the entire variations, suggesting that other oxygen radical-forming factors might be involved.

**Table 1** Level of biomarkers in male and female children

| | 8-oxoGP (%) | 8-oxoGI (RFI) | $hOGG_1$ (%) | $hOGG_1$ (RFI) | Double staining percentage (%) |
|---|---|---|---|---|---|
| Male (*n*=54) | 94.55 (89.14, 94.16)[b] | 61.74 (58.02, 73.57) | 93.29 (88.39, 93.11) | 110.50 (100.39, 125.07) | 86.76 (81.10, 86.90) |
| Female (*n*=28) | 94.98 (90.03, 95.17) | 69.91 (60.28, 78.83) | 91.78 (88.31, 92.86) | 102.51 (85.39, 116.24) | 87.10 (81.14, 87.79) |
| *P*[a] | 0.469 | 0.323 | 0.577 | 0.225 | 0.822 |

A: Non-parameter Mann-Whitney Test, B: Data in cells presented as median (95 % confidence interval).

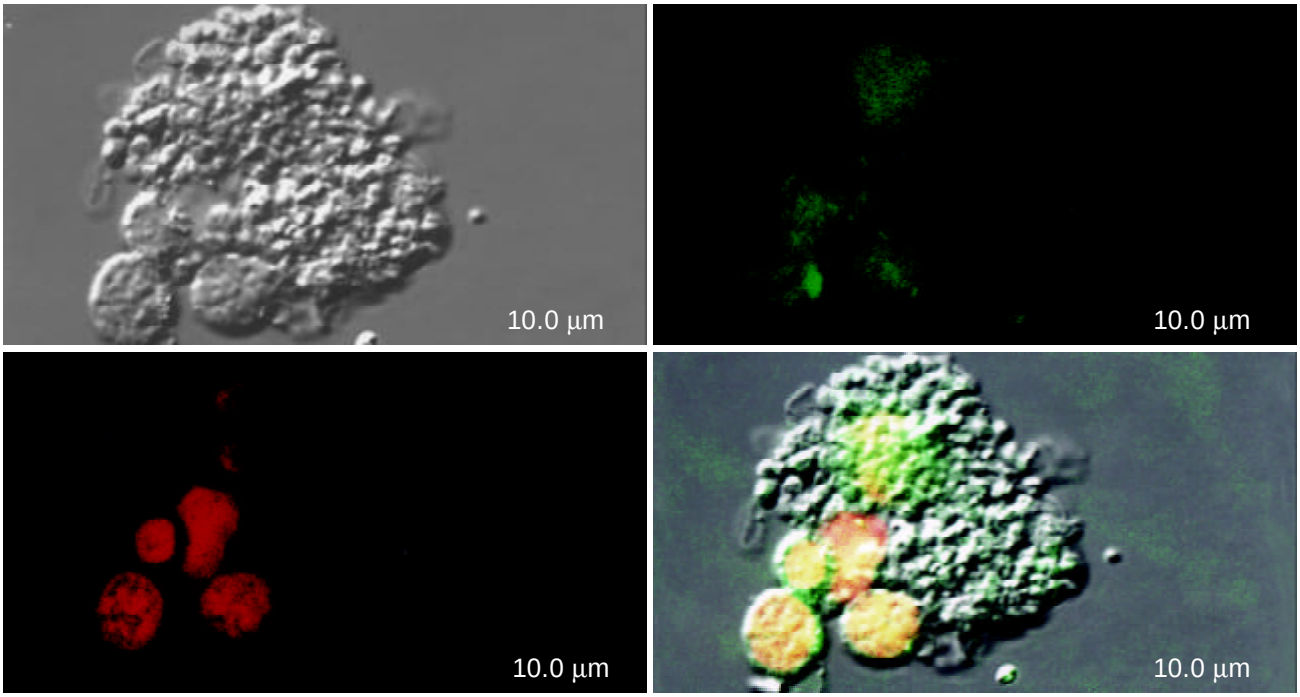



**Figure 1** Representative images of leukocytes stained by 8-oxoG-FITC probes (green) and $hOGG_1$-PE complex (red) under laser confocal microscopy. The bottom-right figure demonstrated that some of the leukocytes were simultaneously stained with both FITC and PE complex.

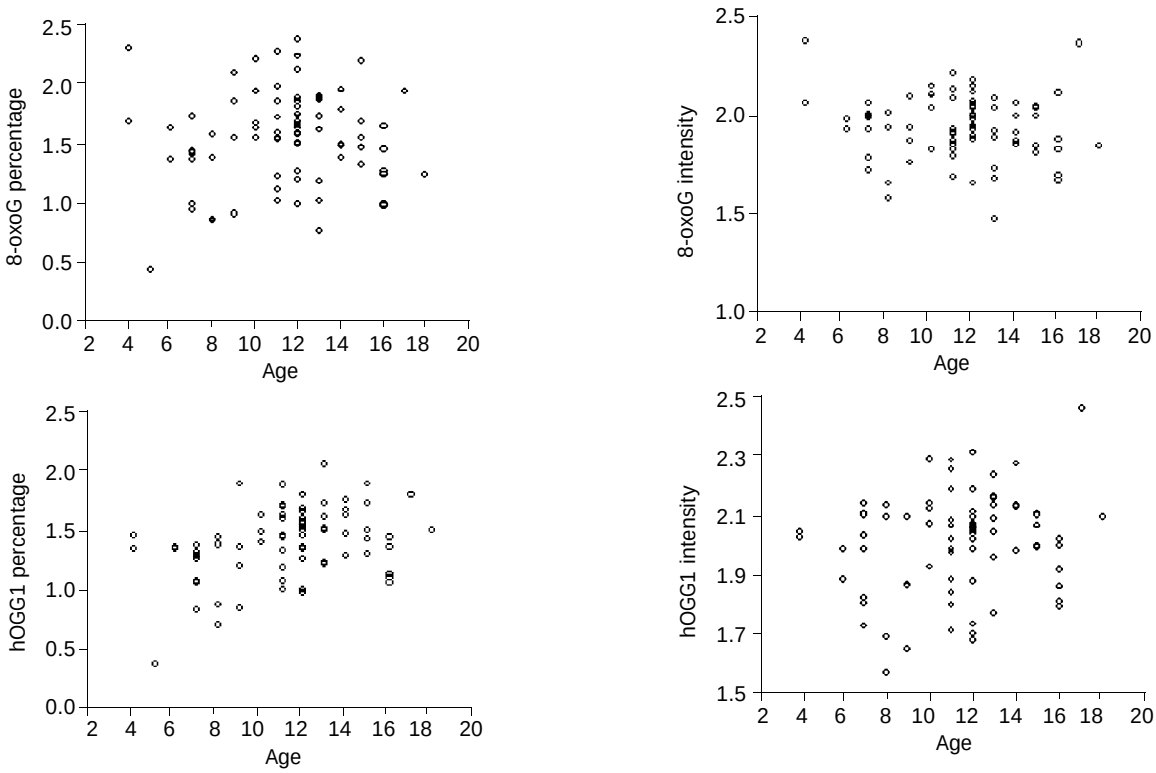



**Figure 2** Scatters of age-biomarkers.

**Table 2** Partial Correlation between biomarkers, age and BMI

| | 8-oxoGP (%) | 8-oxoGI (RFI) | $hOGG_1P$ (%) | $hOGG_1I$ (RFI) | Double staining percentage (%) |
|---|---|---|---|---|---|
| 8-oxoGP (%)[a] | | 0.6987 (0.000)[f] | 0.8333 (0.000)[f] | 0.5299 (0.000)[f] | 0.9553(0.000)[f] |
| 8-oxoGI (RFI)[a] | | | 0.4843 (0.000)[f] | 0.5155 (0.000)[f] | 0.7075(0.000)[f] |
| $hOGG_1P$ (%)[a] | | | | 0.6727 (0.000)[f] | 0.8482(0.000)[f] |
| $hOGG_1I$ (RFI)[a] | | | | | 0.6528(0.000)[f] |
| Age[b] | 0.1832 (0.102) | -0. 0326 (0.773) | 0.3332 (0.002)[f] | 0. 2316 (0.037)[e] | 0.1655(0.140) |
| BMI[b] | -0.3339 (0.002)[f] | -0. 0983 (0.383) | -0. 3111 (0.005)[f] | -0. 0415 (0.713) | -0.2285(0.0040)[f] |
| Age[c] | 0.2414(0.031)[e] | -0.0204 (0.858) | 0.3954 (0.000)[f] | 0.2390 (0.033)[e] | 0.2015(0.073) |
| BMI[d] | -0.3663 (0.001)[f] | -0.0950 (0.402) | -0.3779 (0.001)[f] | -0.0735 (0.517) | -0.2552(0.022)[e] |

A: Adjusted for (gender+age+BMI), B: Adjusted for gender; C:Adjusted for (gender+BMI), D: Adjusted for (gender+age), E: Correlation was significant at the 0.05 level, F: Correlation was significant at the 0.01 level, G: Data presented in cells were correlation coefficient (*P*).

**Table 3** Association between biomarkers and $hOGG_1$-326 SNP (Oneway ANOVA)

| | 8-oxoGP (%) | | | 8-oxoGI (RFI) | | | $hOGG_1P$ (%) | | | $hOGG_1I$ (RFI) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Median | *P* (LSD) | | Median | *P* (LSD) | | Median | *P* (LSD) | | Median | *P* (LSD) | |
| | (95 % CI) | Cys / Ser | Ser / Ser | (95 % CI) | Cys / Ser | Ser / Ser | (95 % CI) | Cys / Ser | Ser / Ser | (95 % CI) | Cys / Ser | Ser / Ser |
| Cys / Cys | 94.03 | 0.075 | 0.250 | 58.61 | 0.023[a] | 0.252 | 93.37 | 0.633 | 0.699 | 116.72 | 0.714 | 0.329 |
| (*n*=32) | (85.96, 93.78) | | | (50.01, 70.31) | | | (85.58, 93.45) | | | (94.94, 133.28) | | |
| Cys / Ser | 94.73 | | 0.864 | 71.16 | | 0.573 | 93.28 | | 0.452 | 110.90 | | 0.205 |
| (*n*=37) | (91.48, 95.53) | | | (64.25, 80.99) | | | (90.26, 93.35) | | | (97.92, (123.07) | | |
| Ser / Ser | 94.98 | | | 58.73 | | | 91.80 | | | 94.93 | | |
| (*n*=13) | (88.98, 96.61) | | | (52.01, 84.67) | | | (87.18, 93.64) | | | (73.26, 106.82) | | |
| (F) *P* | | (1.751) 0.180 | | | (2.741) 0.071 | | | (0.312) 0.733 | | | (0.821) 0.444 | |

[a]*P*<0.05.

**Table 4** Multiple regression models of predictors on levels of oxidative DNA damage biomarkers

| Predictors in the model | Double staining percentage (%) | | 8-oxoGP | | 8-oxoGI | | $hOGG_1P$ | | $hOGG_1I$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Beta[b] | *P* | Beta | *P* | Beta | *P* | Beta | *P* | Beta | *P* |
| 8-oxoGP | | | | | | | 0.463 | | 0.000[c] | |
| 8-oxoGI | 0.521 | 0.000[c] | | | | | | | 131.226 | 0.000[c] |
| $hOGG_1P$ | | | 0.922 | 0.000[c] | | | | | | |
| $hOGG_1I$ | 0.411 | 0.000[c] | | | 6.591E-02 | 0.008[c] | | | | |
| Age | 0.090 | 0.201 | -0.139 | 0.108 | -0.075 | 0.426 | 2.532E-02 | 0.001[c] | 3.069 | 0.018[c] |
| Gender | -0.027 | 0.693 | -0.081 | 0.336 | -0.106 | 0.253 | 0.058 | 0.474 | 0.104 | 0.262 |
| BMI | -0.046 | 0.496 | -0.055 | 0.511 | -1.048E-02 | 0.050[c] | -0.053 | 0.516 | 0.037 | 0.696 |
| $hOGG_1$ 326SNP | 0.056 | 0.427 | 0.103 | 0.029[c] | 2.370E-03 | 0.000[c] | -0.112 | 0.166 | -15.684 | 0.006[c] |
| Constant | 0.480 | 0.000 | 6.969E-02 | 0.705 | 1.601 | 0.000 | 0.401 | 0.001 | -133.512 | 0.001 |
| $R^2$(F)[d] | 0.667 (79.227)[e] | | 0.470 (35.033)[e] | | 0.377 (15.729)[e] | | 0.509(40.875)[e] | | 0.386(16.369) [e] | |

A: $hOGG_1$ 326SNP: Cys/Cys=1; Cys/Ser=2; Ser/Ser=3; B: Beta: standardized coefficient; C: Variables entered final equation (backward); D: R square of model (F value of ANOVA); E: ANOVA *P* value <0.01.

## DISCUSSION

Population-based researches can provide new clues to etiology of a disease. The unique epidemiology of high mortality rate of HCC in Guangxi Zhuang Autonomous Region, China has provided an ideal population model for the study of oxidative DNA damage and corresponding repair mechanism[1]. Although aflatoxin exposure was not measured in the present study, our earlier reports and others have shown that the dietary intake of aflatoxin in the local residents was high[28,36]. Furthermore, it has been well established that AFB1 causes rapid ROS formation and leads to DNA oxidative damage, which plays a critical role in hepato-carcinogenesis[22]. The subjects of the present study came from a typical HCC high risk community. In order to rule out the possibility of influence from hepatitis virus infection and other potential environmental and occupational factors, such as pesticides, cigarette smoking and alcohol, we have limited the present study to 82 children under the age of 18 with no known liver diseases or other medical history.

### *Methodology of quantification for oxidative DNA damage*

Immunohistochemistry-based approaches have been widely used for the quantification of DNA adducts in various tissues[37], as well as in *in-situ*[38,39] or urinary[40] 8-oxoG quantification. On the other hand, flow cytometry technology allows multi-parameter analyses of heterogeneous cell population, in which immunophenotyping of both surface and cytoplasmic antigens, DNA analysis and functional evaluations are combined. Subsets of cells can be identified and characterized by patterns of maturation antigens and staining intensity[41]. This report is the first study thus far utilizing flow cytometry for simultaneous quantification of both 8-oxoG and a high risk population of HCC as a model. Compared to the immuno-quantification of 8-oxoG using visualized counting of positive-staining cells, the flow cytometry approach we used, has the following advantages. The assessment is more objective compared to other methods as the scoring is not operator dependent, both the number of positive-staining cells and fluorochrome intensity could be quantified simultaneously, the sample required (-$1\times10^6$ cells) is much less, compared to other traditional methods, and high-throughput (it can handle as many as 100 samples per batch, thus eliminating possible batch to batch variations).

Prior to the present investigation, attention was paid during the pilot study to standardize and validate the instrumentation and methodology. Factors such as specificity and performance of the reagents, staining intensity, spectral overlap, and instrument compensation were carefully evaluated[41]. The overall indication is that the flow cytometry determinations of 8-oxoG and $hOGG_1$ are of high reliability. The reproducibility is generally over 90 % with batch-to batch variation of less than 10 %. As for the actual samples from Guangxi, a substantial proportion of leukocytes was found to be positively-stained for both 8-oxoG and $hOGG_1$. These values are much higher compared to normal healthy subjects from Singapore of about 20 % to 40 % (Tao *et al*, unpublished data). Table 2 clearly demonstrates that there were significant correlations between 8-oxoG level and $hOGG_1$ expression, either expressed as percentage or chromophore intensity. Even after confounding factors such as age and BMI were adjusted, there were still significant associations between oxidative damage and $hOGG_1$ expression in leukocytes (Table 2). This finding supports that normal cells have certain defensive mechanism against ubiquitous oxidative DNA damage[9], and $hOGG_1$ works as a housekeeping gene, ubiquitously expressed during cell cycle[42].

Although inter-individual variations of $hOGG_1$ are genetically determined, the present data and several earlier studies suggest that its expression could be influenced by endogenous formation of ROS or environmental carcinogens. Increased 8-oxoG repair activity has been shown to increase in smoker's leukocytes[43] and in lung cells exposed to asbestos[44]. Quantitative assessment of hOGG1 expression in peripheral blood cells can provide information on exposure to environmental carcinogens[45]. The present study has clearly demonstrated that hOGG1 in leukocytes, expressed either in percentage or fluorescence intensity, can indicate the oxidative damage in an HCC high risk population, suggesting that $hOGG_1$ is a useful biomarker for monitoring oxidative stress, in addition to 8oxoG.

### *Gender and oxidative DNA damage*

Based on the epidemiological data obtained earlier in other parts of China as well as in Guangxi Zhuang Autonomous Region, the occurrence of HCC is predominant in men, the ratio of male to female is 4-6:1[1]. However, in the present study, we did not observe any difference in either 8oxoG or $hOGG_1$ level between male and female children (Table 1). According to Loft *et al*.,

adult healthy men aged 40 to 60 excreted 29 % (10-48 %) more 8-oxoG in urine than women[46]. Nevertheless, DNA damage, measured as either percentage of DNA migrated in COMET tail[47] or 8-oxoG excretion in urine[48], was not associated with gender in other two adult populations. In healthy individuals, there was no difference in $hOGG_1$ activity due to gender by means of an 8-oxoG-containing oligonucleotide assay[17]. Since this was a children-based study, further evidences from adult subjects are required to elucidate the role of gender-related factors in hepatocarcinogenesis in this population.

### *Age, BMI and oxidative DNA damage*

An inversed relationship between BMI and urinary excretion of 8-oxoG in adults has been documented[46,49], and was postulated to be due to a higher metabolic rate in lean persons[46]. In the present study, similarly, we observed a negative correlation between BMI and 8-oxoGP, and between BMI and $hOGG_1P$ (Table 2), which was consistent with that in previous reports. There was also a positive correlation between $hOGG_1$ level and age, which was not consistent with that of 8-oxoG (Figure 2, Table 2). This age-dependent increase of $hOGG_1$ in children has never been reported elsewhere. The results indicated that, the age-dependent increase of $hOGG_1$ level can not simply be explained by metabolic rate and BMI.

Using COMET assay to study DNA damage of children in Mexico City, Calderon-Garciduenas[50] showed that there was an age-dependent increase in the percentage of nasal cells with COMET tails >10 microns, implying a dose response relationship between exposure to environment pollutants and increase of age. Drury[51] in a preliminary study of 15 children observed an increase of mean 8-OHdG excretion in urine with postnatal age ($r$=0.80, $P$<0.0001). Nevertheless, the author argued that these changes could also be due to changes in the activity of the enzyme responsible for 8-OHdG excision. According to Bogdanov[52], however, neonates of 50-60 days had a higher 8-OHdG excretion in urine (13.39±0.082 ng/mg creatinine, $n$=150) than children aged 3-9 (4.62±0.091 ng/mg creatinine, $n$=32). So far no report about hOGG1 expression in children is available. Therefore it is not known whether this age-dependency is attributed to growth-related increase of metabolism or dietary increase (accumulation) of exposure to aflatoxin or other hazards, or both. Further studies with a larger population should be able to provide more insights in this preliminary observation.

### *$hOGG_1$-Cys326Ser SNP and oxidative DNA damage*

DNA repair enzyme OGG1 is a DNA glycosylase/AP lyase that has been hypothesized to play an important role in preventing carcinogenesis by repairing oxidative damage to DNA. Specifically, it can efficiently repair 8-oxoG, a major base lesion produced by ROS and formed by endogenous metabolism or exposure to environmental oxidizing agents or genotoxic compounds[10]. In this study we have analyzed the variants of $hOGG_1$-Cys326Ser in 84 children and the results showed that the frequency of $hOGG_1$-Cys326 allele of 54.8 % was similar to that reported by Sugimura[13] and Takezaki[53] conducted in two other Chinese populations (54.5 % to 60.7 %). In the present investigation, individuals with the $hOGG_1$ 326Ser allele rather than $hOGG_1$ 326Cys allele, had a significantly higher concentration of leukocyte 8-oxoG level (Tables 3 and 4).

Kohn *et al*. first described a reduced repair activity of $hOGG_1$-Cys326 protein in a complementation assay system[12]. Nevertheless, this observation was not supported by another study using cell homogenates-cleavage system[17]. A recent study by Janssen *et al* found that DNA repair activity of OGG1 in human lymphocytes was not dependent on the Ser326Cys variants[17]. Paralleling to the results of functional studies, population studies on $hOGG_1$-326 polymorphism and cancer susceptibility thus far were also not conclusive[17-21]. It is however important to note that the proportion of homozygous Ser-Ser individuals is the highest in Melanesians (74.5 %), and German (57.1 %), lower in Australian Caucasians (40 %), Japanese (27.7 %) and even lower in Chinese (12 %)[13].

Since the 8-oxoG level measured in a tissue at time is an integration of a number of parameters including the level of ROS, tissue redox status, cellular antioxidant defense mechanism and DNA repair system[21], our data suggest that the role of $hOGG_1$ SNP, if any, in modulating 8-oxoG level of individuals, may be diluted by other confounders. On the other hand, it is believed that $hOGG_1$ may not be the only gene associated with oxidative damage. An alternative DNA oxidative damage repair pathway to minimize the effects of 8-oxoG in genomes has also been reported recently[54]. Carefully designed studies considering these confounders however, are obviously needed to verify this observation.

From the data obtained in this study, it is concluded that, oxidative damage is significantly correlated with the DNA damage repair enzyme $hOGG_1$, there are positive associations between oxidative damage, repair enzyme $hOGG_1$ and age, while there are reverse relationships between oxidative damage and body mass indexes, and polymorphism of $hOGG_1$ variant appears to influence 8-oxoG level in peripheral leukocytes, but only at a marginal strength. These findings provide some new insights into a better understanding of the complex etiology and molecular events that could lead to the development of HCC. Further study should take into consideration of both long term environment exposure and genetic susceptibility in a larger population.

## ACKNOWLEDGEMENT

We are grateful to Prof. Chia SE and Dr. Dong F for their statistical advice and critical comments on the manuscript. We are also grateful to Ong HY and Ong YB for their technical support (Department of Community, Occupational and Family Medicine, National University of Singapore, Singapore.).

## REFERENCES

1 **Yeh FS**, Mo CC, Luo S, Henderson BE, Tong MJ, Yu MC. A serological case-control study of primary hepatocellular carcinoma in Guangxi, China. *Cancer Res* 1985; **45**: 872-873
2 **Halliwell B**. Can oxidative DNA damage be used as a biomarker of cancer risk in humans? Problems, resolutions and preliminary results from nutritional supplementation studies. *Free Radic Res* 1998; **29**: 469-486
3 **Cerutti PA,** Trump BF. Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* 1991; **3**: 1-7
4 **Ames BN**. Endogenous oxidative DNA damage, aging, and cancer. *Free Radic Res Commun* 1989; **7**: 121-128
5 **Halliwell B**. Effect of diet on cancer development: is oxidative DNA damage a biomarker?(1,2). *Free Radic Biol Med* 2002; **32**: 968-974
6 **De Flora S**, Izzotti A, Randerath K, Randerath E, Bartsch H, Nair J, Balansky R, van Schooten F, Degan P, Fronza G, Walsh D, Lewtas J. DNA adducts and chronic degenerative disease. Pathogenetic relevance and implications in preventive medicine. *Mutat Res* 1996; **366**: 197-238
7 **Kasai H**. Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res* 1997; **387**: 147-163
8 **Hazra TK**, Hill JW, Izumi T, Mitra S. Multiple DNA glycosylases for repair of 8-oxoguanine and their potential *in vivo* functions. *Prog Nucleic Acid Res Mol Biol* 2001; **68**: 193-205
9 **Brozmanova J**, Dudas A, Henriques JA. Repair of oxidative DNA damage-an important factor reducing cancer risk. Minireview. *Neoplasma* 2001; **48**: 85-93

10 **Bruner SD**, Norman DP, Verdine GL. Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* 2000; **403**: 859-866

11 **Boiteux S,** Radicella JP. Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress. *Biochimie* 1999; **81**: 59-67

12 **Kohno T**, Shinmura K, Tosaka M, Tani M, Kim SR, Sugimura H, Nohmi T, Kasai H, Yokota J. Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. *Oncogene* 1998; 16: 3219-3225

13 **Sugimura H**, KohnoT, Wakai K, Nagura K, Genka K, Igarashi H, Morris BJ, Baba S, Ohno Y, Gao C, Li Z, Wang J, Takezaki T, Tajima K, Varga T, Sawaguchi T, Lum JK, Martinson JJ, Tsugane S, Iwamasa T, Shinmura K, Yokota J. hOGG1 Ser326Cys polymorphism and lung cancer susceptibility. *Cancer Epidemiol Biomarkers Prev* 1999; **8**: 669-674

14 **Le Marchand L**, Donlon T, Lum-Jones A, Seifried A, Wilkens LR. Association of the hOGG1 Ser326Cys polymorphism with lung cancer risk. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 409-412

15 **Elahi A**, Zheng Z, Park J, Eyring K, McCaffrey T, Lazarus P. The human OGG1 DNA repair enzyme and its association with orolaryngeal cancer risk. *Carcinogenesis* 2002; **23**: 1229-1234

16 **Xing DY**, Tan W, Song N, Lin DX. Ser326Cys polymorphism in hOGG1 gene and risk of esophageal cancer in a Chinese population. *Int J Cancer* 2001; **95**: 140-143

17 **Janssen K**, Schlink K, Gotte W, Hippler B, Kaina B, Oesch F. DNA repair activity of 8-oxoguanine DNA glycosylase 1 (OGG1) in human lymphocytes is not dependent on genetic polymorphism Ser326/Cys326. *Mutat Res* 2001; **486**: 207-216

18 **Xu J**, Zheng SL, Turner A, Isaacs SD, Wiley KE, Hawkins GA, Chang BL, Bleecker ER, Walsh PC, Meyers DA, Isaacs WB. Associations between hOGG1 sequence variants and prostate cancer susceptibility. *Cancer Res* 2002; **62**: 2253-2257

19 **Wikman H**, Risch A, Klimek F, Schmezer P, Spiegelhalder B, Dienemann H, Kayser K, Schulz V, Drings P, Bartsch H. hOGG1 polymorphism and loss of heterozygosity (LOH): significance for lung cancer susceptibility in a Caucasian population. *Int J Cancer* 2000; **88**: 932-937

20 Hanaoka T, Sugimura H, Nagura K, Ihara M, Li XJ, Hamada GS, Nishimoto I, Kowalski LP, Yokota J, Tsugane S. hOGG1 exon7 polymorphism and gastric cancer in case-control studies of Japanese Brazilians and non-Japanese Brazilians. *Cancer Lett* 2001; **170**: 53-61

21 **Hardie LJ**, Briggs JA, Davidson LA, Allan JM, King RF, Williams GI, Wild CP. The effect of hOGG1 and glutathione peroxidase I genotypes and 3p chromosomal loss on 8-hydroxydeoxyguanosine levels in lung cancer. *Carcinogenesis* 2000; **21**: 167-172

22 **Shen HM,** Ong CN. Mutations of the p53 tumor suppressor gene and ras oncogenes in aflatoxin hepatocarcinogenesis. *Mutat Res* 1996; **366**: 23-44

23 **Smela ME**, Currier SS, Bailey EA, Essigmann JM. The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. *Carcinogenesis* 2001; **22**: 535-545

24 **Hagen TM**, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1994; **91**: 12808-12812

25 **Moriya K**, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Todoroki T, Kimura S, Koike K. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; **61**: 4365-4370

26 **National Cancer Control Office**. Atlas of Cancer Mortality in The People's Republic of China. Nanjing Institute of Geography, *China Map Press Shanghai* 1979

27 **Huang TR**, Yu JH, Zhang ZHQ. Analysis on epidemic feature and secular trend of primary liver cancer in Guangxi. *Guangxi Medical J* 2000; **22**: 677-679

28 **Wang JS**, Huang T, Su J, Liang F, Wei Z, Liang Y, Luo H, Kuang SY, Qian GS, Sun G, He X, Kensler TW, Groopman JD. Hepatocellular Carcinoma and Aflatoxin Exposure in Zhuqing Village, Fusui County, People's Republic of China. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 143-146

29 **Peng T**, Li LQ, Lin JL, Lu YF, Wu S, Liang ST, Xiao Q, Liao QH. p53 gene 249codon mutation in recurrent hepatocellular carcinoma from Guangxi province. *Chinese J General Surgery* 2000; **15**: 17

30 **Deng Z**, Pan L, Ma Y. Sequence alterations in p53 gene of hepatocellular carcinoma from high aflatoxin risk area in Guangxi. *Zhonghua Zhongliu Zazhi* 1997; **19**: 18-21

31 **Stern MC**, Umbach DM, Yu MC, London SJ, Zhang ZQ, Taylor JA. Hepatitis B, aflatoxin B(1), and p53 codon 249 mutation in hepatocellular carcinomas from Guangxi, People's Republic of China, and a meta-analysis of existing studies. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 617-625

32 **Struthers L**, Patel R, Clark J, Thomas S. Direct detection of 8-oxodeoxyguanosine and 8-oxoguanine by avidin and its analogues. *Anal Biochem* 1998; **255**: 20-31

33 **Sattler U**, Calsou P, Boiteux S, Salles B. Detection of oxidative base DNA damage by a new biochemical assay. *Arch Biochem Biophys* 2000; **376**: 26-33

34 **Lenka N**, Lu ZJ, Sasse P, Hescheler J, Fleischmann BK. Quantitation and functional characterization of neural cells derived from ES cells using nestin enhancer-mediated targeting *in vitro*. *J Cell Sci* 2002; **115**: 1471-1485

35 **Forsberg L**, de Faire U, Morgenstern R. Low yield of polymorphisms from EST blast searching: analysis of genes related to oxidative stress and verification of the P197L polymorphism in GPX1. *Hum Mutat* 1999; **13**: 294-300

36 **Zhu JQ**, Zhang LS, Hu X, Xiao Y, Chen JS, Xu YC, Fremy J, Chu FS. Correlation of dietary aflatoxin B1 levels with excretion of aflatoxin M1 in human urine. *Cancer Res* 1987; **47**: 1848-1852

37 **Poirier MC**, Santella RM, Weston A. Carcinogen macromolecular adducts and their measurement. *Carcinogenesis* 2000; **21**: 353-359

38 **Kitada T**, Seki S, Iwai S, Yamada T, Sakaguchi H, Wakasa K. In situ detection of oxidative DNA damage, 8-hydroxydeoxyguanosine, in chronic human liver disease. *J Hepatol* 2001; **35**: 613-618

39 **Otani K**, Shimizu S, Chijiiwa K, Yamaguchi K, Noshiro H, Tanaka M. Immunohistochemical detection of 8-hydroxy-2'-deoxyguanosine in gallbladder epithelium of patients with pancreaticobiliary maljunction. *Eur J Gastroenterol Hepatol* 2001; **13**: 1363-1369

40 **Saito S**, Yamauchi H, Hasui Y, Kurashige J, Ochi H, Yoshida K. Quantitative determination of urinary 8-hydroxydeoxyguanosine (8-OH-dg) by using ELISA. *Res Commun Mol Pathol Pharmacol* 2000; **107**: 39-44

41 **Owens MA**, Vall HG, Hurley AA, Wormsley SB. Validation and quality control of immunophenotyping in clinical flow cytometry. *J Immunol Methods* 2000; **243**: 33-50

42 **Dhenaut A**, Boiteux S, Radicella JP. Characterization of the hOGG1 promoter and its expression during the cell cycle. *Mutat Res* 2000; **461**: 109-118

43 **Asami S**, Hirano T, Yamaguchi R, Tomioka Y, Itoh H, Kasai H. Increase of a type of oxidative DNA damage, 8-hydroxyguanine, and its repair activity in human leukocytes by cigarette smoking. *Cancer Res* 1996; **56**: 2546-2549

44 **Kim HN**, Morimoto Y, Tsuda T, Ootsuyama Y, Hirohashi M, Hirano T, Tanaka I, Lim Y, Yun IG, Kasai H. Changes in DNA 8-hydroxyguanine levels, 8-hydroxyguanine repair activity, and hOGG1 and hMTH1 mRNA expression in human lung alveolar epithelial cells induced by crocidolite asbestos. *Carcinogenesis* 2001; **22**: 265-269

45 **Hanaoka T**, Yamano Y, Hashimoto H, Kagawa J, Tsugane S. A preliminary evaluation of intra- and interindividual variations of hOGG1 messenger RNA levels in peripheral blood cells as determined by a real-time polymerase chain reaction technique. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 1255-1258

46 **Loft S**, Vistisen K, Ewertz M, Tjonneland A, Overvad K, Poulsen HE. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* 1992; **13**: 2241-2247

47 **Giovannelli L**, Saieva C, Masala G, Testa G, Salvini S, Pitozzi V, Riboli E, Dolara P, Palli D. Nutritional and lifestyle determinants of DNA oxidative damage: a study in a Mediterranean population. *Carcinogenesis* 2002; **23**: 1483-1489

48 **Witherell HL**, Hiatt RA, Replogle M, Parsonnet J. *Helicobacter pylori* infection and urinary excretion of 8-hydroxy-2-deoxyguanosine, an oxidative DNA adduct. *Cancer Epidemiol Biomarkers Prev* 1998; **7**: 91-96

49 **Kasai H**, Iwamoto-Tanaka N, Miyamoto T, Kawanami K, Kawanami S, Kido R, Ikeda M. Life style and urinary 8-hydroxydeoxyguanosine, a marker of oxidative dna damage: effects of exercise, working conditions, meat intake, body mass index, and smoking. *Jpn J Cancer Res* 2001; **92**: 9-15

50 **Calderon-Garciduenas L**, Wen-Wang L, Zhang YJ, Rodriguez-Alcaraz A, Osnaya N, Villarreal-Calderon A, Santella RM. 8-hydroxy-2'-deoxyguanosine, a major mutagenic oxidative DNA lesion, and DNA strand breaks in nasal respiratory epithelium of children exposed to urban pollution. *Environ Health Perspect* 1999; **107**: 469-474

51 **Drury JA**, Jeffers G, Cooke RW. Urinary 8-hydroxydeoxyguanosine in infants and children. *Free Radic Res* 1998; **28**: 423-428

52 **Bogdanov MB**, Beal MF, McCabe DR, Griffin RM, Matson WR. A carbon column-based liquid chromatography electrochemical approach to routine 8-hydroxy-2'-deoxyguanosine measurements in urine and other biologic matrices: a one-year evaluation of methods. *Free Radic Biol Med* 1999; **27**: 647-666

53 **Takezaki T**, Gao CM, Wu JZ, Li ZY, Wang JD, Ding JH, Liu YT, Hu X, Xu TL, Tajima K, Sugimura H. hOGG1 Ser (326) Cys polymorphism and modification by environmental factors of stomach cancer risk in Chinese. *Int J Cancer* 2002; **99**: 624-627

54 **Klungland A**, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T, Barnes DE. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A* 1999; **96**: 13300-13305

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2194-2197
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Total vascular exclusion technique for resection of hepatocellular carcinoma

Zhen-Yu Yin, Xiao-Ming Wang, Ren-Xiang Yu, Bai-Meng Zhang, Ke-Ke Yu, Ning Li, Jie-Shou Li

**Zhen-Yu Yin, Ning Li, Jie-Shou Li,** Institute of General Surgery, School of Medicine, Nanjing University, Nanjing 210093, Jiangsu Province, China
**Zhen-Yu Yin, Xiao-Ming Wang, Ren-Xiang Yu, Bai-Meng Zhang, Ke-Ke Yu,** Department of General Surgery, Zhongshan Hospital, Xiamen 361004, Fujian Province, China
**Correspondence to:** Zhen-Yu Yin, Department of General Surgery, Zhongshan Hospital, Xiamen 361004, Fujian Province, China. davidmd@sohu.com
**Telephone:** +86-592-2292045 **Fax:** +86-592-2212328
**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To improve the low resection rate, poor prognosis and to control the massive hemorrhage during operation, total vascular exclusion (TVE) technique was used in hepatectomies of advanced and complicated hepatocellular carcinomas (HCCs).

**METHODS:** Five hundred and thirty patients with HCCs were admitted in our hospital. They were divided into TVE technique group (group A: $n$=78), Pringle maneuver method group (group B: $n$=176) and unresectable group (group C: $n$=276). The clinical, operative, pathological parameters and outcome of the patients were statistically evaluated.

**RESULTS:** Group A had a significantly higher resection rate than group B (accounting for 47.92 % and 33.21 % respectively). There was no significant difference in blood loss, blood transfusion and perioperative mortality between groups A and B. Both groups had the similar median disease free survival time (14.6 *vs* 16.3 months) and 1 year survival rate (92.9 % *vs* 95.5 %). The TVE group had a medial survival time of 40.5 months and its 5-year survival rate was 34.6 %.

**CONCLUSION:** As compared with Pringle maneuver method, the total vascular exclusion is a safe and effective technique to increase the total resection rate of advanced and complicated HCCs.

Yin ZY, Wang XM, Yu RX, Zhang BM, Yu KK, Li N, Li JS. Total vascular exclusion technique for resection of hepatocellular carcinoma. *World J Gastroenterol* 2003; 9(10): 2194-2197
http://www.wjgnet.com/1007-9327/9/2194.asp

## INTRODUCTION

At present, operations including tumor resection and liver transplantation offer the only chance of cure for the patients with HCCs[1], and hepatectomy remains the normal choice when liver transplantation is not available. HCCs we met were mostly advanced with a low resection rate and had a high risk of lethal blood loss during operation as well as a high mortality after operation especially when the liver was affected by chronic hepatitis or cirrhosis[2-7]. To avoid excessive bleeding and blood transfusion, several methods to limit bleeding have been developed since hepatic portal clamping was successfully performed by Pringle in 1908, which led to the development of total vascular exclusion (TVE) of the liver by Heaney in 1966. Since then, Huguet and his colleagues have better characterized and widely advocated the use of TVE, which can greatly reduce the risk of massive hemorrhage and air embolism[8].

Pringle maneuver method is routinely used in hepatectomy to control blood loss during HCCs operation, TVE is there fore controversial. To the present, no study has documented its safety and efficacy as compared with Pringle maneuver method in resection of HCCs. This study was review our experiences with resection of HCCs by TVE and Pringle maneuver methods.

## MATERIALS AND METHODS

### *Patients*

From January 1994 to January 2002, 530 patients with HCCs were admitted in our hospital, they were divided into 3 groups. Group A: 78 patients with complicated HCCs underwent total vascular exclusive hepatectomies. Group B: 176 patients with HCCs underwent Pringle maneuver hepatectomies. Group C: 276 patients with unresectable HCCs underwent conservative treatments such as transhepatic artery embolization.

### *Diagnosis and vascular exclusion selection*

HCCs were diagnosed by examinations such as serum tumor marker α-fetal protein, B-type ultrasound, plain or enhanced spiral computed tomography, magnetic resonance imaging before operation. The diagnosis was confirmed by pathologic examination after operation.

Pringle maneuver method was routinely used in the hepatectomy in our center, and the TVE was only used in advanced and complicated HCC patients such as massive tumors needing major hepatectomy, tumor closing or invading the major blood vessels of liver, tumor in the caudate lobe and multiple tumors.

### *Perioperative care and treatment*

Preoperative care: A venous catheter was introduced one week before operation for routine parenteral nutrition to improve the patient' s liver function, nutritional status and coagulation condition. During perioperative care period, frozen plasma should be infused and blood transfusion must be strictly controlled. Blood loss and ascites production during the operation were balanced by infusing fresh frozen plasma. Intraoperative blood transfusion was given only if the hematocrit value was below 0.30. To prevent bleeding, hemostatic drugs such as fibrinogen and thrombinogen were always intravenously given. The main aim during the first few postoperative days was to restore the liver function and prevent hepatic failure. We used 20 % human albumin 100-200 ml/day to maintain the serum protein level. Glucose solution was given for the energy, and short-term antibiotics, histamine blockers were also administered. Appropriate oral intake was restored as soon as possible. The blood discharge from the drain was

carefully monitored. When bleeding exceeded 100 mL/h, an emergency laparotomy was performed. The patients were taken care of in an intensive care unit for the first 24 to 48 hrs with their the life signs inspected. Immediate postoperative treatments included hemostasis, prophylaxis antibiotic treatment and total parental nutritional support.

### *Operative technique*

The surgical technique was described previously[2,8]. In general, a bilateral subcostal incision with or without an upward midline extension was used, and intraoperative ultrasound was routinely used to determine location of the tumor, or possible tumor modules in the contralateral lobe and the exact relationship between the tumor and the major liver blood vessels. In group A, all the hepatic ligaments were divided to allow complete mobilization of the liver and exposure of the whole retrohepatic vena cava. TVE was prepared by carefully dissecting the suprahepatic and infrahepatic vena cava, and right adrenal veins and accessory hepatic veins were ligated if necessary to allow complete venous control during clamping. Clamps were always applied in the following sequence: hepatoduodenal ligament, infrahepatic vena cava and suprahepatic vena cava. During transection of the liver, 5 min interval was always allowed in every 15-20 min TVE until the transection was over. After the resection was completed, the clamps were removed in reverse order of their application. Pringle maneuver method was applied in group B at the time of liver transection and consisted of cross-clamping the hepatoduodenal ligament until the liver transection was completed. If the time was more than 20 min, the clamp was released for about 5 min until the operation was completed. Hemostasis of the raw surface of the liver was assured by biological fibrin glue and exact suture. Closed drainage was routinely used before closure of the incision.

### *Data collection and analysis*

All medical records of the patients of the three groups were reviewed retrospectively. Major hepatectomy was defined as resection of two or more liver segments according to Goldsmith and Woodburne, while minor hepatectomy was defined as resection of only one segment[2]. Tumor closing or invading the major liver blood vessels was defined as the distance between them which was less than 1 cm.

The values were expressed as median (range) and cases (percent). The overall survival after hepatic resection was calculated by the Kaplan-Meier method. Statistical evaluations were performed by using unpaired Student *t* test and chi-spuare analysis, and comparison was made by log rank analysis. Statistical significance was determined by a *P* value of less than 0.05. Calculations were made with SPSS computer software (Chicago, IL).

## RESULTS

As shown in Table 1, the two groups (groups A and B) of patients were similar in terms of age, sex. Both groups had similar high HBV infectious rate. Although the operative time and blood exclusive time were long in TVE group, no significant difference was found in blood loss and blood transfusion between groups A and B. More major hepatectomies and caudate lobe hepatectomies were performed in group A than in group B.

The pathologic data are shown in Table 2. There was a significant difference in the size of tumors between groups A and B. From the data, the rate of tumor closing or invading the major liver blood vessels in group A was higher than that in group B. The patients with HCCs in group A had a higher probability in their advanced stage, about 70 % of the patients were TNM stage 3 or 4 in group A, while only about 32 % in group B. Although higher cirrhosis rate, multiple tumor possibility, more caudate lobe location and higher risk of tumor rupture were found during operation in group A than in group B, the possibilities of tumor free resection margin in the two groups were similar.

**Table 1** General clinical data

| Clinical parameters | Group A (*n*=78) | Group B (*n*=176) |
|---|---|---|
| Age (yr) | 51.72 (36-71) | 49.66 (14-74) |
| Male | 69 (88.64 %) | 147 (83.52 %) |
| HbsAg (+) | 67 (85.89 %) | 151 (85.79 %) |
| Child-Push grade | | |
| Grade A | 41 (52.54 %)[a] | 124 (70.45 %) |
| Grade B | 28 (35.90 %)[a] | 38 (21.59 %) |
| Grade C | 9 (11.53 %) | 14 (8.0 %) |
| Total resection rate | 47.92%(254 / 530)[a] | 33.21 %(176 / 530) |
| Procedure time (min) | 268 (150-325)[a] | 178(128-356) |
| Blood exclusion time (min) | 25.4 (12-55)[a] | 14.2(8-28) |
| Blood loss (ml) | 818 (250-2800) | 725(180-2400) |
| Blood transfusion (ml) | 690 (0-2400) | 620 (0-2600) |
| Total hospital stay time (d) | 29.4 (12-35) | 19.8 (10-39) |
| Re-operation | 11 (14.10 %) | 16 (9.09 %) |
| Emergency operation | 5 (6.41 %) | 9 (5.11 %) |
| Local hepatectomy | 12 (15.38 %)[a] | 48 (27.28 %) |
| Minor hepatectomy | 21 (26.92 %)[a] | 96 (54.55 %) |
| Major hepatectomy | 45 (57.69 %)[a] | 32 (18.19 %) |
| Caudate lobe hepatectomy | 4 (5.13 %)[a] | 0 (0) |

[a]*P*<0.05 *vs* statistically significant when compared with group B.

**Table 2** Pathologic data

| Clinical parameters | Group A (*n*=78) | Group B (*n*=176) |
|---|---|---|
| The mass | | |
| Median diameter (cm) | 11.58 (6.2-24.6)[a] | 6.25 (1.8-12.7) |
| >or =5 cm | 72 (92.30 %)[a] | 118 (67.05 %) |
| >or =10 cm | 53 (67.95 %)[a] | 28 (15.90 %) |
| Close or invade vana cana | 23 (29.49 %)[a] | 17 (9.65 %) |
| Close or invade major hepatic vein | 21 (26.92 %)[a] | 13 (7.39 %) |
| Close or invade major bile duct | 11 (14.10 %)[a] | 8 (4.55 %) |
| Close or invade major port vein | 32 (41.03 %)[a] | 22 (12.5 %) |
| TNM tumor stage | | |
| Stage 1 | 6 (7.69 %)[a] | 49 (27.84 %) |
| Stage 2 | 12 (15.38 %)[a] | 71 (40.34 %) |
| Stage 3 | 36 (46.15 %)[a] | 32 (18.19 %) |
| Stage 4a | 20 (25.64 %)[a] | 22 (12.5 %) |
| Stage 4b | 4 (5.13 %)[a] | 2 (1.14 %) |
| Cirrhosis | 59 (75.64 %) | 120 (68.18 %) |
| Multiple tumor | 8 (10.25 %)[a] | 6 (3.41 %) |
| Caudate lobe tumor | 4 (5.13 %)[a] | 0 (0) |
| Tumor free resection margin | 74 (94.87 %) | 173 (98.3 %) |
| Tumor rupture during operation | 11 (14.10 %)[a] | 13 (7.39 %) |

[a]*P*<0.05 *vs* statistically significant when compared with group B.

Bleeding was most common short-term complication after hepatectomy, but there was no significant difference between the two groups, accounting for 20.5 % and 16.48 %, respectively. Although the complications in group A including bile leakage, ascites, pleural effusion, jaundice, hepatic failure were significantly higher than those in group B, the reoperation

rate within 24 h after operation and perioperative mortality were similar in the two groups. The data are shown in Table 3.

The prognostic data are shown in Table 4. As compared with Pringle maneuver technique, the hepatectomy of TVE might lead to similar median disease-free survival time and short-term survival rate (92.3 % *vs* 95.5 % of one year survival rate, $P>0.05$). The median survival time and long-term survival rate in group A were significantly lower than those in group B, but obviously higher than those in group C. The results showed that the Pringle maneuver group had a higher incidence of remote metastasis than the other two groups.

**Table 3** Postoperative complications

| Clinical parameters | Group A | Group B |
|---|---|---|
| Bleeding | 16 (20.51 %) | 29 (16.48 %) |
| Bile leakage | 6(7.69 %)[a] | 8 (4.55 %) |
| Infection (including abscess) | 12 (15.38 %) | 23 (13.68) |
| Pleural effusion | 31 (39.74 %)[a] | 34 (19.32 %) |
| Reoperation within 24 h after operation | 3 (3.85 %) | 6 (3.40 %) |
| Ascites | 16 (20.51 %)[a] | 20 (11.36 %) |
| Jaundice | 6 (7.69 %)[a] | 5 (2.84 %) |
| Hepatic failure | 6 (7.69 %)[a] | 5 (2.84 %) |
| Total morbidity | 40 (51.28 %)[a] | 45 (25.57 %) |
| Perioperative mortality | 2 (2.56 %) | 5 (2.84 %) |

[a]$P<0.05$ *vs* statistically significant when compared with group B.

**Table 4** Outcome after hepatic resection

| Clinical parameters | Group A | Group B | Group C |
|---|---|---|---|
| Median disease-free survival (m) | 14.6 (8-25) | 16.3 (9-37) | - |
| Median survival (m) | 40.5 (28-52) | 57.6 (33-84) | 8.8 (5-31) |
| Cumulative survival rate | | | |
| 1 year survival | 92.3 %[b] | 95.5 %[b] | 29.9 % |
| 3 year survival | 51.3 %[ab] | 69.6 %[b] | 2.9 % |
| 5 year survival | 34.6 %[ab] | 48.0 %[b] | 0 |
| Remote metastasis rate | 16.27%[a] | 23.9%[b] | 14.8 % |

[a]$P<0.05$ *vs* statistically significant when compared with group B; [b]$P<0.05$ *vs* statistically significant when compared with group C.

## DISCUSSION

With the advances in surgical technique, the mortality rate of hepatectomy today is less than 5 %[2]. Despite of the satisfactory outcome of hepatectomy for HCC[9], hepatectomy of advanced and complicated HCC remains a major surgical challenge, especially when underlying liver cirrhosis is present[10-12]. HCC is mostly resulted from hepatitis virus infection and liver cirrhosis, and the conventional approach used in hepatcetomy will always lead to excessive bleeding and high risk of perioperative mortality[13,14]. Perioperative transfusion has been found to promote recurrence of HCC and to result in short disease-free and overall survivals[15], the highlight of surgery of advanced and complicated HCC is thus to prevent massive bleeding and blood transfusion in hepatectomies[2]. HCCs we met were always in their terminal stage with a diameter larger than 10 cm. Since the Pringle maneuver method does not prevent hepatic venous bleeding or air embolism, new ways of vascular exclusion has to be devised. The TVE technique has been widely accepted in resection of advanced and complicated HCCs since it was introduced by Heaney in 1966 and modified in clinical practice[3,7,8,16]. In general, the TVE technique is used predominantly for major resections or centrally placed lesions or in cases with blood vessels involved.

TVE technique means the total vascular block of the liver during hepatectomy. Though bleeding is decreased in the operation, the TVE technique will prolong the warm ischemia time of the liver, so we must emphasize its safety. Complications were noted in the total vascular exclusive group, which was regarded to be corresponded to the higher incidence of complex resections[17]. Berney and his colleagues showed that the risk factors for postoperative complications were the duration of surgery and the amount of blood transfused[3]. The frequency of perihepatic infected fluid collections has been reported to be 2 % to 20 %, biliary fistulas occurrence was up to 8 % of patients[18]. Brancatisano showed similar perioperative mortality (2.5 %) in radical major hepatic surgery by TVE but with a lower postoperative complication rate (about 46 %) than that of ours[19].

At present, it is generally accepted that liver resection performed under intermittent warm ischema is a safe and well-tolerated modality in patients with and without cirrhotic livers[20]. Huguet and others showed that hepatocytes could tolerate normothermic ischemia in excess of 1 hour, and ischemia up to 2 hour without major detrimental effects other than transient hepatic failure has never been reported[21,22]. Others suggested that the risks related to hemorrhage were of greater concern than those related to the time of ischemia. Thus, for advanced and complicated HCC, it is safer to continue vascular exclusion, within 1 h, until complete resection is achieved, rather than take the risk of significant bleeding for the sake of a shorter period of ischemia. A prospective randomized trial showed that the postoperative outcome of patients who underwent liver resection with Pringle maneuver method was better than that of those who underwent operation with other methods[2]. The results of our data showed that although the TVE group had a high hepatic failure rate and complication occurrence than the Pringle maneuver group, but the perioperative mortality was not significantly higher, suggesting that the hepatic failure and complication occurrence might be transient and recoverable.

Another major problem of TVE is the hemodynamic change during TVE. As reported before, hemodynamic tolerance to TVE was excellent in most patients[3], and we also found that the patients could adapt well and quickly to the haemodynamic changes observed after total vascular exclusion. On the bases of the reported complications of spinal cord ischemia, renal failure or aortic injury after routine aortic clamping[23] and the excellent hemodynamic tolerance obtained without aortic occlusion, we applied the modified TVE without aortic exclusion.

Advanced and complicated HCCs were formerly regarded as contraindications of operation for their high mortality and postoperative recurrent rate, the resection rate of advanced and complicated HCCs was extremely low. It was reported that the resection rate of HCCs was 12-28 %[18, 24,25]. After the use of TVE technique, the resection rate of HCCs increased remarkably, which was also confirmed in our retrospective analysis.

Untreated patiens with HCCs had a median survival time of 6 months, no 5-year survival has been reported. But the surgical treatment prolonged the median survival to 42 months and the 5-year survival to 32 % in some center[26]. Good results could be obtained through an aggressive surgical approach for patients with advanced and complicated HCCs, even for those with tumor thrombi in the portal trunk and vena cava invasion[27]. Comparatively, our perioperative mortality of the patients with advanced and complicated HCCs by TVE was similar to the hepatectomy by Pringle maneuver method and the 5-year survival rate of TVE group after resection was similar to others[28]. The shorter long-term survival rate and median survival time of complicated HCCs by TVE compared with

Pringle maneuver group might be resulted from their poorer conditions such as inflammatory activity, hepatic reserve and tumor characteristics, advanced TNM stage, higher possibility of introgenic tumor rupture, higher rate of metachronous and multicentric liver carcinogenesis[29-31]. However, the long-term survival rate and median survival time of the TVE group were remarkably higher than those of the unresectable group. By the way, the remote metastasis rate of the Pringle maneuver group was higher than that of the TVE group and unresectable group, and this result needs to be further studied. In addition, the potentially harmful effect on the metabolic function of hepatocytes should be mentioned in future study.

In summary, hepatectomy by TVE is a safe and effective technique in surgical treatment of advanced and complicated HCC as compared with Pringle maneuver technique. It can increase the resection rate of HCCs, reduce the massive bleeding during operation and increase the survival rate of advanced and complicated HCCs. This series of HCCs demonstrate that an aggressive policy of liver resection by TVE can be adopted as a feasible therapeutic option without excess mortality.

## REFERENCES


1 **Wakabayashi H**, Yachida S, Maeba T, Maeta H. Indications for portal vein embolization combined with major hepatic resection for advanced-stage hepatocellular carcinomas. A preliminary clinical study. *Dig Surg* 2000; **17**: 587-594
2 **Man K**, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Prospective evaluation of Pringle maneuver in hepatectomy for liver tumors by a randomized study. *Ann Surg* 1997; **226**: 704-711
3 **Berney T**, Mentha G, Morel P. Total vascular exclusion of the liver for the resection of lesions in contact with the vena cava or the hepatic veins. *Br J Surg* 1998; **85**: 485-488
4 **Shimada M**, Matsumata T, Akazawa K, Kamakura T, Itasaka H, Sugimachi K, Nose Y. Estimation of risk of major complications after hepatic resection. *Am J Surg* 1994; **167**: 399-403
5 **Torzilli G**, Makuuchi M, Inoue K, Takayama T, Sakamoto Y, Sugawara Y, Kubota K, Zucchi A. No-mortality liver resection for hepatocellular carcinoma in cirrhotic and noncirrhotic patients: Is there a way? A prospective analysis of our approach. *Arch Surg* 1999; **134**: 984-992
6 **Rui JA**, Wang SB, Chen SG, Zhou L. Right trisectionectomy for primary liver cancer. *World J Gastroenterol* 2003; **9**: 706-709
7 **Qin LX**, Tang ZY. The prognostic significance of clinical and pathological features in hepatocellular carcinoma. *World J Gastroenterol* 2002; **8**: 193-199
8 **Huguet C,** Addario-Chieco P, Gavelli A, Arrigo E, Harb J, Clement RR. Technique of hepatic vascular exclusion for extensive liver resection. *Am J Surg* 1992; **163**: 602-605
9 **Makuuchi M**, Takayama T, Kubota K, Kimura W, Midorikawa Y, Miyagawa S, Kawasaki S. Hepatic resection for hepatocellular carcinoma- Japanese experience. *Hepatogastroenterology* 1998; **45**(Suppl 3): 1267-1274
10 **Capussotti L**, Polastri R. Operative risks of major hepatic resections. *Hepatogastroenterology* 1998; **45**: 184-190
11 **Farges O**, Malassagne B, Flejou JF, Balzan S, Sauvanet A, Belghiti J. Risk of major liver resection in patients with underlying chronic liver disease: a reappraisal. *Ann Surg* 1999; **229**: 210-215
12 **Tang ZY**. Hepatocellular carcinoma-cause, treatment and metastasis. *World J Gastroenterol* 2001; **7**: 445-454
13 **Liu CL**, Fan ST, Lo CM, Tung-Ping Poon R, Wong J. Anterior approach for major right hepatic resection for large hepatocellular carcinoma. *Ann Surg* 2000; **232**: 25-31
14 **Tjandra JJ**, Fan ST, Wong J. Peri-operative mortality in hepatic resection. *Aust Nz J Surg* 1991; **61**: 201-206
15 **Hanazaki K**, Kajikawa S, Shimozawa N, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J. Hepatic resection for large hepatocellular carcinoma. *Am J Surg* 2001; **181**: 347-353
16 **Stephen MS**, Gallagher PJ, Sheil AG, Sheldon DM, Storey DW. Hepatic resection with vascular isolation and routine supraceliac aortic clamping. *AM J Surg* 1996; **171**: 351-355
17 **Buell JF**, Koffron A, Yoshida A, Hanaway M, Lo A, Layman R, Cronin DC, Posner MC, Millis JM. Is any method of vascular control superior in hepatic resection of metastatic cancers? Longmire clamping, pringle maneuver, and total vascular isolation. *Arch Surg* 2001; **136**: 569-575
18 **Fong Y**, Brennan MF, Brown K, Heffernan N, Blumgart LH. Drainage is unnecessary after elective liver resection. *Am J Surg* 1996: **171**: 158-162
19 **Brancatisano R**, Isia A, Habib N. Is radical hepatic surgery safe? *Am J Surg* 1998; **175**: 161-163
20 **Wu CC**, Hwang CR, Liu TJ, P'eng FK. Effects and limitations of prolonged intermittent ischaemia for hepatic resection of the cirrhotic liver. *Br J Surg* 1996; **83**: 121-124
21 **Huguet C**, Gavelli A, Chieco PA, Bona S, Harb J, Joseph JM, Jobard J, Gramaglia M, Lasserre M. Liver ischemia for hepatic resection: where is the limit? *Surgery* 1992; **111**: 251-259
22 **Hannoun L**, Borie D, Delva E, Jones D, Vaillant JC, Nordlinger B, Parc R. Liver resection with normothermic iscaemia exceeding 1 h. *Br J Surg* 1993; **80**: 1161-1165
23 **Emre S**, Schwartz ME, Katz E, Miller CM. Liver resection under total vascular isolation. Variations on a theme. *Ann Surg* 1993; **217**: 15-19
24 **Makuuchi M**, Mori T, Gunven P, Yamazaki S, Hasegawa H. Safety of hemihepatic vascular occlusion during resection of the liver. *Surg Gynecol Obstet* 1987; **164**: 155-158
25 **Hu RH**, Lee PH, Yu SC, Dai HC, Sheu JC, Lai MY, Hsu HC, Chen DS. Surgical resection for recurrent heapatocullar carcinoma: prognosis and analysis of risk factors. *Surgery* 1996; **120**: 23-29
26 **Nagorney DM**, van Heerden JA, Ilstrup DM, Adson MA. Primary hepatic malignancy: surgical management and determinants of survival. *Surgery* 1989; **106**: 740-748
27 **Madariaga JR**, Fung J, Gutierrez J, Bueno J, Iwatsuki S. Liver resection combined with excision of vena cava. *J Am Coll Surg* 2000; **191**: 244-250
28 **Buell JF**, Rosen S, Yoshida A, Labow D, Limsrichamrern S, Cronin DC, Bruce DS, Wen M, Michelassi F, Millis JM, Posner MC. Hepatic resection: effective treatment for primary and secondary tumors. *Surgery* 2000; **128**: 686-693
29 **Fan ST**. What patients can survive disease free after complete resection for hepatocellular carcinoma? A multivariate analysis. *Jpn J Clin Oncol* 2000; **30**: 75-81
30 **Sakon M**, Umeshita K, Nagano H, Eguchi H, Kishimoto S, Miyamoto A, Ohshima S, Dono K, Nakamori S, Gotoh M, Monden M. Clinical significance of hepatic resection in hepatocellular carcinoma: Analysis by disease-free survival curves. *Arch Surg* 2000; **135**: 1456-1459
31 **Hanazaki K**, Kajikawa S, Shimozawa N, Mihara M, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J. A 15-year retrospective study of hepatic resection for stage IV-A hepatocellular carcinoma shows value in hepatitis B negative patients. *Am J Surg* 2002; **183**: 89-94


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2198-2201
World Journal of Gastroenterology
 ISSN 1007-9327

• LIVER CANCER •

# Role of multiphase scans by multirow-detector helical CT in detecting small hepatocellular carcinoma

Hong Zhao, Kang-Rong Zhou, Fu-Hua Yan

**Hong Zhao,** Department of Radiology, First Hospital, Shanxi Medical College, Taiyuan 030001, Shanxi Province, China
**Kang-Rong Zhou, Fu-Hua Yan,** Department of Radiology, Zhongshang Hospital, Fudan University, Shanghai 200032, China
**Supported by** the Ministry Public Health Programme, No.97030220
**Correspondence to:** Dr. Hong Zhao, Department of Radiology, First Hospital, Shanxi Medical College, Taiyuan 030001, Shanxi Province, China. zhaohongmd@sina.com
**Telephone:** +86-351-4044111 Ext 24781
**Received:** 2003-03-12 **Accepted:** 2003-04-14

## Abstract

**AIM:** To evaluate the role of multiphasic scanning by multirow-detector helical CT (MDCT) in detecing small hypervascular hepatocellular carcinoma (SHCC).

**METHODS:** Multiphasic scanning was carried out in 75 patients with SHCC with Marconi MX8000 CT scanner. The early arterial phase (EAP), late arterial phase (LAP) and the portal venous phase (PVP) scans were started at 21 s, 34 s and 85 s respectively. The mean difference of CT values between tumor and liver parenchyma for each scanning phase was measured, and the sensitivity of detection of SHCC in each of these phases and in the combined phase was calculated and statistically analyzed.

**RESULTS:** The mean difference of CT values between tumor and liver parenchyma was significant in 71 lesions$\geqslant$1 cm in three phases ($P$<0.05). In 91 tumor foci, the detectability of SHCC was 45.1 %, 83.5 % and 92.3 % in EAP, LAP and double arterial phases (DAP), respectively. The early arterial phase plus the portal venous phase and the double arterial phase plus the portal venous phase were 94.5 %, 97.8 %, respectively. Whereas the detectability in LAP plus PVP and in DAP plus PVP had no statistical difference.

**CONCLUSION:** The utility of faster speed and thinner slice MDCT and multiphase scanning protocol can improve the detectability of hypervascular small hepatocellular carcinoma. Among which LAP is superior to EAP in depicting the lesions.

Zhao H, Zhou KR, Yan FH. Role of multiphase scans by multirow-detector helical CT in detecting small hepatocellular carcinoma. *World J Gastroenterol* 2003; 9(10): 2198-2201
http://www.wjgnet.com/1007-9327/9/2198.asp

## INTRODUCTION

It has been recognized that the majority of hepatocellular carcinomas (HCC) are hypervascular. During the hepatic arterial phase (AP), hypervascular lesions will be greatly enhanced, and become iso- or hypodense in the portal venous phase (PVP), which is a sensitive and specific feature for diagnosing SHCC. A biphasic hepatic acquisition helical CT scanning technique has become a standard method for clinical diagnosis of SHCC. Some studies and clinical applications have shown that the arterial dominant phase using a single row detector helical CT (SDCT) is effective for detection of SHCC[1-6], but by using this kind of scanner, the scanning of the whole liver takes approximately 20 s with a great difference in scanning time from subdiaphragmatic region to the right lower border of the liver. So that, some lesions are not conspicuously enhanced within the time.

Recently, a new generation of MDCT has been used in clinical practice. The scanning time can be shortened to 0.5 s. If the four-detector array CT scanner is used, the entire hepatic acquisition can be accomplished in a very short period (4-8 s). Therefore, to makes use of the advantages of faster scanning speed of MDCT, we can create a double arterial phase (DAP) scanning technique and compare the detectability of SHCC in the early arterial phase (EAP), late arterial phase (LAP) and portal venous phase (PVP).

## MATERIALS AND METHODS

### *Patients*

From September 2001 to July 2002, 75 patients (67 men, 8 women, mean age 49 years) with 91 lesions were enrolled in this study. All the patients were proved or suspected to have SHCC who had undergone imaging diagnostics as US, SDCT or MRI. Among them, 40 cases were confirmed by surgical operation or biopsy, 11 recurred cases were postoperatively diagnosed, and the other 24 cases were diagnosed by clinical data, such as history of liver diseases, evaluation of serum AFP and other imaging modalities.

### *Methods*

Multiphasic CT scans of liver were performed with Marconi MX8000 CT scanner using the following parameters, namely 0.5-0.75 s scanning time, 6.5 mm thick section, 23.3 mm/s table speed, 120 KVp and 200-250 mA.

Before examination, 800-1 000 ml water was taken as oral contrast. The whole liver scanning was followed by nonionic contrast enhancement with a dose of 1.5 ml/kg and an injection rate of 3 ml/s via the antecubital vein. Multiphase acquisition was performed with a scanning delay set for EAP, LAP and PVP at 21 s, 34 s and 85 s, respectively. Each of the whole liver scanning by cephalad-caudal orientation was completed in 4-8 s with breath held.

### *Imaging analysis*

CT attenuations of 71/91 tumor foci with diameter$\geqslant$1 cm were measured in all images, and the difference in density of the tumor foci and surrounding hepatic parenchyma was calculated during the enhanced three phases.

Based on the enhancement and the comparison with adjacent liver parenchyma, the tumor foci were described as hyper-, iso- and hypoattenuation, only hyper- and hypoattenuation lesions could be detected and the number of tumor foci was recorded blindly by two radiologists.

### *Statistical analysis*

The statistical analysis was done by SPSS10.0 software, Chi-square test and analysis of variance.

## RESULTS

Among the ninety-one tumor foci identified on the images, 20 of them were less than 1 cm in diameter. The size of the other 71 lesions varied from 10 mm to 30 mm (mean 21.3 mm.) The maximum contrast of tumor-to-liver of the majority lesions during the late arterial phase is shown in Table 1. The difference of tumor-to-liver contrast among the EAP, LAP and PVP was statistically significant ($P$<0.05).

**Table 1** Mean attenuation difference of tumor-to-liver in all phases

| Scanning phases | No of SHCCs | X±SD |
|---|---|---|
| Noncontrast | 71 | 15.75±7.0[a] |
| Early arterial phase | 71 | 11.0±9.4[a] |
| Late arterial phase | 71 | 20.2±14.7[a] |
| Portal venous phase | 71 | 18.0±10.68 |

Note: The mean attenuation difference value of each phase was an absolute value. [a]$P$<0.05.

The sensitivity of tumor foci detection in each scanning phase is shown in Table 2.

**Table 2** Sensitivity of SHCC detection for each scanning phase

| Scanning phases | Sensitivity (%) ($n$=91) |
|---|---|
| EAP | 45.1(41/91) |
| LAP | 83.5(76/91) |
| DAP | 92.3(84/91) |
| PVP | 78.0(71/91) |
| EAP+PVP | 84.6(77/91) |
| LAP+PVP | 94.5(86/91) |
| EAP+LAP+PVP | 97.8(89/91) |

Table 2 shows that there was a significant difference in the sensitivity of SHCCs detection between EAP and LAP (45.1 % *vs* 83.5 %). Considering the importance of different phases, the sensitivity of the combined phase was 92.3 %, 94.5 %, and 97.8 % for DAP, LAP plus PVP and DAP plus PVP, respectively, which was higher than that of each phase. (Figures 1-3).

The comparison of detectability is shown in Table 3. The EAP had a lower sensitivity for SHCCs compared with others ($P$<0.05). A notably statistical significance was found in LAP plus PVP and EAP plus PVP ($P$<0.05), but no statistical significance was observed in LAP plus PVP and DAP plus PVP.

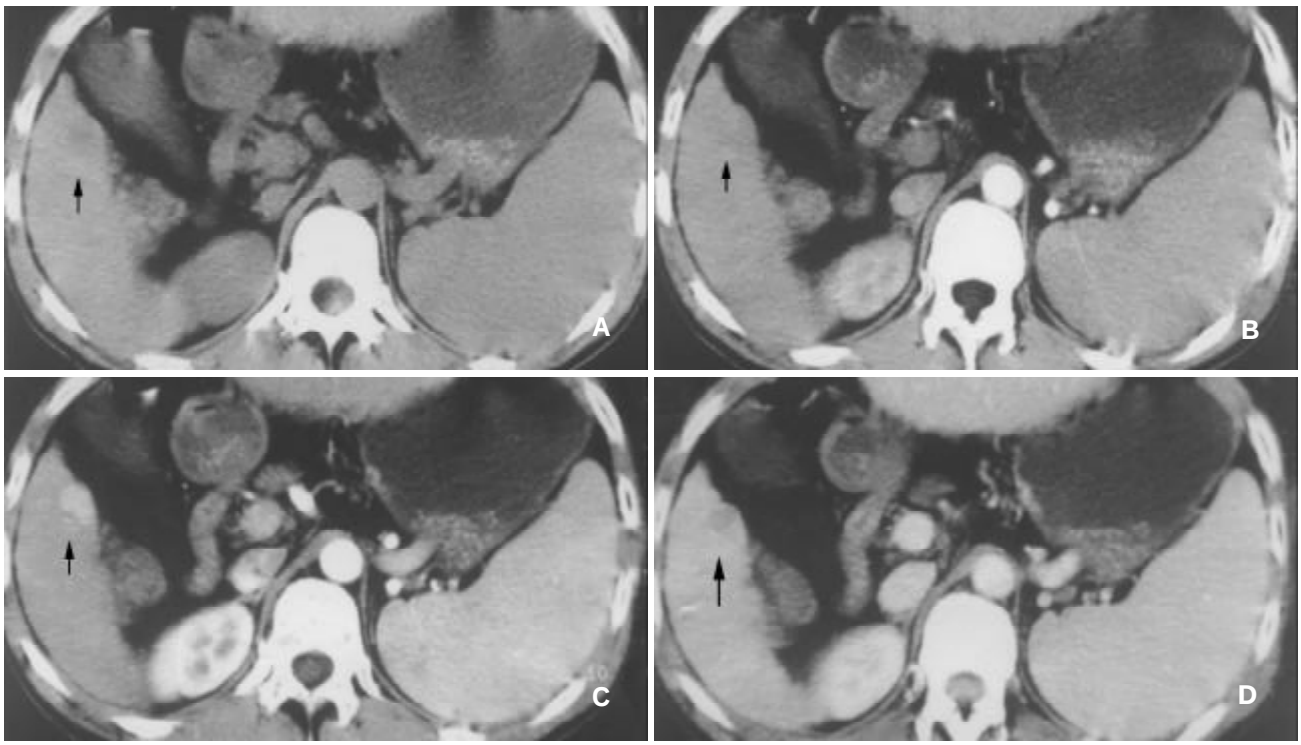



**Figure 1** SHCC with size of 1.5 cm in diameter. A: Precontrast image shows hypoattenuating lesion. B:The early arterial phase image shows nonenhanced lesion. C: In the late arterial phase,the lesions enhanced. D: In the portal venous phase, the lesions enhanced dropped down.

**Table 3** Comparison of $X^2$ value between each scanning phase

| Scanning phases | LAP | ELAP | PVP | EAP+PVP | LAP+PVP | EAP+LAP+PVP |
|---|---|---|---|---|---|---|
| EAP | $P$<0.001[a] | $P$<0.0018 | $P$<0.001[a] | $P$<0.001[a] | $P$<0.001[a] | $P$<0.001[a] |
| LAP | | $P$=0.088 | $P$=0.258 | $P$=0.831 | $P$=0.033[a] | $P$=0.006[a] |
| ELAP | | | $P$=0.088 | $P$=0.135 | $P$=0.669 | $P$=0.285 |
| PVP | | | | $P$=0.200 | $P$<0.001[a] | $P$<0.001[a] |
| EAP+PVP | | | | | $P$=0.053 | $P$=0.010[a] |
| LAP+PVP | | | | | | $P$=0.521 |

Note: All data represented the comparison between each scanning phase using McNemar Test. [a]$P$<0.05 was considered significant.

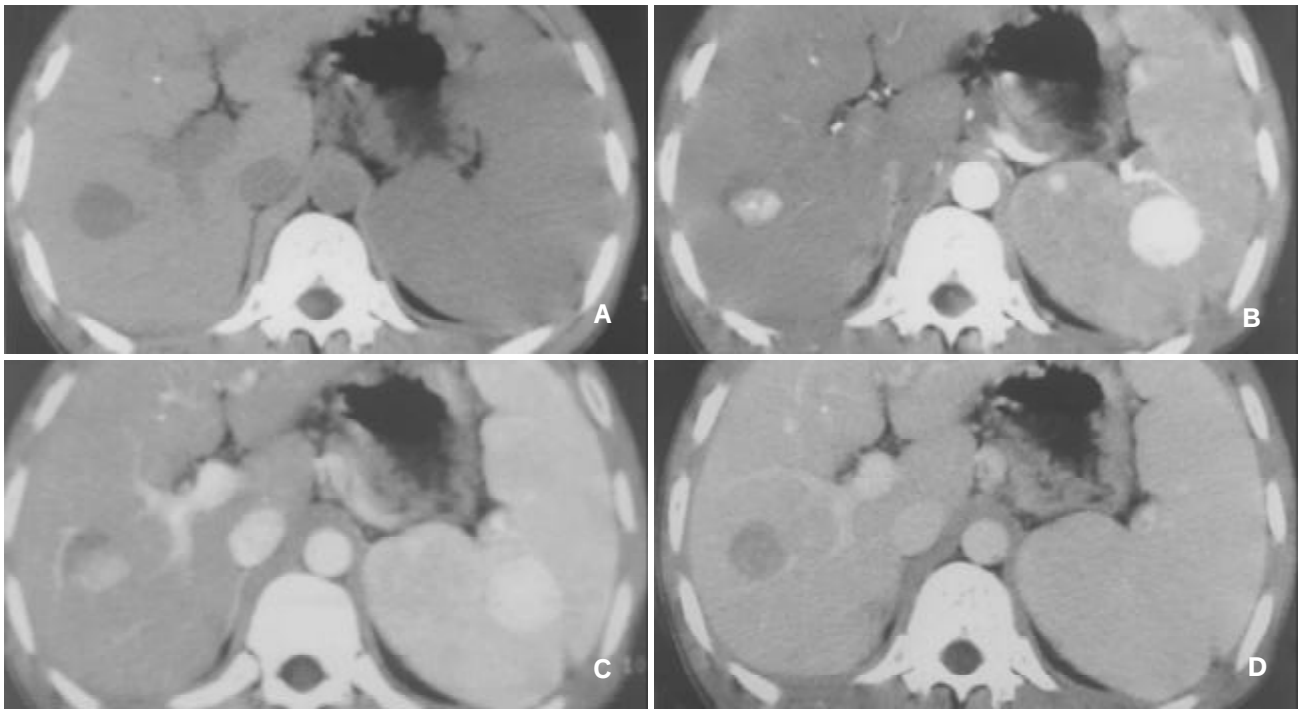



**Figure 2** SHCC of 2.0 cm in diameter, A: Precontrast image shows hypoattenuating lesion. B: CT scan obtained at the same level as in the early arterial phase, Tumor intensely enhanced. C: In the late arterial phase, the lesions showed enhancement yet. D: In the portal venous phase, the lesions enhancement dropped down.

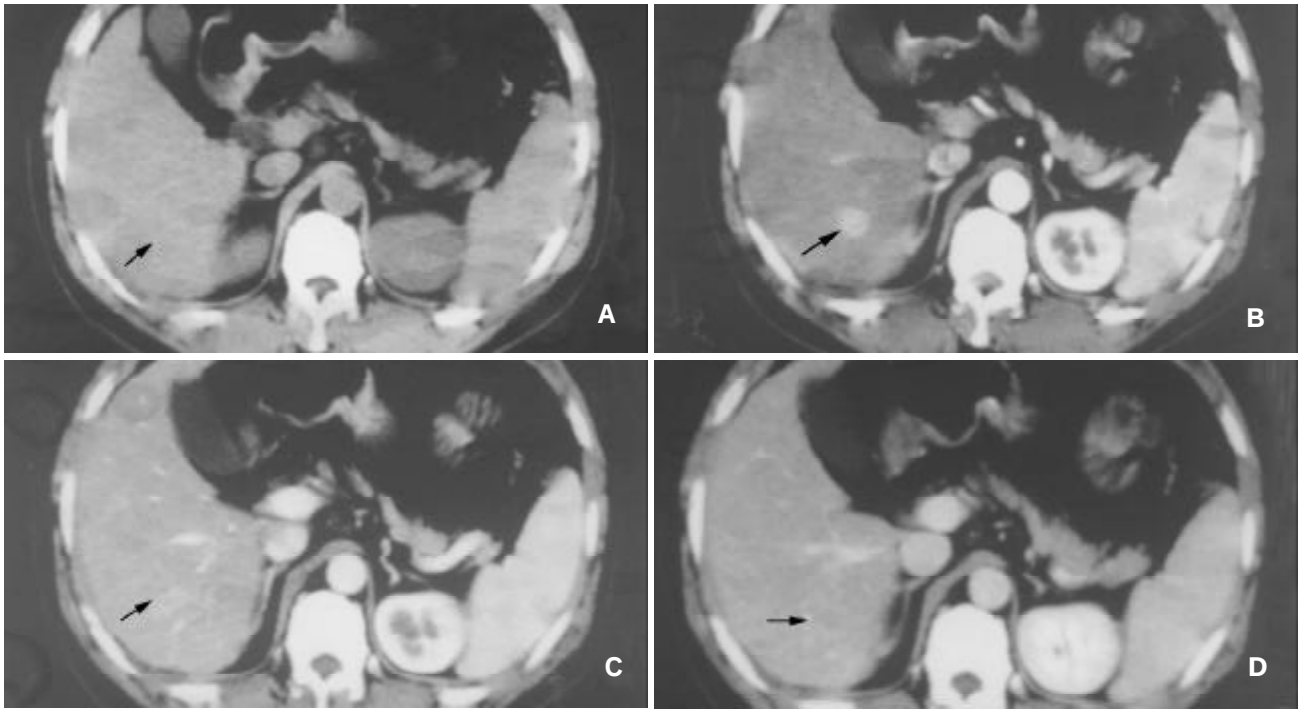



**Figure 3** SHCC of 1.5 cm in diameter, A: Hypoattenuating lesion showed in plance image. B: CT scan obtained at the same level as in the early arterial phase, Tumor intensely enhanced. C: In the late arterial phase tumor becomes isodense (arrow). D: In the portal venous phase, the tumor turned to hypodense.

## DISCUSSION

The biphasic acquisition helical CT scanning technique has become a standard method for the detection of suspected hypervascular hepatomas. But the single-row detector helical CT (SDCT) has a slow scanning speed (0.8-1 s) and acquires only one section of CT data with each rotation of the X-ray tube. The whole liver scanning using SDCT only takes 20-25 s. It is recognized that the liver has a dual blood supply, the duration of the virtual hepatic arterial phase equals the interval from the beginning of the contrast inflow into the liver from arteries to the beginning of the contrast inflow from the portal vein. It is so short that SDCT is impossible to cover the whole liver during the real hepatic arterial phase[1-6].

Application of MDCT technology in clinical practice has brought about a decisive breakthrough, which is an important milestone in helical CT technologic revolution. The major attribution of MDCT is faster Z-axis coverage speed and improves the longitudinal resolution. MDCT can acquire multiple sections of CT data with each rotation of the X-ray tube and scan the whole liver in 4-8 s, thus imaging of the whole liver can be completed

during the virtual arterial phase[7-13], even twice of the whole liver scanning can be accomplished during the virtual arterial phase. In our study the images in EAP showed intense enhancement of hepatic artery, and minimum enhancement of portal vein, and none of the hepatic parenchyma. The images in the late arterial phase demonstrated substantial portal vein, slight parenchyma enhancement, and no hepatic vein enhancement. The images in PVP showed hepatic veins enhancement, which were not yet enhanced during the early and late arterial phases[14-17].

Our previous studies[18] of continuously dynamic enhancement at the levels of hepatic artery, aorta and hepatic parenchyma, as well as at the level of SHCC foci showed that the arterial phase scanning was started at 16 s (12-22 s) and ended at 40 s. The mean duration of the arterial phase was 23 s. SHCCs were enhanced most markedly in the arterial phase. Due to its hypervascular nature , the mean peak time of the enhancement of SHCC foci was 45.4 s (31-56 s), and the maximum difference of the enhancement between tumor and liver parenchyma occurred at 36 s (28-48 s). Although SHCCs enhanced maximally at 45.4 s, the liver enhancement increased gradually and became quite obvious at that time. The conspicuity depended upon the enhancement difference of the tumor to the liver. Hence the optimally delayed scanning time should be the period when the maximum difference of enhancement occurred between tumor and liver and should not be at the peak time of the tumor enhancement[1,19,20]. This was the theoretical basis on which we set the EAP, LAP and PVP (or parenchyma phase) at 21 s, 34 s and 85 s, respectively. The delayed scanning time at different phases set up by us was in consistency with that reported by Murakami *et al*. They decided the delayed scanning timing for EAP (19.4s) using a mini bolus test, and for LAP (34.9 s), just a 5 s interscan delay after the end of the first pass scanning for table increment patients' movement and patients' respiration[15,16].

This study showed that SHCCs were enhanced during the arterial phase gradually and declined during the portal venous phase (Figure 1). But the mean enhancement difference was greater in LAP than that in EAP ($P$<0.05) (Table 1). We presume that this reflects the time interval for distribution of contrast-enhanced hepatic arterial blood into the tumor neovasculature and diffusion into the interstices of the tumor[15-22].

The results of our study showed that the sensitivity of detection of SHCC foci ($n$=91) was 45.1 % and 83.5 % for EAP and LAP. So the late arterial phase was very important for the detection of tumor foci. During the double arterial phase, it could reach 92.3 %, and was higher than that in the portal venous phase ($P$<0.05). This result indicated that the arterial phase was superior to the portal venous phase in the detectability of SHCCs and was similar to that reported by other authors using biphasic scanning of SDCT[15,16]. But the CT attenuation difference of enhancement of tumors and liver between LAP and EAP led to an extremely significant difference ($P$<0.001) in the detectability between LAP and EAP (83.5 % *vs* 45.1 %). Therefor, LAP is the best way for demonstrating SHCCs, which represents the advantage of triphasic scanning using MDCT. In addition, MDCT scans allow hepatic imaging with least image thickness and acquire the data in the early arterial phase, later arterial and portal venous phases and improve the sensitivity for depicting SHCC[15,16].

Since DAP combined with PVP had the highest detectability (97.8 %), but with no statistical difference when compared with LAP plus PVP (94.5 %). We consider the latter is more practical.

## REFERENCES


1 **Baron RL**, Oliver JH 3rd, Dodd GD 3rd, Nalesnik M, Holbert BL, Carr B. Hepatocellular carcinoma: evaluation with biphasic, contrast-enhanced, helical CT. *Radiology* 1996; **199**: 505-511

2 **Oliver JH 3rd**, Baron RL, Federle MP, Rockette HE Jr. Detecting hepatocellular carcinoma: value of unenhanced or arterial phase CT imaging or both used in conjunction with conventional portal venous phase contrast-enhanced CT imaging. *Am J Roentgenol* 1996; **167**:71-77

3 **Oliver JH** 3rd, Baron RL. Helical biphasic contrast-enhanced CT of the liver: technique, indications, interpretation, and pitfalls. *Radiology* 1996; **201**: 1-14

4 **Oliver JH** 3rd, Baron RL, Federle MP, Jones BC, Sheng R. Hypervascular liver metastases: do unenhanced and hepatic arterial phase CT images effect tumor detection? *Radiology* 1997; **205**: 709-715

5 **Paulson EK**, McDermott VG, Keogan MT, Delong DM, Frederick MG, Nelson RC. Carcinoid metastases to the liver: role of triple-phase helical CT. *Radiology* 1998; **206**: 143-150

6 **Mitsuzaki K**, Yamashita Y, Ogata I, Nishiharu T, Urata J, Takahashi M. Multiple-phase helical CT of the liver for detecting small hepatomas in patients with liver cirrhosis: contrast-injection protocol and optimal timing. *Am J Roentgenol* 1996; **167**: 753-757

7 **Berland L**, Smith K. Multidetector-array CT: Once again, technology creates new opportunities. *Radiology* 1998; **209**: 327-329

8 **Lewis MA**. Multislice CT. Opportunities and challenges. *British J Radiol* 2001; **74**: 779-781

9 **Spielmann AL**, Nelson RC, Lowry CR, Johnson GA, Sundaramoothy G, Sheafor DH, Paulson EK. Liver: Single breath-hold dynamic subtraction CT with multi-detector row helical technology-feasibility study. *Radiology* 2002; **222**: 278-283

10 **Hu H**, He HD, Foley WD, Fox SH. Four multidetector-row helical CT: Image quality and volume coverage speed. *Radiology* 2000; **215**: 55-62

11 **Fuchs T**, Kachelrieb M, Kalender WA. Technical advances in multi-slice spiral CT. *European J Radiol* 2000; **36**: 69-73

12 **Hu H**. Multi-slice helical CT: Scan and reconstruction. *Med Phys* 1999; **26**: 5-18

13 **Tadafumi S**, Toshimasa M, Kazuhiro Y,Kazuhiro S, Isamu N. Helical CT of the liver with computer-assisted bolus-tracking technology: Scan delay of arterial phase scanning and effect of flow rates. *J Comput Assist Tomogr* 2000; **24**: 219-223

14 **Sandstede JJW**, Tschammler A, Beer M, Vogelsang C, Wittenberg G, Hahn D. Optimization of automatic blous tracking for timing of the arterial phase of helical liver CT. *Eur Radiol* 2001; **11**: 1396-1400

15 **Till BR**, Rupert PW, Florian G. Timing of the hepatic arterial phase during contrast-enhanced computed tomography of the liver: Assessment of normal values in 25 volunteers. *Invest Radiol* 2000; **35**: 486-492

16 **Foley WD**, Mallisee TA, Hohenwalter MD, Wilson CR, Quiroz FA, Taylor AJ. Multiphase hepatic CT with a multirow detector CT scanner. *AJR* 2000; **175**: 679-685

17 **Murakami T**, Kim T, Takamura M, Hori M, Takahashi S, Federle MP, Tsuda K, Osuga K, Kawata S, Nakamura H, Kudo M. Hypervascular hepatocellular carcinoma: Detection with double arterial phase multi-detector row helical CT[1]. *Radiology* 2001; **218**: 763-767

18 **Zhou KR**, Yan FH, Tu BW. Evaluation of the arterial phase of biphase enhanced SCT in the diagnosis of small HCC. *Chin J Hepatol* 1999; **7**: 135-137

19 **Ohashi I,** Hanafusa K, Yoshida T. Small hepatocellular carcinomas: two-phase dynamic incremental CT in detection and evaluation. *Radiology* 1993; **189**: 851-855

20 **Hollett MD**, Jeffrey RB Jr, Nino-Murcia M, Jorgensen MJ, Harris DP. Dual-phase helical CT of the liver: value of arterial phase scans in the detection of small (<or=1.5 cm) malignant hepatic neoplasms. *Am J Roentgenol* 1995; **164**: 897-884

21 **Ichikawa T**, Kitamura T, Nakajima H, Sou H, Tsukamoto T, Ikenaga S, Araki T. Hypervascular hepatocellular carcinoma: Can double arterial phase imaging with multidetector CT improve tumor depiction in the cirrhotic liver? *AJR* 2002; **179**: 751-758

22 **Larson RE**, Semelka RC, Bagley AS, Molina PL, Brown ED, Lee JKT. Hypervascular malignant liver lesions: comparison of various MR imaging pulse sequences and dynamic CT[1]. *Radiology* 1994; **192**: 393-399


**Edited by** Wu XN and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2202-2206
World Journal of Gastroenterology
 ISSN 1007-9327

• COLORECTAL CANCER •

# Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma

Po Zhao, Ying-Chuan Hu, Ian C. Talbot

**Po Zhao,** Department of Pathology, Chinese PLA General Hospital, Beijing 100853, Beijing, China and Academic Department of Pathology, St. Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Harrow, HA1 3UJ, London, UK
**Ying-Chuan Hu,** Department of Pathology, Huaxi Medical College, Sichuan University, Chengdu 610041, Sichuan Province, China
**Ian C. Talbot,** Academic Department of Pathology, St. Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Harrow, HA1 3UJ, London, UK
**Correspondence to:** Dr. Po Zhao, Department of Pathology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China. zhaopo@plagh.com.cn or Professor Ian C. Talbot, Academic Department of Pathology, St Mark' s Hospital, Cancer Research UK, Colorectal Cancer Unit, Watford Road, Harrow HA1 3UJ, London, UK. i.talbot@cancer.org.uk
**Telephone:** +86-10-66937954 **Fax:** +86-10-68181689
**Received:** 2003-03-02 **Accepted:** 2003-05-16

## Abstract

**AIM:** To describe the correlation between innunostaining patterns of p16 and CDK4 and prognosis in colorectal carcinoma.

**METHODS:** Paraffin sections of 74 cases of colorectal carcinoma were analysed immunohistochemically for expression of p16 and CDK4 proteins.

**RESULTS:** Most carcinomas showed stronger p16 and CDK4 immunostaining in the cytoplasm than the adenomas or the adjacent normal mucosa. Strong immunostaining of p16 was a predictor for better prognosis whereas strong cytoplasmic immunostaining of CDK4 was a predictor for poor prognosis. Both p16 and CDK4 immunostainings were correlated with histological grade or Dukes' stage.

**CONCLUSION:** These results support the experimental evidence that interaction of expression of p16 and CDK4 may play an important role in the Rb/p16 pathway, and the expression paterns of CDK4 and p16 may be imperative in the development of colorectal carcinoma, thus becoming a new prognostic marker in colorectal cancer.

Zhao P, Hu YC, Talbot IC. Expressing patterns of p16 and CDK4 correlated to prognosis in colorectal carcinoma. *World J Gastroenterol* 2003; 9(10): 2202-2206
http://www.wjgnet.com/1007-9327/9/2202.asp

## INTRODUCTION

p16 gene, an important tumor suppressor gene, regulates cell proliferation negatively through inhibition of the kinase activity of cyclin-dependent kinase 4/6 (CDK4/6), which promotes phosphorylation and therefore inactivation of a very important tumor suppressor product, the retinoblastoma (Rb) gene protein[1-4]. The Rb/p16 tumor-suppressor pathway is abrogated frequently in multiple types of human tumors, either through inactivation of Rb or p16 tumor-suppressor protein, or through overexpression of cyclin D1 or cyclin-dependent kinase 4 (CDK4) oncoproteins. But no deletion and only quite a low frequency of mutation on p16 gene have been found in colorectal cancer since this gene was identified in 1994[1,5]. CpG islands are areas rich in CpG dinucleotides, which are found within the promotors of about 60 % of human genes. These CpG islands normally lack DNA methylation, regardless of the expression status of the gene[6]. Methylation of promotor usually leads to irreversible inhibition of gene transcription[7]. It has become apparent that *de novo* methylation is an important alternate mechanism to code region mutation for inactivating tumor suppressor genes during neoplasia[8-11]. Herman *et al*.[9] previously reported that there was methylation of p16 gene in 40 % of primary colorectal cancers and 92 % of colorectal cancer cell lines, in which all the p16 genes were inactivited by Southern analysis. Gonzalez-Zulueta *et al*.[10] found the evidence of methylation of the 5' CpG island not only in cancer tissues but also in normal colonic tissues by the more sensitive PCR-based assay, in which methylation of p16 exon 1 was detected in 1 of 10 (10 %) colon carcinomas and methylation of p16 exon 2 was detected in 7 of 10 (70 %) colon cancers. It is interesting that in 50 % of the colon normal/tumor matched cases, p16 was methylated and not expressed in the normal tissues but unmethylated and highly expressed in the tumor tissues. It also suggested that methylation of exon 1, but not exon 2, of p16 was associated with transcriptional silencing although the CpG island in exon 2 of p16 underwent extensive *de novo* methylation in colorectal carcinoma. More recently, Ahuja *et al*.[12] found that 16 (34 %) of 47 colorectal cancers exhibited methylation in exon 1 of the p16 gene, which was associated with microsatellite instability. Transcriptional silencing of p16 gene could be due to either gene deletion or methylation in exon 1. In either event, there would be no expression product in the cells. Therefore, we used an immunohistochemical method to detect p16 protein level *in situ* and compared with its binding oncoprotein, CDK4, as a simple way to investigate their possible interaction in the development and prognosis of colorectal carcinoma. We also detected the mRNA levels of both p16 and CDK4 genes and checked the methylation status on the promoter region of p16 gene to confirm their expression results in immunocytochemistry.

## MATERIALS AND METHODS

### *Colorectal carcinoma*

Seventy-four cases of colorectal carcinoma were randomly and retrospectively selected from the files of the Academic Department of Pathology, St. Mark' s Hospital, London, UK and the Department of Pathology, the former West China University of Medical Sciences, China. Specimens obtained at surgery were routinely fixed in 10 % neutral formalin and embedded in paraffin. The clinical stage was determined according to the Dukes' stages A, B and C. The histological grade of tumors was also determined according to the WHO criteria as follows: grade I as well differentiated, grade II as moderately differentiated, and grade III as poorly differentiated. Follow-up data on 5-year survival rate in 32 cases were available for further analysis with variables.

### *Immunohistochemical staining*

Immunohistochemical staining for p16 and CDK4 was performed according to the standard ABC method except that the pressure for cooking procedure was used for antigen retrieval pretreatment[13, 14]. Serial 4 μm thick sections were cut and dewaxed in xylene and rehydrated in a graded ethanol series. The sections were immersed in 3 % hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. and rinsed in running water. After that, sections were immersed in boiling 1 mM EDTA-NaOH (pH 8.0) buffer in a pressure cooker. The pressure cooker was then sealed and brought to full pressure. The heating time was 2 minutes which began only when full pressure was reached. At 2 minutes, the cooker was depressured and cooled under running water. The lid was then removed, and the hot buffer was flushed out with cold water from a running tap. The cooled sections were washed twice in PBS before immunohistochemical staining, then immersed in 0.05 % avidin for 30 minutes to block any possible endogenous biotin exposed to heating. Prior to immunohistochemical staining, the sections were first incubated with 10 % horse serum for monoclonal antibody and 10 % goat serum for polyclonal antibody respectively for 15 minutes to block non-specific binding. The primary monoclonal mouse antibody against human p16 protein (Pierce, USA) and polyclonal rabbit antibody against human CDK4 protein (Santa Cruz Biotechnology, USA) were diluted in 200 with 0.01 M PBS (pH 7.2), respectively. Then the sections were allowed to react by the standard ABC method using a VECTASTAIN Elite PK-6100 kit (Vector Labratories, Inc., USA), as directed by the manufacturer. A previously known positive pancreatic carcinoma was used as a positive control. The primary antibody was replaced by 0.01 M PBS or 10 % serum as a negative control. Normal colon mucosal tissue was used as a normal control.

### *Evaluation of score*

The slides were examined and data regarding staining positivity were recorded before the clinical outcome was available. When p16 or CDK4 protein expression was scored, both the extent and intensity of immunopositivity were considered, according to Hao *et al*[15]. The intensity of positivity was scored as follows: 0 as negative, 1 as weak, 2 as moderate, 3 as strong as normal liver. The extent of positivity was scored as follows: 0 <5, 1>5-25 %, 2>25-50 %, 3>50-75 %, and 4>75 % of the tumor cells in the respective lesions. The final score was determined by multiplying the intensity of positivity and the extent of positivity scores, yielding a range from 0 to 12. Scores 9-12 were defined as strong staining pattern (++), 5-8 as weak staining pattern (+), and 0-4 as markedly reduced or negative expression (-).

### *RNA extraction and RT-PCR*

Total RNA was isolated from the frozen tissues of 10 paired normal and tumor samples using the total RNA isolation system (Promega) and treated with RNase-free DNase I (GeneHunter). Equal amount (0.2 μg) of total RNA from each sample was added into the access RT-PCR system (Promega) which carried on both cDNA syntheses by reverse transcription and PCR in a same tube with a total volume of 25 μl reaction solution according to the manufacturer' s directions. The primer pairs used to amplify p16 and CDK4 genes were as follows. The sequence of p16 sense primer was 5' -CCCGCTTTCGTAGTTTTCAT-3' and that of antisense primer was 5' -TTATTTGAGCTTTGGTTCTG-3' [16] and that of CDK4 sense primer was 5' -ATGGCTGCCACTCGATAT GAACCC-3' and antisense primer, 5' -GTACCAGAGCGT AACCACCACAGG-3' [17]. In addition, primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene were used as an internal control to ensure the quality of the templet RNA and reagents in reactions. The sequence of sense primer was 5' -TGGTATCGTGGAAGGACTCATGAC-3' and antisense primer was 5' -ATGCCAGTGAGCTTCCCGTT CAGC-3' [16]. cDNA for each sample was synthesized at 48 ℃ for 45 min, followed by at 94 ℃ for 2 min to inactivate reverse transcriptase and was subjected to 40 cycles of amplification under the conditions described before[16,17] except for modified extension step during each cycle at 68 ℃ for 2 min according to the access RT-PCR system' s direction. Water controls, including primers and all reagents except for RNA in PCR reactions, were setup for potential DNA contamination.

### *Methylation-specific PCR*

Samples from 24 cases of colorectal carcinoma with residual adenoma were selected for DNA extraction. DNA was extracted from these cases according to the conventional methods. The methylation status on the promoter region of p16 was assessed by methylation-specific PCR (MSP) as described previously[9] Genomic DNAs from the primary tumors were subjected to bisulfate modification using the CpGenome DNA modification kit (Intergen, New York, NY). Treatment of genomic DNA with sodium bisulfate converted unmethylated cytosines (but not methylated cytosines) to uracil, which was then converted to thymidine during the subsequent PCR step, yielding sequence differences between methylated DNA. PCR primers distinguishing these methylated and unmethylated DNA sequences were used. Primer sequences of p16 genes[9] for both the methylated and unmethylated forms were 5' -TTATTAGAGGGTGGGGCGGATCGC-3' /5' -GACCCCGAACCGCGACCGTAA-3' and 5' -TTATTAGAGGGTGGGGTGGATTGT-3' /5' -CAACCCCAAACCACAACCATAA-3' , with annealing temperature at 65 ℃ and at 60 ℃, and the expected PCR product sizes were 150 bp and 151 bp, respectively. For PCR amplification, 2 μl of bisulfate-modified DNA was added in a final volume of 25 μl PCR mixture containing 1×PCR buffer, $MgCl_2$, deoxynucleotide triphosphates, and primers (100 pmol each per reaction), and 1 unit of AmpiTaq Gold (Applied Biosystems, Branchburg, NJ). Amplification was performed at 95 ℃ for 12 min, 35 cycles at 95 ℃ for 1 min, the specific annealing temperature for both methylated and unmethylated at 65 ℃ and at 60 ℃ for 1 min, and at 72 ℃ for 1 min, followed by a final 7-min extension at 72 ℃. PCR products (15 μl) were loaded onto a 10 % nondenaturing polyacrylamide gel, stained with ethidium bromide after 2 hours of electrophoresis, visualized under UV illumination. MSP for all samples was repeated to confirm their methylation status.

### *Statistical analysis*

Fisher' s exact test (two sided) and Pearson Chi square test for trends in proportions were used to assess the associations between Fhit expression and pathological indices. A $P<0.05$ was considered statistically significant.

## RESULTS

The results are summarized in Tables 1-2.

The 5-year survival of the 32 patients according to the available data was significantly shorter in patients with Dukes' C tumors than in those with Dukes' A and B tumors ($P<0.01$).

### *p16 expression in colorectal carcinoma*

In 61 patients in whom non-neoplastic mucosa was detected adjacent to the carcinoma, weak p16 expression was observed in the nuclei and moderate p16 expression only in the cytoplasm around nuclei. Most of the cytoplasm of goblet cells filled with

mucus was negative (Figures 1A, B). Of the 74 specimens examined, 73 (98.6 %) were p16-positive (Table 1). Expression of p16 was almost always observed in the cytoplasm but only sporadically in the nuclei. Of the p16 positive specimens, 53 showed a strong expression pattern and 20 a weak expression pattern (Figure 1C). Strong p16 expressions were detected in 15 (78.9 %) patients who survived more than 5 years, while weak p16 expressions were detected in 8 (61.5 %) patients who survived less than 5 years after operation (Table 1). The prognosis was significantly better in patients with tumors in strong expression pattern of p16 ($P$<0.001).

**Table 1** Relationship between p16 expression and clinicopathological features

| Clinicopathological features | No. of patients | p16 Immunostaining patterns | | |
|---|---|---|---|---|
| | | Strong | Weak | Negative |
| Dukes' stage | | | | |
| A | 23 | 16 | 7 | 0 |
| B | 22 | 16 | 6 | 0 |
| C | 29 | 21 | 7 | 1 |
| Histological grade | | | | |
| I | 24 | 17 | 7 | 0 |
| II | 41 | 30 | 10 | 1 |
| III | 9 | 6 | 3 | 0 |
| Total | 74 | 53 | 20 | 1 |
| 5-year survival rate | | | | |
| Alive | 20 | 15 | 5 | 0 |
| Dead | 12 | 4 | 8 | 0 |
| Total | 32 | 19 | 13 | 0 |

### *CDK4 expression in colorectal carcinoma*

In 49 of 61 non-neoplastic mucosaea adjacent to the carcinoma, CDK4 nuclear and cytoplasmic immunoreactivity was a little weaker than the p16 staining, but otherwise identical. The remaining 12 were negative. Expression of CDK4 was observed predominantly in the cytoplasm (Figure 1D). All the 74 specimens showed CDK4 expression (Table 2). Of the CDK-positive specimens, 33 showed a strong expressing pattern and 41 yielded a weak expressing pattern. Tumors with strong CDK4 expression were found in 4 (28.6 %) patients who had survived for more than 5 years after operation, whereas those with weak CDK4 expression were found only in 2 (11.1 %) patients who died within 5 years after operation. The prognosis was significantly poorers in patients with cancers in strong expression of CDK4 ($P$<0.001).

**Table 2** Relationship between CDK4 expression and clinicopathological features

| Clinicopathological features | No. of patients | CDK4 Immunostaining patterns | | |
|---|---|---|---|---|
| | | Strong | Weak | Negative |
| Dukes' stage | | | | |
| A | 23 | 9 | 14 | 0 |
| B | 22 | 10 | 12 | 0 |
| C | 29 | 14 | 15 | 0 |
| Histological grade | | | | |
| I | 24 | 8 | 16 | 0 |
| II | 41 | 19 | 22 | 0 |
| III | 9 | 6 | 3 | 0 |
| Total | 74 | 33 | 41 | 0 |
| 5-year survival rate | | | | |
| Alive | 20 | 4 | 16 | 0 |
| Dead | 12 | 10 | 2 | 0 |
| Total | 32 | 14 | 18 | 0 |

### *Correlation between p16 and CDK4 expression*

Of the 74 tumors with CDK4 expression (strong in 33, weak in 41), p16 expression was also found in all but one (strong in 53, weak in 20). There was a significantly reverse correlation between p16 and CDK4 expression patterns ($P$<0.001).

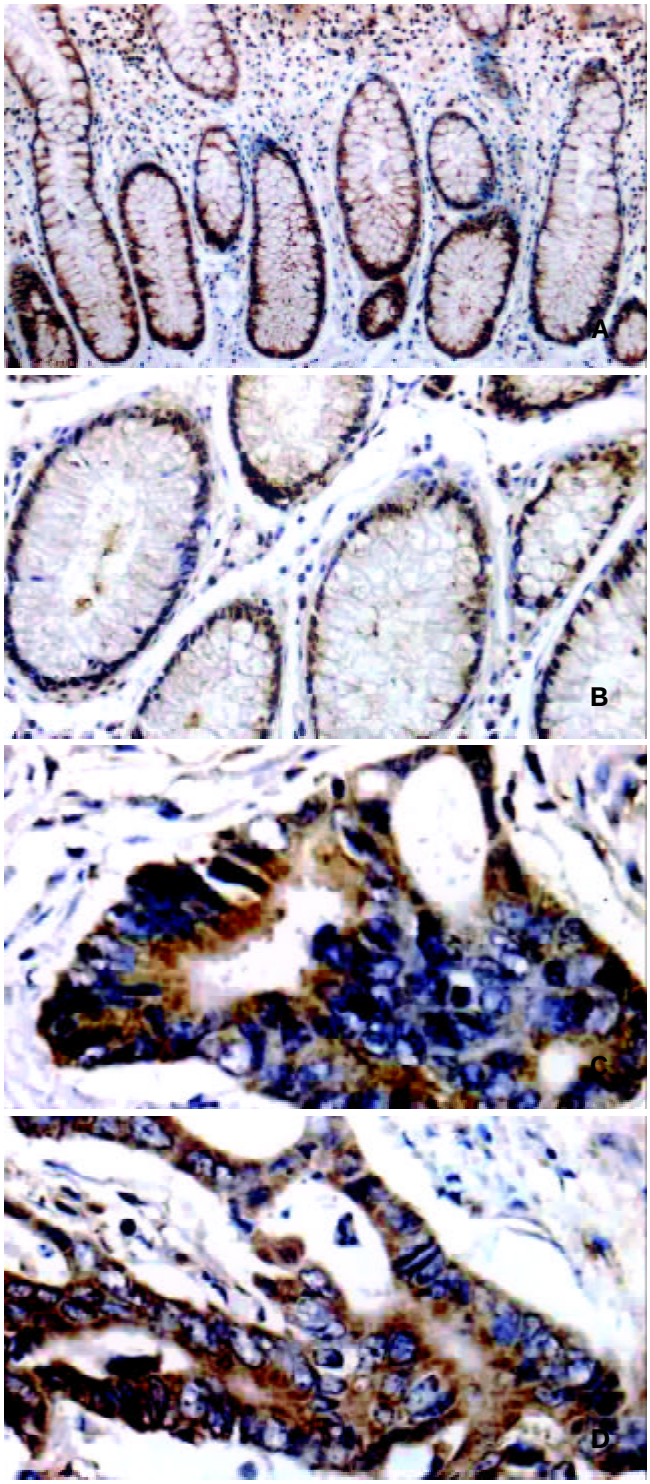



**Figure 1** Expression patterns for p16 and CDK4 gene proteins. A, B: nuclear/cytoplasmic pattern of p16 protein in normal crypts (SP×100, 200), C: overexpression pattern of p16 in the cytoplasm of colon cancer cells (SP×400), D: overexpression pattern of CDK4 in the cytoplasm of colon cancer cells (SP×400).

### *Correlation between p16 or CDK4 expression and clinicopathological features*

In our study, a significant correlation was found between p16 or CDK4 expression patterns and the tumor stage ($P$<0.001;

*P*<0.001) or the histological grade (*P*<0.001; *P*<0.001) (Tables 1, 2). The prognosis was significantly poorer in patients with weak p16 expression pattern (*P*<0.001), or with strong CDK4 expression pattern (*P*<0.001).

### *Results of RT-PCR and MSP*

We investigated the mRNA expression of p16 and CDK4 genes in 10 frozen normal-tumor paired tissues. The results showed that the level of mRNA expressions of p16 (9/10) and CDK4 (10/10) of cancer tissues rose in accordence with that of proteins of p16 and CDK4 in immunostaining (Figures 2A, B). We also determined the frequency of promoter methylation of p16 gene by MSP-PCR, but the result showed no positively methylated signal in all the 24 cases of colorectal carcinoma with residual adenoma (Figure 2C).

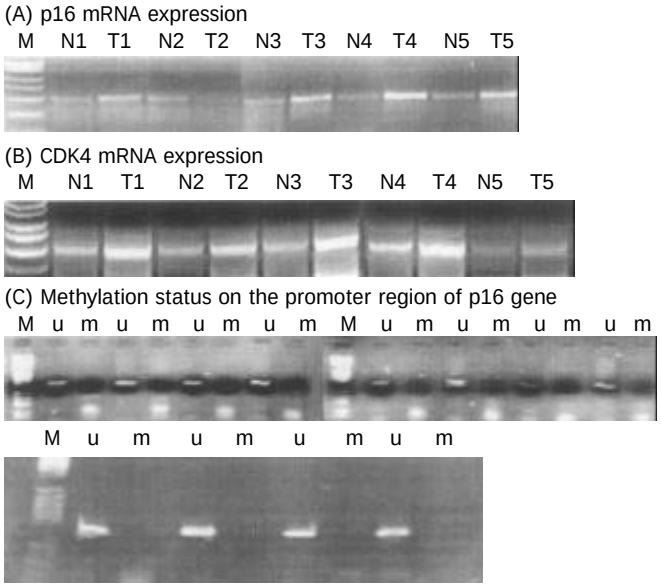



**Figure 2** Results in p16 and CDK4 mRNA expressions as well as p16 gene methylation status on the promoter region. (A) and (B): mRNA levels of both p16 and CDK4 genes were elevated in colorectal carcinoma (T), compared with their matched normal (N) tissues. (C) There was no methylation detected on p16 promoter region in all 24 cases of cancer tissue with residue adenoma. u: unmethylated, m: methylated, M: molecular marker (PBR322 / Hae III).

## DISCUSSION

Loss of expression of proteins such as p16 could occur as a consequence of either homozygous gene deletion or gene methylation. One aim of this study was to assess the extent to which both homozygous deletion and methylation on promoter region of p16 gene occurred with loss of gene expression in colorectal carcinoma. All carcinomas but one in this study showed the expression of p16 protein, confirmed not only by immunocytochemistry but also by RT-PCR and MSP, suggesting that loss of p16 gene expression is infrequent in primary colorectal carcinoma. The localization of p16 protein was similar to that of CDK4, almost always in the cytoplasm of adenocarcinoma cells. This indicates that the main site of interaction between p16 and CDK4 in adenocarcinoma cells is the cytoplasm. This contrasts with the localization of p16 protein in astrocytoma[18], in which nuclear expression is always more intense than any cytoplasmic expression. Based on these findings, our results indicate that CDK4 may contribute to phosphorylation of pRB, and to the loss of regulating function of p16 protein in nuclei, leading to the development of colorectal carcinoma. Induction of p16 overexpression would act as a brake at G1/S transition through pRB phosphorylation by CDK4 overexpression on the one hand, but predominant mislocation or only in cytoplasm might also imply the function of p16 in the nuclei, its ability to regulate transcriptions of other important genes related to proliferation and angiogenesis[19-21], was lost in colorectal carcinogenesis on the other hand.

The previous results[22-26] suggest that the deletion or mutational inactivity of both p16 and Rb proteins may be a rare event in cervical carcinogenesis. Moreover, overexpression of the p16 protein may be a useful diagnostic marker for cervical neoplastic lesions on routine laboratory screening. It was proposed that the expression of two viral oncogenes, E6 and E7, in epithelial stem cells be required to initiate and maintain cervical carcinogenesis and result in significant overexpression of the cellular p16 protein. Since this protein was not expressed in normal cervical squamous epithelia, screening for p16 over-expressing cells could specifically identify dysplastic lesions, and significantly reduced the inter-observer disagreement of the conventional cytological or histological tests. The similar results[28-30] have also been reported in prostate cancer, gastrointestinal stromal tumor and gastritis, in which overexpression of p16 in high grade prostate intraepithelial neoplasia (HGPIN) and cancer was correlated with, but independent of, pathological stage and was associated with early relapse in cancer patients treated with radical prostatectomy. Overexpression of p16 in HGPIN was also an independent predictor of disease relapse and increased the risk of recurrence 2.24-fold, providing the first evidence for a prognostic marker in HGPIN. The aberrant cytoplasmic expression as well as overexpression of p16 in colorectal carcinoma might be due to its binding to CDK4, CDK6 and some unknown proteins, thereby forming a larger volume of molecule which is uneasy to pass through the nuclear membrane. Nuclear expression of p16 would therefore imply extra unbound p16[5, 19], which might play other important roles in inhibiting transcription of genes related to tumor progression in the nuclei. For instance, VEGF for angiogenesis is besides or independent of binding CDK4/6[18-20]. It could be that CDK4 overexpression is an initial event, which is followed by a reactive overexpression of p16 in colorectal carcinoma, thus the oncoprotein function of CDK4 is inhibited to some extent by binding to p16. In our study, the histological grade, Dukes' stage and prognosis in colorectal carcinoma were closely related to p16 or CDK4 expression (*P*<0.001), respectively, suggesting that the expression of p16 or CDK4 protein may be a useful diagnostic and prognostic marker for colorectal neoplastic lesions. A more extended study is required to confirm the value of aberrant cytoplasmic expression or overexpression of p16 protein as a prognostic marker.

## REFERENCES

1 **Kamb A,** Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, Skolnick MH. A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 1994; **264:** 436-440

2 **Serrano M,** Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D / CDK4. *Nature* 1993; **366:** 704-707

3 **Marx J.** New tumor suppressor may rival p53. *Science* 1994; **264:** 344-345

4 **Nobori T,** Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994; **368:** 753-756

5 **Okamoto A,** Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, Harris CC. Mutations and altered expression of $p16^{INK4}$ in human cancer. *Proc Natl Acad Sci U S A* 1994; **91:** 11045-11049

6 **Bird AP.** CpG-rich islands and the function of DNA methylation. *Nature* 1986; **321:** 209-213

7 **Eden S,** Cedar H. Role of DNA methylation in the regulation of transcription. *Curr Opin Genet Dev* 1994; **4:** 255-259
8 **Sakai T,** Toguchida J, Ohtani N, Yandell DW, Rapaport JM, Dryja TD. Allele-specific hypermethylation of the retinoblastoma tumor-suppressor gene. *Am J Hum Genet* 1991; **48:** 880-888
9 **Herman JG,** Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrent DNA methylation in all common human cancers. *Cancer Res* 1995; **55:** 4525-4530
10 **Gonzalez-Zulueta M,** Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, Jones PA. Methylation of the 5'CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res* 1995; **55:** 4531-4535
11 **Herman JG,** Latif F, Weng Y, Lerman MI, Zbarl B, Liu S, Samib D, Duan DS, Gnarra JR, Linehan WM, Baylin SB. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 1994; **91:** 9700-9704
12 **Ahuja N,** Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa JP. Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* 1997; **57:** 3370-3374
13 **Norton AJ,** Jordan S, Yeomans P. Brief, high-temperature heat denaturation (pressure cooking): a simple and effective method of antigen retrieval for routinely processed tissues. *J Pathol* 1994; **173:** 371-379
14 **Pileri SA,** Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, Ascani S, Santini D, Piccaluga PP, Leone O, Damiani S, Ercolessi C, Sandri F, Pieri F, Leoncini L, Falini B. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J Pathol* 1997; **183:** 116-123
15 **Hao XP,** Willis JE, Pretlow TG, Rao JS, MacLennan GT, Talbot IC, Pretlow TP. Loss of fragile histidine triad expression in colorectal carcinomas and premalignant lesions. *Cancer Res* 2000; **60:** 18-21
16 **Chen YJ,** Shih LS, Chen YM. Quantitative analysis of CDKN2, p53 and retinoblastoma mRNA in human gastric carcinoma. *Int J Oncol* 1998; **13:** 249-254
17 **Ohtsuki F,** Yamamoto M, Nakagawa T, Tanizawa T, Wada H. Granulocyte-macrophage colony-stimulating factor abrogates transforming growth factor-b1-mediated cell cycle arrest by upregulating cyclin D2/Cdk6. *Br J Haematol* 1997; **98:** 520-527
18 **Rao LS,** Miller DC, Newcomb EW. Correlative immunohistochemistry and molecular genetic study of the inactivation of the $p16^{INK4A}$ genes in astrocytomas. *Diagn Mol Pathol* 1997; **6:** 115-122
19 **Emig R,** Magener A, Ehemann V, Meyer A, Stilgenbauer F, Volkmann M, Wallwiener D, Sinn HP. Aberrant cytoplasmic expression of the p16 protein in breast cancer is associated with accelerated tumour proliferation. *Br J Cancer* 1998; **78:** 1661-1668
20 **Harada H,** Nakagawa K, Iwata S, Saito M, Kumon Y, Sakaki S, Sato K, Hamada K. Restoration of wild-type p16 down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human gliomas. *Cancer Res* 1999; **59:** 3783-3789
21 **Bartkova J,** Lukas J, Guldberg P, Alsner J, Kirkin AF, Zeuthen J, Bartek J. The P16-cyclinD/Cdk4-PRb pathway as a functional unit frequently altered in melanoma pathogenesis. *Cancer Res* 1996; **56:** 5475-5483
22 **Klaes R,** Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001; **92:** 276-284
23 **Klaes R,** Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol* 2002; **26:** 1389-1399
24 **von Knebel Doeberitz M.** New molecular tools for efficient screening of cervical cancer. *Dis Markers* 2001; **17:** 123-128
25 **Sano T,** Oyama T, Kashiwabara K, Fukuda T, Nakajima T. Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. *Pathol Int* 1998; **48:** 580-585
26 **Negri G,** Egarter-Vigl E, Kasal A, Romano F, Haitel A, Mian C. p16INK4a is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors: an immunohistochemical study with immunocytochemical correlations. *Am J Surg Pathol* 2003; **27:** 187-193
27 **Mc Cluggage WG,** Jenkins D. p16 immunoreactivity may assist in the distinction between endometrial and endocervical adenocarcinoma. *Int J Gynecol Pathol* 2003; **22:** 231-235
28 **Henshall SM,** Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, Delprado W, Stricker PD, Grygiel JJ, Sutherland RL. Overexpression of the cell cycle inhibitor p16INK4A in high-grade prostatic intraepithelial neoplasia predicts early relapse in prostate cancer patients. *Clin Cancer Res* 2001; **7:** 544-550
29 **Schneider-Stock R,** Boltze C, Lasota J, Miettinen M, Peters B, Pross M, Roessner A, Gunther T. High prognostic value of p16INK4 alterations in gastrointestinal stromal tumors. *J Clin Oncol* 2003; **21:** 1688-1697
30 **Shirin H,** Hibshoosh H, Kawabata Y, Weinstein IB, Moss SF. p16Ink4a is overexpressed in *H pylori*-associated gastritis and is correlated with increased epithelial apoptosis. *Helicobacter* 2003; **8:** 66-71


**Edited by** Ma JY and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2207-2210
World Journal of Gastroenterology
 ISSN 1007-9327

• COLORECTAL CANCER •

# Prognostic factors in 165 elderly colorectal cancer patients

Ke-Jun Nan, Hai-Xia Qin, Guang Yang

**Ke-Jun Nan, Hai-Xia Qin,** Department of Oncology, First Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China
**Guang Yang,** Medical College, Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China
**Correspondence to:** Ke-Jun Nan, Department of Oncology, First Hospital of Xi' an Jiaotong University, 1 Jiankang Xilu, Xi' an 710061, Shaanxi Province, China. qinhaixia100925@sina.com.cn
**Telephone:** +86-29-5324086 **Fax:** +86-29-5324086
**Received:** 2003-06-28 **Accepted:** 2003-07-24

## Abstract

**AIM:** To analyse the prognostic factors in 165 colorectal patients aged ⩾70.

**METHODS:** One hundred and sixty-five elderly patients with colorectal cancer diagnosed by histology were entered into the retrospective study between 1994 and 2001. Patients were given optimal operation alone, chemotherapy after operation, or chemotherapy alone according to tumor stage, histology, physical strength, and co-morbid problems. Survival rate was calculated by Kaplan-Meier method, and compared with meaningful variances by Log-rank method. Prognostic factors were analyzed by Cox regression.

**RESULTS:** The 1,2,3,4,5 year survival rate (all-cause mortality) was 87.76 %, 65.96 %, 52.05 %, 42.77 %, 40.51 %, respectively. The mean survival time was 41.89±2.33 months (95 % CI: 37.33-46.45 months), and the median survival time was 37 months. Univariate analysis showed that factors such as age, nodal metastasis, treatment method, Duke's stage, gross findings, kind of histology, and degree of differentiation had influences on the survival rate. Multivariate analysis showed that factors such as treatment method, Duke's stage, kind of histology and degree of differentiation were independent prognostic factors.

**CONCLUSION:** This study suggests that the prognosis of elderly colorectal cancer patients is influenced by several factors. Most of elderly patients can endure surgery and/or chemotherapy, and have a long-time survival and good quality of life.

Nan KJ, Qin HX, Yang G. Prognostic factors in 165 elderly colorectal cancer patients. *World J Gastroenterol* 2003; 9(10): 2207-2210
http://www.wjgnet.com/1007-9327/9/2207.asp

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors in the world[1-5]. In China it is the fifth of malignant tumor, and the third of alimentary tract malignant tumor[6, 7]. Its incidence rises with increasing age[8,9]. Currently, the majority of colon and rectum tumors arise in patients aged 70 and over. Improvements in public health, nutrition and the prevention and treatment measures have prolonged the life of elderly individuals. The average life expectancy of a 70-year-old man can be prolonged ten years and of a 70-year-old woman 15 years. As a result, there will be a rise in the prevalence of CRC in elderly patients in the coming decades. But the elderly always have some co-morbid problems, and their clinical, pathological characteristics are different from young patients, so how to rationally treat CRC cancer of the old becomes very important.

## MATERIALS AND METHODS

### *Clinical data*

There were 205 elderly colorectal patients (aged ⩾70) in the First Hospital of Xi' an Jiaotong University from 1994 to 2001, accounting for 12.1 % of the total colorectal cancer patients. One hundred and sixty-five cases were enrolled into the study that had full histology, clinical and follow-up records. There were 105 males, and 60 females (sex ratio was 1.75:1). All patients were aged from 70 to 91 (the median 74 years), and the mean age was 74.67±0.54 years. The latent period ranged from 1 day to 4.5 years, and the mean latent time was 6.21±0.69 months. One hundred and three tumors were located in colon, and 68 in rectum, respectively, including 3 simultaneous double-tumors and 3 different time double-tumors. In the 129 resection specimens, the dimension was smaller than 5 cm in 74 and ⩾5 cm in 55. Forty-six patients had adenomatous polyps simultaneously. One hundred and forty-five had co-morbidity, among them 73 had cardiovascular diseases, 24 respiratory tract disorders, and 17 cerebral vessel disorders, 16 diabetes mellitus, and 15 other diseases.

### *Treatment methods*

**Operation** 118 Duke's A, B, C, D stage patients received curative resection, 27 received palliate resection, and 4 received only emergency surgery. 16 cases received no surgery because of their reluctance or bad status.

**Chemotherapy** Patients who conformed with the requirements were given chemotherapy: PS 0-2 with Duke's C or D or high risk Duke's B tumors (having any characteristic such as perforated or obstructed tumors, T4 tumors, poor differentiation in histology, extra-mural vascular invasion, or mucinous differentiation). They were given 5-Fu/CF+L-OHP every 21 days, L-OHP 80-100 mg· $m^{-2}$ >2 h iv $d_1$, LV 200 mg· $m^{-2}$· $d^{-1}$ 2 h iv $d_{1-5}$, 5-Fu 400 mg· $m^{-2}$· $d^{-1}$ iv $d_{1-5}$. Forty-seven patients with Duke's B and C tumors had 197 cycles of chemotherapy. Patients with Duke's D tumor accepted 154 cycles of chemotherapy. Among them, 15 cases received post-operation chemotherapy, and 28 cases pure chemotherapy.

### *Statistical analysis*

Kaplan-Meier method was used to calculate survival rate, and Log rank test was used in the univariate analysis. Cox regression Model was used in the multivariate analysis. The SPSS 10.0 for windows was used for all the statistical analyses.

## RESULTS

### *General information*

In this study, the 1, 2, 3, 4, 5 year survival rate (all-cause mortality) was 87.76 %, 65.96 %, 52.05 %, 42.77 %, 40.51 %, respectively. The mean survival time was 41.89±2.33 months (95 % CI: 37.33-46.45 months), and the median survival time

was 37 months. The mortality of surgery was 2.8 % (4/145), and death occurred due to perforation, pulmonary infection, and cachexia, respectively. The rate of chemotherapy side effect was 87.3 %, but besides 1 $IV^0$ diarrhea and 1 $IV^0$ myelosupression, all others were 0-$III^0$.

### *Univariate analysis*

There were several risk factors by univariate analysis as shown in Table 1. But other factors had nothing to do with prognosis, such as gender, latent period, tumor size, co-morbid problems, precancerous lesion, and bloody stool.

**Table 1** Results of univariate analysis

| Variance | *n* | 1 year survival rate (%) | 3 year survival rate (%) | 5 year survival rate (%) | *P* value |
|---|---|---|---|---|---|
| Age | | | | | 0.0332 |
| 70-75 | 96 | 91.49 | 59.40 | 48.62 | |
| 75-91 | 69 | 82.58 | 41.79 | 28.84 | |
| Treatment | | | | | |
| Curative resection+ chemotherapy | 47 | 95.67 | 78.24 | 60.72 | |
| Curative resection | 71 | 95.65 | 62.62 | 55.01 | |
| Palliate resection + chemotherapy | 21 | 71.43 | 11.11 | 0 | <0.005 |
| Palliate resection | 6 | 50.00 | 0 | 0 | |
| Chemotherapy | 16 | 75 | 0 | 0 | |
| Emergency surgery | 4 | 0 | 0 | 0 | |
| Duke's stage | | | | | |
| A | 8 | 100 | 75 | 37.5 | |
| B | 35 | 100 | 79.16 | 64.57 | <0.005 |
| C | 72 | 97.16 | 70.55 | 56.05 | |
| D | 50 | 63.88 | 6.74 | 0 | |
| Gross finding | | | | | |
| Ulcer form | 66 | 92.40 | 64.88 | 53.52 | |
| Projection form | 57 | 92.88 | 58.86 | 49.15 | <0.005 |
| Infiltration form | 6 | 100 | 50 | 33.33 | |
| Type of histology | | | | | |
| Tubular adenoma | 130 | 91.41 | 55.33 | 44.77 | |
| Mucinous adenocarcinoma | 23 | 73.91 | 29.12 | 23.29 | 0.0018 |
| Other types | 12 | 75.0 | 56.25 | 0 | |
| Differentiation of tumor | | | | | |
| High | 10 | 100 | 74.07 | 44.44 | |
| Middle | 123 | 95.87 | 57.84 | 47.55 | <0.005 |
| Low | 32 | 52.78 | 22.62 | 14.14 | |
| Nodular metastasis | | | | | |
| No | 43 | 100 | 79.62 | 67.24 | 0.0008 |
| Yes | 122 | 83.52 | 42.69 | 31.21 | |

**Table 2** Results of multivariate analysis for elderly colorectal cancer

| Factors | Regression coefficient | Standard error | OR | OR 95 % CI | | *P value* |
|---|---|---|---|---|---|---|
| | | | | Lower | Upper | |
| Treatment method | 2.028 | 0.356 | 7.603 | 3.782 | 15.284 | 0.000 |
| Duke's stage | 0.648 | 0.295 | 0.523 | 0.293 | 0.933 | 0.028 |
| Type of histology | -0.824 | 0.248 | 0.439 | 0.270 | 0.713 | 0.003 |
| Differentiation of tumor | -0.850 | 0.246 | 0.427 | 0.264 | 0.692 | 0.016 |

Value: resection + chemotherapy=0, resection only=1. Duke's A+B stage=0, Duke's C+D stage=1. Other type=0, tubular adenoma=1. Low differentiation=0, middle and high differentiation=1.

### *Multi-variate analysis*

The factors associated with the risk of colorectal cancer at $P<0.05$ were furthered tested using backward stepwise conditional Cox regression. The final model consisted of the variables having a significant association with the risk of elderly colorectal cancer at $P<0.05$. The results are shown in Table 2.

## DISCUSSION

Though the number of elderly CRC patients is increasing, study about their treatment is rare, especially in China. A number of factors may account for the reluctance to receive operation and chemotherapy in elderly CRC patients. For example, family members do not want them to accept operation and chemotherapy in their old age, co-morbid problems may complicate or even preclude operation and chemotherapy, elderly patients may be more susceptible to side-effects of operation and chemotherapy which may decrease their quality of life[10], studies on the efficacy and toxicity of chemotherapy in elderly group are limited[11,12].

Treatment method is the most important prognostic index of elderly CRC in this study. Univariate and multivariate analyses showed that operation combined with post-operation chemotherapy was superior to resection only and chemotherapy only. The curative resection rate of the 165 patients was 71.52 % (118/165), and the total resection rate was 87.88 % (145/165). Operation mortality rate was 2.8 %. So it is evident that age is not the contraindication for surgery[13,14]. Resection should still be the first choice of treatment for elderly colorectal patients[15]. Patients who can endure operation after pre-operation management should receive operation in order to decrease the tumor burden. But acute abdomen is frequent in elderly CRC patients, and emergency operation is frequent accordingly, so active peri-operation preparations are necessary[16,17], such as the selection of optimal anesthesia and operation method to make them more endurable to operation, carefully nursing and supporting to reduce the opportunity of complication. Suitable chemotherapy can prolong the life of advanced patients, and improve their quality of life[18]. In this study, the patients who received chemotherapy lived longer than those who only received operation, and there were no severe side effects. It is obvious that elderly patients can survive chemotherapy and have a long-time survival and good quality of life, if an optimal chemotherapy scheme is given[19-23]. Also, because most of CRC relapse or transfer in the following 3 years of operation, it is necessary to give patients adjuvant chemotherapy[24, 25]. As to the elderly patients, several factors must be taken into consideration when chemotherapy is given, such as suitable chemotherapy regimen with good efficiency and low side effects[26,27], the importance of the first cycle[28], liver and kidney function testing and blood cell calculation before every cycle with heart and lung function evaluated if necessary, active support simultaneously, less or no anti-HT3 used to decrease the rate of constipation, long-cycle and convenient scheme to decrease the in-patient time, and changing scheme when it is inefficient[29].

In this study, univariate and multivariate analyses showed that the patients aged 70-75 years had a longer survival than those aged 75-91 years, which conformed with the study that aging was a risk factor for prognosis[30]. The elder the patient the lower of sensitivity, so older patients are always diagnosed in their late stage, which significantly influences the treatment and prognosis. But another study showed that aging had nothing to do with prognosis[31].

Lymph nodular metastasis is a negative indicator of prognosis for elderly CRC patients[30,32-34]. When cancer cells stay in the celiac lymph node, it is probable that malignant cells have transferred to liver, lung, bone, etc, through lymph

circulation and blood circulation. At last patients may become fatigue, even die of target organ' s dysfunction. However, some researchers hold that lymph nodular metastasis is not correlated with prognosis[25].

Both univariate and multi-variate analyses showed that Duke' s stage was an important prognostic factor in this study[34]. The early the stage, the easy the treatment, and the better the long-survival. Duke' s A and some B stage patients did not need to receive chemotherapy at all, but their survival time was the best at all stages. While Duke' s C and D patients needed both operation and chemotherapy, but their prognosis was always poor. Precise pre-operation staging is beneficial to the optimal treatment, which has something to do with the prognosis[35,36]. So doctors must carefully and precisely evaluate the Duke' s stage before and during operation so as to give elderly CRC patients the optimal treatment.

Univariate analysis showed that gross finding was another prognostic factor[34]. In this study, ulcer patients had a longer survival than those with infiltration, which was similar to the result of L.Roncucci' s study[37]. Infiltration type and prominence type always result in intestinal obstruction, which makes the patients have to accept emergency operation. Also, both of them can make patients emaciation, which decreases the endurance to necessary treatment, because of dysfunction of digestion and absorbance. Furthermore, infiltration type always has a poor differentiation in histology.

Univariate and multi-variate analyses showed that histology type and differentiation influenced prognosis in this study[34, 38]. Tubular adenocarcinoma had a better prognosis than mucinous adenocarcinoma. The latter had a poorer differentiation, and this kind of cancer cells had a tendency to transfer to distant sites, which leads to a poor survival. However the high and middle differentiations had a better prognosis and quality of life[30, 39].

In conclusion there are several factors influencing the prognosis of elderly CRC patients. When selecting treatment method, doctors have to take PS, Duke' s stage, age, gross finding, histology type, differentiation, lymph node metastasis into consideration. Besides the clinical characteristics that influence the prognosis of elderly CRC patients, other molecular markers such as oncogene mutation, change of chromosome and vascular endothelial growth factor are also significant prognostic factors[40-46]. Searching for new biology markers and better treatment methods will be one part of the future study.

## REFERENCES


1 **Zhang YL,** Zhang ZS, Wu BP, Zhou DY. Early diagnosis for colorectal cancer in China. *World J Gastroenterol* 2002; **8**: 21-25

2 **Thiis-Evensen E,** Hoff GS, Sauar J, Majak BM, Vatn MH. Flexible sigmoidoscopy or colonoscopy as a screening modality for colorectal adenomas in older age groups? Findings in a cohort of the normal population aged 63-72 years. *Gut* 1999; **45**: 834-839

3 **Li S,** Nie Z, Li N, Li J, Zhang P, Yang Z, Mu S, Du Y, Hu J, Yuan S, Qu H, Zhang T, Wang S, Dong E, Qi D. Colorectal cancer screening for the natural population of Beijing with sequential fecal occult blood test: a multicenter study. *Chin Med J* 2003; **116**: 200-202

4 **Repetto L,** Venturino A, Fratino L, Serraino D, Troisi G, Gianni W, Pietropaolo M. Geriatric oncology: a clinical approach to the older patient with cancer. *Eur J Cancer* 2003; **39**: 870-880

5 **Gatta G,** Faivre J, Capocaccia R, Ponz de Leon M. Survival of colorectal cancer patients in Europe during the period 1978-1989. *Eur J Cancer* 1998; **34**: 2176-2183

6 **Tang ZY.** Xiandai Zhongliuxue. 2ed. *ShangHai: ShangHai Medical University Press* 2000: 776

7 **Li L,** Lu F, Zhang S. Analyses of variation trend and short-term detection of Chinese malignant tumor mortality during twenty years. *Zhonghua Zhongliu Zazhi* 1997; **19**: 3-9

8 **Wymenga AN,** Slaets JP, Sleijfer DT. Treatment of cancer in old age, shortcomings and challenges. *Neth J Med* 2001; **59**: 259-266

9 **Franceschi S,** La Vecchia C. Cancer epidemiology in the elderly. *Crit Rev Oncol Hematol* 2001; **39**: 219-226

10 **De Marco MF,** Janssen-Heijnen ML, van der Heijden LH, Coebergh JW. Comorbidity and colorectal cancer according to subsite and stage: a population-based study. *Eur J Cancer* 2000; **36**: 95-99

11 Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. Meta-Analysis Group In Cancer. *J Clin Oncol* 1998; **16**: 3537-3541

12 **Daniele B,** Simmonds PD, Best LY, Ross PJ, Cunningham D. Should chemotherapy be used as a treatment of advanced colorectal carcinoma (ACC) in patients over 70 years of age? *Eur J Cancer* 1999; **35**: 1640-1649

13 Surgery for colorectal cancer in elderly patients: a systematic review. Colorectal Cancer Collaborative Group. *Lancet* 2000; **356**: 968-974

14 **Platell C,** Lim D, Tajudeen N, Tan JL, Wong K. Dose surgical sub-specialization influence survival in patients with colorectal cancer? *World J Gastroenterol* 2003; **9**: 961-964

15 **Smith JJ,** Lee J, Burke C, Contractor KB, Dawson PM. Major colorectal cancer resection should not be denied to the elderly. *Eur J Surg Oncol* 2002; **28**: 661-666

16 **Catena F,** Pasqualini E, Tonini V, Avanzolini A, Campione O. Emergency surgery of colorectal cancer in patients older than 80 years of age. *Ann Ital Chir* 2002; **73**: 173-177

17 **Catena F,** Pasqualini E, Tonini V, Avanzolini A, Campione O. Emergency surgery for patients with colorectal cancer over 90 years of age. *Hepatogastroenterology* 2002; **49**: 1538-1539

18 **Magne N,** Francois E, Broisin L, Guardiola E, Ramaioli A, Ferrero JM, Namer M. Palliative 5-fluorouracil-based chemotherapy for advanced colorectal cancer in the elderly: results of a 10-year experience. *Am J Clin Oncol* 2002; **25**: 126-130

19 **Liu LX,** Zhang WH, Jiang HC, Zhu AL, Wu LF, Qi SY, Piao DX. Arterial chemotherapy of 5-fluorouracil and mitomycin C in the treatment of liver metastases of colorectal cancer. *World J Gastroenterol* 2002; **8**: 663-667

20 **Romiti A,** Tonini G, Santini D, Di Seri M, Masciangelo R, Mezi S, Veri A, Santuari L, Vincenzi B, Brescia A, Marchei P, Frati L, Tomao S. Tolerability of Raltitrexed ('Tomudex') in elderly patients with colorectal cancer. *Anticancer Res* 2002; **22**: 3071-3076

21 **Mattioli R,** Lippe P, Recchia F, Massacesi C, Imperatori L, De Filippis S, Rosselli M, Gattafoni P, Casadei V, Consales D. Advanced colorectal cancer in elderly patients: tolerance and efficacy of leucovorin and fluorouracil bolus plus continuous infusion. *Anticancer Res* 2001; **21**: 489-492

22 **Kohne CH,** Grothey A, Bokemeyer C, Bontke N, Aapro M. Chemotherapy in elderly patients with colorectal cancer. *Ann Oncol* 2001; **12**: 435-442

23 **Feliu J,** Mel JR, Camps C, Escudero P, Aparicio J, Menendez D, Garcia Giron C, Rodriguez MR, Sanchez JJ, Gonzalez Baron M. Raltitrexed in the treatment of elderly patients with advanced colorectal cancer: an active and low toxicity regimen. *Eur J Cancer* 2002; **38**:1204-1211

24 **Cunningham D,** Haller D, Miles A. The effective management of colorectal cancer. 1st ed. *London: Aesculapius Medical Press* 2000: 75-81

25 **Allen PJ,** Kemeny N, Jarnagin W, DeMatteo R, Blumgart L, Fong Y. Importance of response to neoadjuvant chemotherapy in patients undergoing resection of synchronous colorectal liver metastases. *J Gastrointest Surg* 2003; **7**: 109-115

26 **Honecker F,** Wedding U, Kolb G, Bokemeyer C. Chemotherapy of colorectal cancer-which therapy is justified for elderly patients? *Onkologie* 2001; **24**: 87-94

27 **Cunningham D,** Zalcberg J, Maroun J, James R, Clarke S, Maughan TS, Vincent M, Schulz J, Gonzalez Baron M, Facchini T. Efficacy, tolerability and management of raltitrexed (Tomudex) monotherapy in patients with advanced colorectal cancer. a review of phase II/III trials. *Eur J Cancer* 2002; **38**: 478-486

28 **Tsalic M,** Bar-Sela G, Beny A, Visel B, Haim N. Severe toxicity related to the 5-fluorouracil/leucovorin combination (the Mayo Clinic regimen): a prospective study in colorectal cancer patients. *Am J Clin Oncol* 2003; **26**: 103-106

29 **Corsi DC,** Ciaparrone M, Zannoni G, Mancini M, Cassano A, Specchia M, Pozzo C, Martini M, Barone C. Predictive value of

thymidylate synthase expression in resected metastases of colorectal cancer. *Eur J Cancer* 2002; **38**: 527-534

30 **Tsigris C,** Karayiannakis AJ, Zbar A, Syrigos KN, Baibas N, Diamantis T, Alexiou D. Clinical significance of serum and urinary c-erbB-2 levels in colorectal cancer. *Cancer Lett* 2002; **184**: 215-222

31 **Maisey NR,** Norman A, Watson M, Allen MJ, Hill ME, Cunningham D. Baseline quality of life predicts survival in patients with advanced colorectal cancer. *Eur J Cancer* 2002; **38**: 1351-1357

32 **Stief CG,** Jonas U, Raab R. Long-term follow-up after surgery for advanced colorectal carcinoma involving the urogenital tract. *Eur Urol* 2002; **41**: 546-550

33 **Steup WH,** Moriya Y, van de Velde CJ. Patterns of lymphatic spread in rectal cancer. A topographical analysis on lymph node metastases. *Eur J Cancer* 2002; **38**: 911-918

34 **Wang JP,** Yang ZL, Wang L, Dong WG, Huang YH, Qin JZ, Zhan WH. Multi-variate regression analysis of clinicopathological characteristics and prognosis of colorectal cancer. *Zhonghua Zhongliu Zazhi* 2003; **25**: 59-61

35 **Walker J,** Quirke P. Prognosis and response to therapy in colorectal cancer. *Eur J Cancer* 2002; **38**: 880-886

36 **Gu J,** Ma ZL, Li Y, Li M, Xu GW. Angiography for diagnosis and treatment of colorectal cancer. *World J Gastroenterol* 2003; **9**: 288-290

37 **Roncucci L,** Fante R, Losi L, Di Gregorio C, Micheli A, Benatti P, Madenis N, Ganazzi D, Cassinadri MT, Lauriola P, Ponz de Leon M. Survival for colon and rectal cancer in a population-based cancer registry. *Eur J Cancer* 1996; **32A**: 295-302

38 **Galindo Gallego M,** Fernandez Acenero MJ, Sanz Ortega J, Aljama A. Vascular enumeration as a prognosticator for colorectal carcinoma. *Eur J Cancer* 2000; **36**: 55-60

39 **Massacesi C,** Norman A, Price T, Hill M, Ross P, Cunningham D. A clinical nomogram for predicting long-term survival in advanced colorectal cancer. *Eur J Cancer* 2000; **36**: 2044-2052

40 **Yi J,** Wang ZW, Cang H, Chen YY, Zhao R, Yu BM, Tang XM. p16 gene methylation in colorectal cancers associated with Duke's staging. *World J Gastroenterol* 2001; **7**: 722-725

41 **Weber JC,** Nakano H, Bachellier P, Oussoultzoglou E, Inoue K, Shimura H, Wolf P, Chenard-Neu MP, Jaeck D. Is a proliferation index of cancer cells a reliable prognostic factor after hepatectomy in patients with colorectal liver metastases? *Am J Surg* 2001; **182**: 81-88

42 **Lee JC,** Wang ST, Chow NH, Yang HB. Investigation of the prognostic value of coexpressed erbB family members for the survival of colorectal cancer patients after curative surgery. *Eur J Cancer* 2002; **38**: 1065-1071

43 **Bouzourene H,** Gervaz P, Cerottini JP, Benhattar J, Chaubert P, Saraga E, Pampallona S, Bosman FT, Givel JC. p53 and Ki-ras as prognostic factors for Dukes' stage B colorectal cancer. *Eur J Cancer* 2000; **36**: 1008-1015

44 **Zhou W,** Goodman SN, Galizia G, Lieto E, Ferraraccio F, Pignatelli C, Purdie CA, Piris J, Morris R, Harrison DJ, Paty PB, Culliford A, Romans KE, Montgomery EA, Choti MA, Kinzler KW, Vogelstein B. Counting alleles to predict recurrence of early-stage colorectal cancers. *Lancet* 2002; **359**: 219-225

45 **Lin LJ,** Zheng CQ, Jin Y, Ma Y, Jiang WG, Ma T. Expression of survivin protein in human colorectal carcinogenesis. *World J Gastroenterol* 2003; **9**: 974-977

46 **Zheng S,** Han MY, Xiao ZX, Peng JP, Dong Q. Clinical significance of vascular endothelial growth factor expression and neovascularization in colorectal carcinoma. *World J Gastroenterol* 2003; **9**: 1227-1230

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2211-2215
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

# Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products

Ying Ma, Shun-Quan Lin, Yi Gao, Mei Li, Wen-Xin Luo, Jun Zhang, Ning-Shao Xia

**Ying Ma, Yi Gao, Wen-Xin Luo, Jun Zhang, Ning-Shao Xia,** The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province, China
**Ying Ma,** Ministry of Education Key Laboratory for Marine Environmental Science, Center for Marine Environmental Science, Xiamen University, Xiamen 361005, Fujian Province, China
**Shun-Quan Lin,** College of Horticulture, South China Agricultural University, Guangzhou, 510642, Guangdong Province, China
**Mei Li,** Xiamen Overseas Chinese Subtropical Plant Introduction Garden, Xiamen 361002, Fujian Province, China
**Supported by** a grant from the Natural Science Foundation of Fujian Province, No. C9910004 and Xiamen Kaili Biologic Product Limited Company
**Correspondence to:** Ning-Shao Xia, The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, Xiamen University, Xiamen 361005, Fujian Province, China. nsxia@jingxian.xmu.edu.cn
**Telephone:** +86-592-2184110 **Fax:** +86-592-2184110
**Received:** 2003-03-12 **Accepted:** 2003-07-15

## Abstract

**AIM:** To transfer hepatitis E virus (HEV) ORF2 partial gene to tomato plants, to investigate its expression in transformants and the immunoactivity of expression products, and to explore the feasibility of developing a new type of plant-derived HEV oral vaccine.

**METHODS:** Plant binary expression vector p1301E2, carrying a fragment of HEV open reading frame-2 (named HEV-E2), was constructed by linking the fragment to a constitutive CaMV35s promoter and nos terminator, then directly introduced into *Agrobacterium tumefaciens* EHA105. With leaf-disc method, tomato plants medicated by EHA105 were transformed and hygromycin-resistant plantlets were obtained in selective medium containing hygromycin. The presence and integration of foreign DNA in transgenic tomato genome were confirmed by Gus gene expression, PCR amplification and Southern dot blotting. The immunoactivity of recombinant protein extracted from transformed plants was examined by enzyme-linked immunosorbant assay (ELISA) using a monoclonal antibody specifically against HEV. ELISA was also used to estimate the recombinant protein content in leaves and fruits of the transformants.

**RESULTS:** Seven positive lines of HEV-E2-transgenic tomato plants confirmed by PCR and Southern blotting were obtained and the immunoactivity of recombinant protein could be detected in extracts of transformants. The expression levels of recombinant protein were 61.22 ng/g fresh weight in fruits and 6.37-47.9 ng/g fresh weight in leaves of the transformants.

**CONCLUSION:** HEV-E2 gene was correctly expressed in transgenic tomatoes and the recombinant antigen derived from them has normal immunoactivity. Transgenic tomatoes may hold a good promise for producing a new type of low-cost oral vaccine for hepatitis E virus.

Ma Y, Lin SQ, Gao Y, Li M, Luo WX, Zhang J, Xia NS. Expression of ORF2 partial gene of hepatitis E virus in tomatoes and immunoactivity of expression products. *World J Gastroenterol* 2003; 9(10): 2211-2215
http://www.wjgnet.com/1007-9327/9/2211.asp

## INTRODUCTION

Research on using plants for expression and delivery of oral vaccine has attracted much academic attention and has become a hot spot of study since 1990 when Curtiss *et al*. first reported the expression of *Streptococcus mutans* surface protein antigen A(SpaA) in tobacco, and great progress has been made since then[1]. So far, more than 10 viral epitopes and subunits of bacterial toxins have been successfully expressed in plants, mainly including hepatitis B surface antigen (HBsAg)[2-9], *E.coli* heat-labile enterotoxin B subunit (LT-B)[10-15], cholera toxin B subunit (CT-B)[16], Norwalk virus capsid protein (NVCP)[17,18], rabies virus glycoprotein[19], *etc*. The involved plant receptors are mostly mode species: tobacco, potato, *etc*., some fruits and vegetables that can be consumed raw, tomato, banana, lettuce and lupine have also been attempted. Significant breakthrough has been achieved in increasing the expression of vaccine components in plants. At present, the plant-derived oral vaccines from potato for HBsAg and LT-B are undergoing clinical trials[13,14], but there has been no report about plant-derived oral vaccine for hepatitis E virus (HEV).

HEV is the major cause of acute, enteric non-A, non-B (NANB) hepatitis in the world, and large outbreaks occur primarily in underdeveloped countries. China is one of the high epidemic areas. The highest infection rate is in young adults (15-40 years). Although only 1 % to 3 % of non-pregnant patients with HEV infection progress to fatal fulminant hepatitis, the mortality can be as high as 20 % in pregnant patients[20]. There is neither effective antiviral drugs nor vaccine against HEV available for commercial use at present. Since HEV pathogen is difficult to culture, it is not easy to develop the live attenuated strains for vaccine. A promising approach is to develop recombinant subunit vaccines. Compared with gene engineering vaccine for hepatitis B, the study of hepatitis E recombinant vaccine was only a recent endeavor, but some progress has been made.

ORF2-encoded protein of HEV is the most promising subunit vaccine candidate because it possesses good antigenicity. So far, HEV ORF2 gene or its fragments have been expressed in prokaryote cells[21-24], insect cells[25], animal cells[26], and *Pichia pastoris*[27], *etc*., and the expression products possessed immunogenicity. HEV vaccine is about to undergo clinical trials[28].

Since hepatitis E occurs primarily in developing countries and impoverished regions with poor environmental sanitation, where administration of various medicinal vaccines may be hampered by the relatively high cost. Plant-derived hepatitis

E vaccine is a promising approach that may solve the problem because of its low cost for delivery and administration, and its safety for humans.

Tomato is a nutrition-rich fruit that can be consumed raw and easily transformed, so it is an ideal plant carrier for oral vaccine. In this paper we reported that a plant expression vector of HEV antigen gene was constructed and transformed into tomato plants with *Agrobacterium tumefaciens*, and its expression in plants and immunoactivity of the expression product were examined. This study would lay the foundation for further research on the development of a new type of plant-derived hepatitis E oral vaccine or other oral vaccines, and promote their practical application.

## MATERIALS AND METHODS

### *Plant material*

Tomato (*Lycopersium esculentum* CV. "XiuNu") seeds were purchased from Xiamen Nong-You Seed Co., Ltd.

### *Reagents, bacteria and plasmids*

Restriction endonucleases and T4 DNA ligase were obtained from Promega Co. Hygromycin and X-gluc staining solution from Calbiochem-novabiochem Co. and Amres Co., respectively. Double antibody sandwich-ELISA kit was provided by Beijing Wantai Biological Pharmaceutical Co. *Agrobacterium tumefaciens* strain EHA105 was kindly presented by Professor Zhang Qi-fa, Huazhong Agricultural University. Plasmid pBPFΩ7 containing CaMV35s promoter and nos terminator, and plant binary plasmid pCAMBIA1301 containing hygromycin-resistant gene, kanamycin-resistant gene and Gus gene, were constructed and preserved in our laboratory.

### *Construction of plant binary expression vector*

An 810 bp DNA fragment (named E2) of HEV ORF2 region, located between amino acid residue 394 and 604[23], was obtained by a PCR-based assembly from the patient's serum and inserted into pBPFΩ7 between CaMV35S promoter and nos terminator at *Bam*HI/*Eco*RI site to form pBE2. The fragment containing "P35S+Ω+E2+Tnos" was isolated by gel extraction from plasmid pBE2 after *Pst*I restrictive digestion and then subcloned into plasmid pCAMBIA1301 that had been digested by the same restriction endonuclease to yield the reconstructed plant binary expression plasmid p1301E2 (Figure 1). Confirmed by restriction digestion, p1301E2 was directly introduced into *Agrobacterium tumefaciens* strain EHA105 by freeze-thaw method.

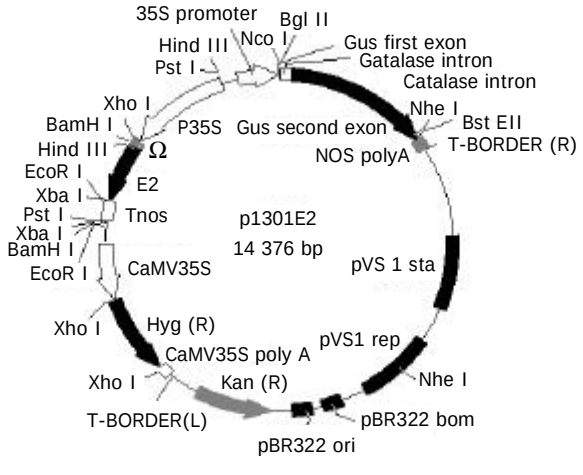



**Figure 1** Structure of plasmid p1301E2.

### *Plant transformation and regeneration*

Tomato was transformed through leaf discs mediated by *Agrobacterium tumefaciens* EHA105 with p1301E2. Shoots were generated from transformed callus after 3-4 weeks selected on medium containing 20 mg of hygromycin (Hyg) and 300 mg of cefotaxime per liter. The rooting was obtained in medium containing 20 mg of Hyg per liter, and the plantlets was transplanted to soil, and watered with 1/2 MS medium.

### *Analysis of Gus gene expression*

Both transformed and untransformed tissues were cut from tomato plants, immerged into Gus reaction buffer (X-gluc staining solution) for 12 to 24 hours at 37 ℃, then bleached with absolute alcohol, observed and photographed under dissecting microscope.

### *Analysis of HEV-E2 gene integration*

**PCR amplification** Genomic DNAs extracted from leaves of tomato plants by CTAB[29] were used as PCR templates. The forward primer HEFP and reverse primer HERP were: 5' -GGA TCC ATA TGC AGC TGT TCT ACT CTC GTC-3' and 5' -CTC GAG AAA TAA ACT ATA ACT CCC GA-3', respectively (synthesized by BioAsia Co., Shanghai). PCR reaction was performed using 50 ng of template DNA, 0.5 μM of each primer in a total volume of 30 μl. Cycling parameters were at 94 ℃ for 10 min, followed by 35 cycles at 94 ℃ for 50 s, at 57 ℃ for 50 s, and at 72 ℃ for 50 s, and a final extension at 72 ℃ for 7 min.

**Southern dot blotting** It was performed as reported previously[29].

### *Analysis of HEV-E2 gene expression*

**ELISA** Total soluble proteins were extracted from leaf and fruit tissues as described[29], and HEV-E2 recombinant protein was detected by HEV enzyme-linked immunosorbant assay (ELISA) kit, the protocol and positive determination were performed according to the instructions supplied with the kit. The expression levels of HEV-E2 in transformants were quantified by ELISA. The extract of transformant was diluted several fold until it could reach the same OD value (measurement wavelength: 450 nm) as the standard HEV-E2 protein (1 ng/mg), then HEV-E2 expression levels in transformants could be calculated according to the sampling quantity, and diluting times.

## RESULTS

### *Regeneration of transgenic tomato plants*

The plasmids for expression of HEV-E2 in plants allowed morphogenesis of transformants on selective medium containing hygromycin. Through about 1 month of selection, 7 independent Hyg-resistant plantlets were obtained. These transformants bore fruits and the seeds were collected. The flowers and fruits of the transformants resembled those of wild-type tomato plants and the seeds were plump. Each fruit produced approximately 70 seeds. This demonstrated that the introduction of foreign gene into tomato plants did not influence the normal growth and development of the transformants (Figure 2).

### *Gus expression in transgenic plants*

Untransformed tissues appeared colourless when stained with Gus reaction solution and bleached with absolute alcohol, whereas transformed tissues presented blue spots even after bleached with absolute alcohol, the spots were generally large in size, with a deep blue color (Figure 3). The results indicated that Gus gene was stably integrated into the genomic DNA of transformed tomatoes.

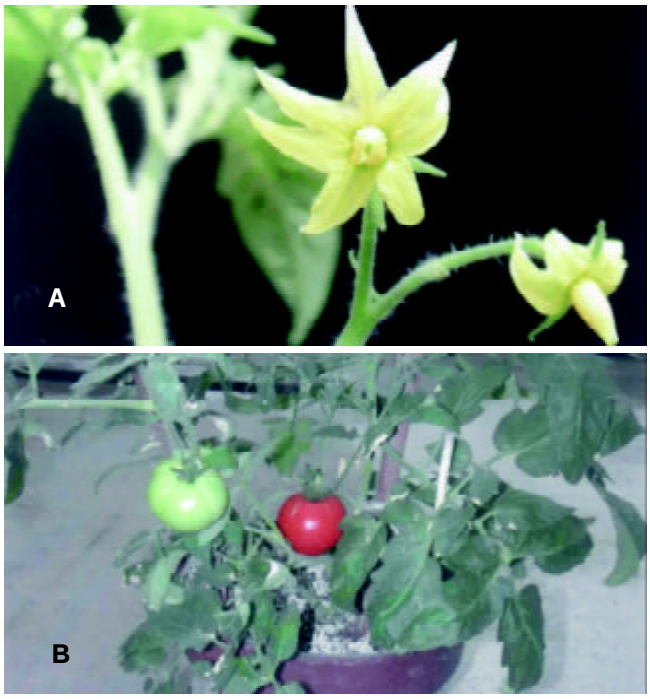



**Figure 2** Transgenic plants in greenhouse. A: Flowers of transformants, B: Fruits of transformants.

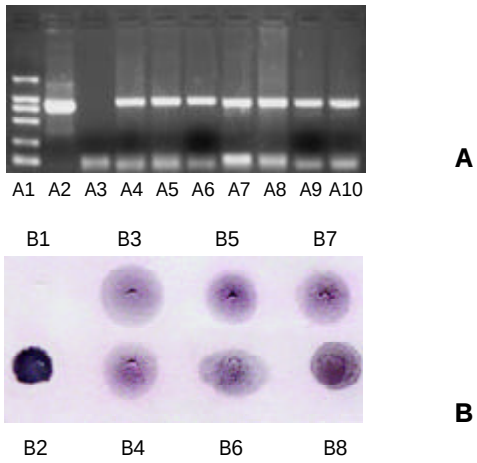



**Figure 4** Analysis of HEV-E2 gene integration. A: PCR amplification of genomic DNA of tomato plants, B: Southern dot blotting of genomic DNA of tomato plants. A1: DL-2000 marker, A2: Positive control (p1301E2), A3: Total DNA of wild-type control plant, A4-A10: Total DNA of independent transformants. B1: Total DNA of wild-type control plant, B2: Positive control (p1301E2), B3-B8: Total DNA of independent transformants.

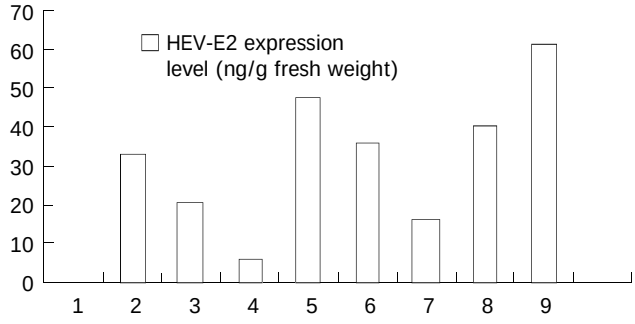



**Figure 5** HEV-E2 expression levels in transgenic tomato plants. 1: Leaves of wild-type control plant, 2-8: Leaves of seven independent transformants, 9: The mixed flesh tissue of 3 independent transformant fruits.

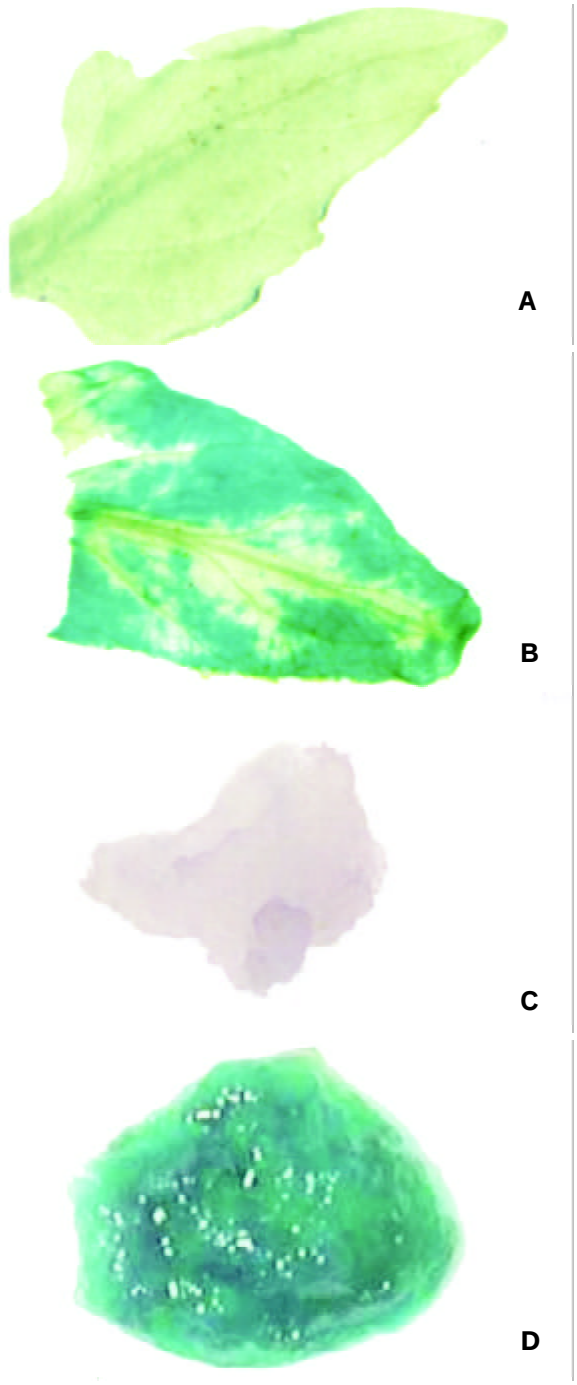



**Figure 3** Analysis of Gus gene expression. A: Leaf of untransformed tomato plant, B: Leaf of transformed tomato plant, C: Flesh of untransformed tomato, D: Flesh of transformed tomato.

### *PCR amplification*

The expected 810 bp fragments were amplified from 7 transformed lines of tomatoes, the length of the fragments was identical with that amplified from p1301E2 (positive control), whereas there was no PCR product in wild-type tomato plants (negative control). The results primarily verified that the target gene was integrated into the genomic DNA of transformed tomatoes (Figure 4A).

### *Southern dot blotting*

To further verify the integration of foreign gene into tomato plants, the total genomic DNA of transformed tomatoes was hybridized by a DIG-labeled probe (encompassing the coding region of HEV-E2 gene) generated by PCR amplification from p1301E2 with HEFP and HERP primers, and all the

transformants gave the same hybridization spots as the positive control(p1301E2) did, whereas the untransformed plant showed no detectable hybridization signal (Figure 4B). Thus the results further confirmed that the target gene was integrated into the tomato plants.

### *Expression of HEV-E2 gene in transgenic tomato plants*

The leaf extracts of 7 lines of transformants and the mixed fruit tissue extracts from 3 transformants were tested by ELISA for the presence of HEV-E2 expression products, and foreign protein could be detected in all of the examined samples. The reaction was specific because wild-type tomato showed no detectable expression products. The result demonstrated that HEV-E2 gene was expressed in transformed tissue. The expression level of HEV-E2 protein was lower as compared to HBsAg in transgenic tomatoes[8], the maximal expression level was only 47.9 ng/g fresh weight in leaves. The expression levels were different between different transformants and between different organs of the same plant, which indicated that the inserting site of foreign DNA into the plant genome was random. The expression in fruits (61.22 ng/g fresh weight) was higher than that in leaves, which was similar to that of HBsAg in transgenic tomatoes[8]. The higher expression of target protein in edible tissue mightbe helpful for producing oral vaccine.

## DISCUSSION

The most striking advantage of using tomato as oral vaccine vector is that the plant-derived vaccine is cheap and tomato is a freshly-eaten fruit, which allows the target population to acquire immunity at the same time when they enjoy the delicious fruits. But accumulation of protein in tomato itself is low, the expression levels of foreign protein in it are much lower. This will make it difficult when administering the plant-derived vaccine. However, the expression of foreign protein in plants might be increased by several modifications, including the use of stronger promoters, the use of plant-derived leader sequences and signal peptide, and targeting the protein for retention in edible tissue and so on[1]. For example, Mason *et al*[2] increased the level of HBsAg 11 fold in transgenic tobacco by linking CaMV35S promoter to the tobacco etch virus (TEV) 5' leader sequence (acts as a translational enhancer). In 1998, Mason *et al*[12] increased the expression of LT-B in transgenic potatoes 3-14 times by designing and constructing a plant-optimized synthetic gene encoding LT-B. Tackaberry *et al*[30] reported the synthesis of recombinant glycoprotein B which expressed specifically in tobacco seeds, with expression level reaching 70-146 ng/mg extracted protein. Lauterslager *et al*[11] made a synthetic gene coding for LT-B and optimized it for expression specifically in potato tubers and accumulation in endoplasmic reticulum, which resulted in a high expression level about 13 μg/g fresh weight of LT-B in potato tubers. Besides, by using chloroplast expression system, Cosa *et al*[31] introduced foreign genes into the chromoplast genome, which enabled the foreign protein to accumulate at 46 % of the total soluble protein in leaves of transgenic plants. All these studies provided us successful experiences in improving expression level of HEV-E2 in transgenic tomatoes. In our study, we only inserted an enhancer Ω in the promoter, there were many reconstruction possibilities to achieve high expression. Currently we are engaged in researches on increasing expression level of foreign proteins, and the animal trials of oral immunization of mice with transgenic tomatoes expressing HEV-E2 are also in progress.

We have successfully introduced HEV-E2 gene into tomatoes and identified the expression protein. The expression product possessed HEV specific antigenicity in transgenic plants. Previous studies also demonstrated that plant-derived vaccines were safe and functional. It is cheap to produce and store, easy to deliver and administer, it has many advantages over other vaccines. Although this technology is not likely to produce great commercial value in the short term, the present advance in plant genetic engineering demonstrates that there is a tremendous potential to develop various low-cost recombinant vaccines by using plants.

## REFERENCES


1 **Ma Y,** Zhang J, Lin SQ, Xia NS. Genetic Engineering vaccines produced by transgenic plants. *Xiamen Daxue Xuebao* 2001; **40:** S71-77

2 **Mason HS,** Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. *Proc Natl Acad Sci U S A* 1992; **89:** 11745-11749

3 **Thanavala Y,** Yang YF, Lyons P, Mason HS, Arntzen C. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. *Proc Natl Acad Sci U S A* 1995; **92:** 3358–3361

4 **Kong Q,** Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc Natl Acad Sci U S A* 2001; **98:** 11539–11544

5 **Kapusta J,** Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki AB. Plant-derived edible vaccine against HBV. *FASEB J* 1999; **13:** 1796-1799

6 **Liu DH.** Plant as a system for production of pharmaceutical proteins. *Shengwu Jishu Tongbao* 1999; **4:** 1-5

7 **Zhao CH,** Wang R, Zhao CS, Wang GL, Tian B. Expression of human hepatitis B virus surface antigen gene with and without preS in transgenic tomato. *Nongye Shengwu Jishu Xuebao* 2000; **8:** 85-88

8 **Ma Y,** Lin SQ, Gao Y, Zhang J, Lu LX, Xia NS. Transformation of HBsAg (hepatitis B virus surface antigen) into tomato plants. *Fujian Nonglin Daxue Xuebao* 2002; **31:** 223-227

9 **Gao Y,** Ma Y, Li M, Cheng T, Li SW, Zhang J, Xia NS. Oral immunization of animals with transgenic cherry tomatillo expressing HBsAg. *World J Gastroenterol* 2003; **9:** 996-1002

10 **Haq TA,** Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 1995; **268:** 714–716

11 **Lauterslager TG,** Florack DE, van der Wal TJ, Molthoff JW, Langeveld JP, Bosch D, Boersma WJ, Hilgers LA. Oral immunization of naive and primed animals with transgenic potato tubers expressing LT-B. *Vaccine* 2001; **19:** 2749-2755

12 **Mason HS,** Haq TA, Clements JD, Artzen CJ. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 1998; **16:** 1336–1343

13 **Tacket CO,** Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat Med* 1998; **4:** 607-609

14 **Tacket CO,** Reid RH, Boedeker EC, Losonsky G, Nataro JP, Bhagat H, Edelman R. Enteral immunization and challenge of volunteers given enterotoxigenic *E. coli* CFA / II encapsulated in biodegradable microspheres. *Vaccine* 1994; **12:** 1270-1274

15 **Streatfield SJ,** Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA. Plant-based vaccines: unique advantages. *Vaccine* 2001; **19:** 2742-2748

16 **Arakawa T,** Chong DK, Langridge WH. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat Biotechnol* 1998; **16:** 292-297

17 **Mason HS,** Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc Natl Acad Sci U S A* 1996; **93:** 5335-5340

18 **Tacket CO,** Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. *J Infect Dis* 2000; **182:** 302-305

19 **McGarvey PB,** Hammond J, Dienelt MM, Hooper DC, Fu ZF,

Dietzschold B, Koprowski H, Michaels FH. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biotechnology* 1995; **13:** 1484-1487

20 **Skidmore S**. Overview of Hepatitis E Virus. *Curr Infect Dis Rep* 2002; **4**: 118-123

21 **Zhang M**, Zhao H, Jiang Y. Expression of hepatitis E virus structural gene in E. coli. *Zhonghua Shiyan He Linchuangbing Duxue Zazhi* 1999; **13**: 130-132

22 **Bi S,** Lu J, Jiang L, Huang G, Pan H, Jiang Y, Zhang M, Shen X. Preliminary evidence that a hepatitis E virus (HEV) ORF2 recombinant protein protects cynomolgus macaques against challenge with wild-type HEV. *Zhonghua Shiyan He Linchuangbing Duxue Zazhi* 2002; **16:** 31-32

23 **Li SW**, Zhang J, He ZQ, Ge SX, Gu Y, Lin J, Liu RS, Xia NS. The study of aggregate of the ORF2 peptide of hepatitis E virus expressed in Escherichia coli. *Shengwu Gongcheng Xuebao* 2002; **18**: 463-467

24 **Zhang M**, Yi Y, Zhan M, Liu C, Bi S. Expression of thermal stable, soluble hepatitis E virus recombinant antigen. *Zhonghua Shiyan He Linchuangbing Duxue Zazhi* 2002; **16**: 20-22

25 **Zhang M**, Emerson SU, Nguyen H, Engle R, Govindarajan S, Blackwelder WC, Gerin J, Purcell RH. Recombinant vaccine against hepatitis E: duration of protective immunity in rhesus macaques. *Vaccine* 2002; **20**: 3285-3291

26 **Jameel S,** Zafrullah M, Ozdener MH, Panda SK. Expression in animal cells and characterization of the hepatitis E virus structural proteins. *J Virol* 1996; **70:** 207-216

27 **Tong Y,** Zhan M, Lu J, Bai Y, Bi S. Immunogenicity of recombinant HEV ORF2 protein expressed in pichia pastoris. *Zhonghua Shiyan He Linchuangbing Duxue Zazhi* 2002; **16:** 23-26

28 **Ling X.** The development of hepatitis E vaccine in China. *Zhonghua Yixue Zazhi* 2001; **81:** 120

29 **Wang GL,** Fang HJ. Principle and technology of plant genetic engineering, 1st ed, Beijing: *Science Press* 1998

30 **Tackaberry ES,** Dudani AK, Prior F, Tocchi M, Sardana R, Altosaar I, Ganz PR. Development of biopharmaceuticals in plant expression systems: cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. *Vaccine* 1999; **17:** 3020-3029

31 **De Cosa B,** Moar W, Lee SB, Miller M, Daniell H. Overex pression of the Bt Cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nat Biotechnol* 2001; **19:** 71-74

**Edited by** Zhu LH

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2216-2220
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

# Lethiferous effects of a recombinant vector carrying thymidine kinase suicide gene on 2.2.15 cells via a self-modulating mechanism

Quan-Cheng Kan, Zu-Jiang Yu, Yan-Chang Lei, Lian-Jie Hao, Dong-Liang Yang

**Quan-Cheng Kan, Yan-Chang Lei, Lian-Jie Hao, Dong-Liang Yang,** Division of Clinical Immunology, Tongji Hospital; Institute of Immunolgy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
**Zu-Jiang Yu,** Department of Infectious Diseases, First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China
**Supported by** the State Key Basic Research Program of China (973 Program, No. 20014CB51008 ) and National Key R & D Program of China for the 10th Five-Year Plan Period (No. 2001BA705B05)
**Correspondence to:** Dr. Dong-Liang Yang, Division of Clinical Immunology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095# Jiefang Avenue, Wuhan 430030, Hubei Province, China. dlyang@tjh.tjmu.edu.cn
**Telephone:** +86-27-83662894
**Received:** 2003-07-18 **Accepted:** 2003-08-07

## Abstract

**AIM:** To determine the lethiferous effects of a recombinant vector carrying thymidine kinase (TK) suicide gene on 2.2.15 cells and the possible self-modulating mechanism.

**METHODS:** A self-modulated expressive plasmid pcDNA3-SCITK was constructed by inserting the fragments carrying hepatitis B virus antisense-S (HBV-anti-S) gene, hepatitis C virus core (HCV-C) gene, internal ribosome entry site (IRES) element of HCV and TK gene into the eukaryotic vector pcDNA3, in which the expression of TK suicide gene was controlled by the HBV S gene transcription. 2.2.15 cells that carry the full HBV genome and stably express series of HBV antigen were transfected with $pcDNA_3$-SCITK or vector $pcDNA_3$-SCI which was used as the mock plasmid. The HepG2 cells transfected with $pcDNA_3$-SCITK were functioned as the negative control. All the transfected cells were incubated in DMEM medium supplemented with 10 μg/ml. of ganciclovir (GCV). The HBsAg levels in the supernatant of cell culture were detected by ELISA on the 1st, 3rd and 6th day post-transfection. Meanwhile, the morphology of tranfected cells was recorded by the photograph and the survival cell ratio was assessed by the trypan blue exclusion test on the 6th day post-transfection.

**RESULTS:** The structural accuracy of $pcDNA_3$-SCITK was confirmed by restriction endonuclease digestion, PCR with specific primers and DNA sequencing. The HBsAg levels in the supernatant of transfected 2.2.15 cell culture were significantly decreased on the 6th day post-transfection as compared with that of the mock control ($P<0.05$). The lethiferous effect of $pcDNA_3$-SCITK expression on 2.2.15 cells was initially noted on the 3rd day after transfection and aggravated on the 6th day post transfection, in which the majority of transfected 2.2.15 cells were observed shrunken, round in shape and even dead. With assessment by the trypan blue exclusion test, the survival cell ratio on the 6th day post transfection was 95 % in the negative control and only 11 % in the experimental group.

**CONCLUSION:** The results indicate that suicide gene expression of $pcDNA_3$-SCITK can only respond to HBV-S gene transcription, which may be potentially useful in the treatment of HBV infection and its related liver malignancies.

Kan QC, Yu ZJ, Lei YC, Hao LJ, Yang DL. Lethiferous effects of a recombinant vector carrying thymidine kinase suicide gene on 2.2.15 cells via a self-modulating mechanism. *World J Gastroenterol* 2003; 9(10): 2216-2220
http://www.wjgnet.com/1007-9327/9/2216.asp

## INTRODUCTION

Despite the availability of efficient vaccines, chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide[1-7]. The World Health Organization estimates that there are still 350 million chronic carriers of the virus who are at risk of developing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma[8, 9]. This issue is also an important problem in China. The carrier rates of HBsAg are estimated to be as high as more than 10 % of Chinese population and a substantial proportion of liver cancer incidence is associated with chronic HBV infection[10-12]. Currently interferon-alpha (IFN-α), an immunomodulator, and two synthetic nucleoside analogues, lamivudine and adefovir dipivoxil, are the only licensed antiviral agents for the treatment of chronic HBV infection. However, this therapeutical modality is still problematic. In addition to the standard treatment endpoints being achievable only in approximately 20-30 % of those treated with either type of agents, interferon usually confers serious side effects, and lamivudine is associated with the occurrence of viral mutations and drug-resistant strains[13-17]. Thus, there is an urgent need for the development of more effective modalities to deal with HBV infection. As yet, several therapeutic protocols with molecular biological techniques have been tried in experimental studies of this disease in the last few years[1,18-25], although significant progress remains to be made in such kinds of explorations. In the present study, we tried to construct a recombinant vector carrying TK suicide gene and harboring a self-modulating property for gene therapy in an attempt to vanquish HBV infection in a fire-new strategy.

## MATERIALS AND METHODS

### *Materials*

Plasmid $pcDNA_3$-TK containing TK gene and $pcDNA_3$-S carrying HBV-S gene were maintained in our laboratory. Eukaryotic expression vector $pcDNA_3$ was from Invitrogen Company, Netherlands. Purification kit for PCR product was obtained from QIAGEN Company (Germany). Reverse transcriptase and PCR amplification system were purchased from Shenzhen Jingmei Company (China) and Expand PCR kit form Stratgene Company (USA). Taq and Pfu enzymes were purchased from Bio-Star Company (Canada). Restriction endonucleases such as EcoR I, BamH I and HinD III, $T_4$ DNA ligase and ELISA kit for detecting HBsAg were from Huamei Bioengineering Company (Luoyang,

China). All the PCR primers used in the study were synthesized by Takara Ltd (Dalian, China).

### *Preparation of gene fragments*

The fragments of HCV-IRES element and core gene were obtained by RT-PCR. Total HCV RNA was extracted and purified from 100 μl samples of HCV-positive sera using a guanidium isothiocyanate-acid phenol extraction procedure (Jingmei Company, Shenzhen, China). Precipitated RNA was dissolved in 20 μl of diethyl pyrocarbonate-treated water and quantified by UV spectrum analysis. Specific cDNA for HCV IRES element and core gene were amplified respectively with RT-PCR kit by adding their corresponding primers according to the protocols of Reverse Transcription System (Jingmei Company, Shenzhen, China). The primers for the HCV IRES were sequenced as sense: 5' -GCGCGGATCCGGGCGACAC TCCACCATAG-3' (nucleotides [nt] 17 to 36) and antisense: 5' -GCGAATTCGTTTTTCTTTGAGGTTTAGGATTC-3' (nt 347 to 371). The primers for the HCV core gene were 5' -TAGGAATCCTGATGAGCACGAATCCTAAACCTC-3' (sense) and 5' -GCGCGGATCCTTAAGCGGAAGCTGGGA TG-3' (antisense). PCR was performed on the Real-time PCR apparatus (Roche, USA) with a program for 30 cycles at 42 ℃ for 45 min and at 94 ℃ for 5 min followed by at 92 ℃ for 30 s, at 55 ℃ for 30 s and at 72 ℃ for 30 s. After prepared by routine procedures, the PCR products were analyzed by agarose gel electrophoresis and stored at -20 ℃ until use.

The antisense fragment of HBV-S gene lack of initiating codon (ATG) was amplified with the PCR protocol using the oligonucleotide sequence 5' -GCGCGTGCAAGCTT ATAAAACGCCGCAGACACATC-3' (sense) and 5' -ATTCGTGCTCATCAGGATTCCTAGGACCCCTTC-3' (antisense) as primers, and $pcDNA_3$-S as the template. After an initial denaturation step was performed, PCR was carried out for 45 cycles at 95 ℃ for 15 s, at 70 ℃ for 5 s and at 72 ℃ for 15 s each cycle. The PCR products were identified by electrophoresis on an l % of agarose gel stained with ethidium bromide and stored at -20 ℃ until use.

TK gene segment was prepared by digestion of plasmid $pcDNA_3$-TK with EcoR I and purified with the routine procedures.

### *Construction of self-modulated expression vector*

The construction scheme for the self-modulated expression vector that can respond to HBV infection and eliminate the infected hepatic cells is shown in Figure 1. Briefly, ligation of antisense fragment of HBV-S gene with HCV-C gene segment was performed by SOEing PCR[26-28], in which the sense sequence of HCV-C gene primer 5' -TAGGAATCCTGATGA GCACGAATCCTAAACCT**C**-3' and a truncated antisense sequence of HBV-S gene primer 5' -ATTCGTGCTCATCA GGATTCCTAGGACCCC-3' were used as primers. The major SOEing PCR round comprised of 30 cycles at 92 ℃ for 30 s, at 50 ℃ for 45 s and at 72 ℃ for 60 s each cycle, and a final extension at 72 ℃ for 10 min. The ligated PCR products were purified and analyzed by agarose gel electrophoresis and used to link with the HCV-IRES fragment by $T_4$ ligase after both segments were digested by BamH I. The resultant HBV antisense S-HCV C-HCV IRES (SCI) fragment was further amplified by PCR with the major round to be at 92 ℃ for 30 s, at 53 ℃ for 45 s and at 72 ℃ for 60 s for 30 cycles, and the final extension at 72 ℃ for 10 min, in which HBV-S gene sense primer, 5' -GCGCGTGCAAGCTTATAAAACGCC GCAGACACATC-3, and HCV-IRES antisense primer, 5' -GCGAATTCGTTTTTCTTTGAGGTTTAGGATTC-3' , were employed as primers. After digested by corresponding restriction endonucleases, the amplified HBV antisense S-HCV C-HCV IRES fragment was cloned into Hind III and EcoR I sites of $pcDNA_3$ to generate eukaryotic expression plasmid pcDNA3-SCI (Figure 1). In the same way, the expression plasmid $pcDNA_3$-SCITK was yielded by cloning TK fragment into EcoR I site of $pcDNA_3$-SCI. Both of the newly constructed plasmid were confirmed by restriction endonuclease digestion, PCR with specific primers and finally by DNA sequencing (Baosheng Company, Daliang, China).

### *Self-restricted expression of recombinant plasmid*

HepG2 and its derived cell line 2.2.15 that carries the full HBV genome and can stably express series of HBV antigen were maintained in our laboratory. They were cultured in a modified Eagal' s essential medium (Sigima) supplemented with 100 $U \cdot ml^{-1}$ penicillin, 100 $U \cdot ml^{-1}$ streptomycin, and L-glutamine, nonessential amino acids and sodium bicarbonate

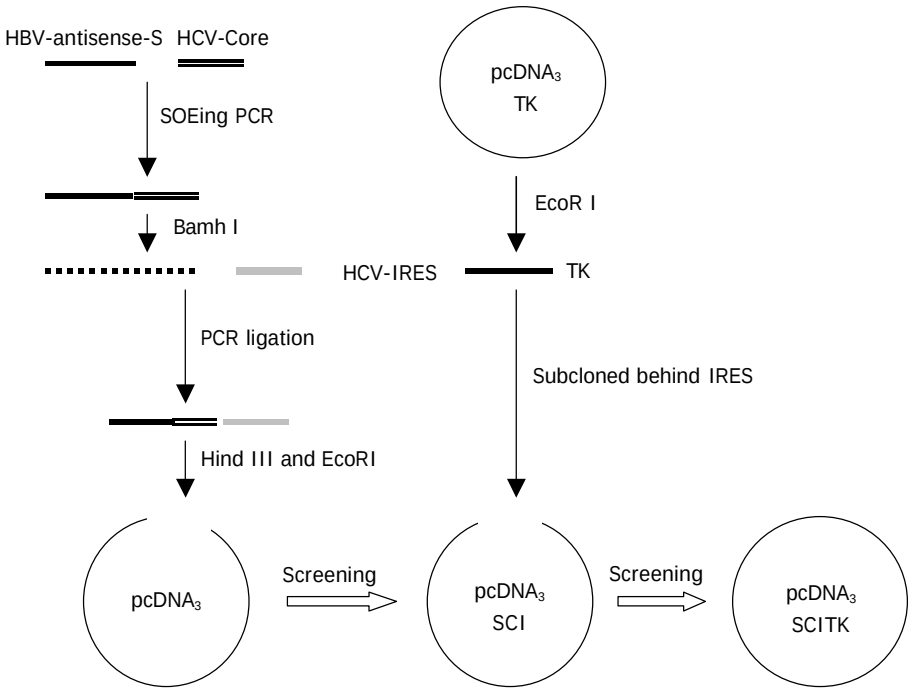



**Figure 1** Construction scheme of recombinant plasmid $pcDNA_3$-SCITK.

in doses recommended by the China Type Culture Collection (Wuhan, China) at 37 ℃ in a humidified atmosphere containing 5 % of $CO_2$. One day prior to transfection, cells were transferred onto an 8-well plate and incubated in complete Dulbecco' s modified Eagle' s medium (DMEM) containing 10 % (v/v) fetal calf serum (FCS) and 10 μg/ml of ganciclovir. When the cells grew to 50-80 % confluence, they were transfected with $pcDNA_3$-SCITK or pcDNA3-SCI plasmid in a ratio of per $10^6$ cells to 1 μg of plasmid DNA by LipofectAMINE transfection reagent following the manufacturer' s instructions. (Invitrogen Co. USA). 2.2.15 cells transfected with $pcDNA_3$-SCITK or $pcDNA_3$-SCI plasmids were designated as the experimental group or the mock group. The HepG2 cells transfected with $pcDNA_3$-SCITK plasmids were functioned as the negative control. All of the transfected cells were maintained in DMEM medium with 10 % (v/v) fetal calf serum (FCS) and 10 μg/ml of ganciclovir for at least 7 days. The HBsAg levels in the supernatant of cell culture were detected by ELISA on the 1st, 3rd and 6th day after transfection. Meanwhile, the morphology of transfected cells were recorded by the photograph and the survival cell ratio was assessed by the trypan blue exclusion test on the 6th day of posttransfection.

### *Statistical analysis*

Experimental data were processed by analysis of variance and *t*-test for comparison between groups. Results were expressed as mean ± SE. $P<0.05$ was selected as the level of statistical significance.

## RESULTS

### *Structural identification of constructed plasmids*

The segment analysis of recombinant plasmid $pcDNA_3$-SCITK by restriction endonuclease digestion and PCR with specific primers demonstrated that the inserted gene sequences in the plasmid were completely consistent with that of the theoretical

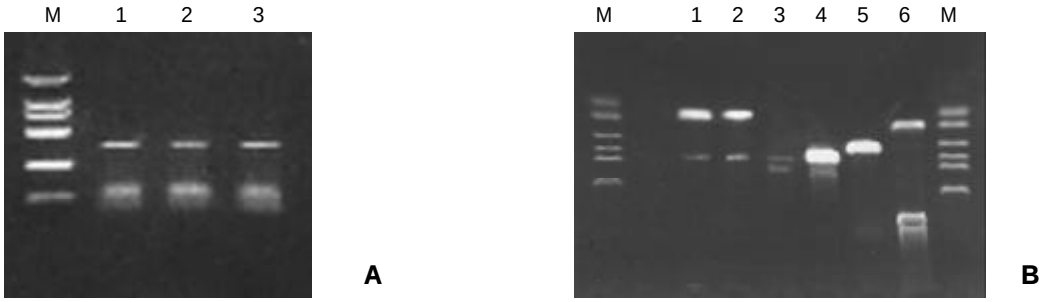
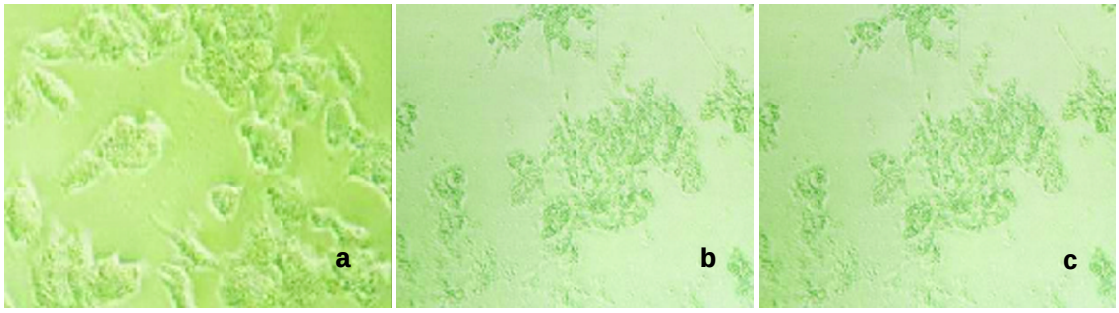
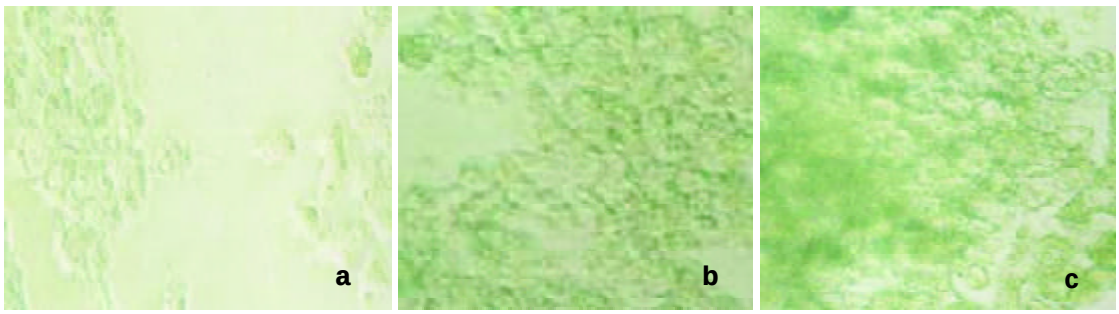



**Figure 2** A: RT-PCR products of HCV IRES element. M: marker (2 kb), Lanes 1-3: HCV IRES element (330 bp). B. Component segment analysis of $pcDNA_3$-SCITK by agarose gel electrophoresis. M: marker (2 kb), Lanes 1-2: SCI-TK fragment demonstrated by PCR with HBV-S gene sense primer and TK antisense primer (5'-ACTTCCGTGGCTTCTTGCTG-3' (nt 150-170). Lane 3: SCI fragment verified by PCR with HBV-S gene sense primer and HCV IRES antisense primer. Lane 4: SI segment from the ligation of HBV-S and HCV-Core gene by SOEing PCR. Lane 5: RT-PCR product of HCV-Core gene. Lane 6: Partial antisense segment of HBV-S gene.




A. Experimental group.




B. Negative control.

**Figure 3** Effects of $pcDNA_3$-SCITK expression on morphological alterations of 2.2.15 and HepG2 cells. Photographs a, b and c in each group exhibited respectively the cell changes on the 1st, 3rd and 6th day post transfection. Lethiferous changes of 2.2.15 cells transfected with $pcDNA_3$-SCITK were noted initially on the 3rd day of posttransfection and aggravated on the 6th day after transfection, in which the majority of cells were observed shrunken, rounded in shape and even dead. However, the negative contrl HepG2 cells grew well.

calculations as shown in Figure 2, which were further confirmed by the DNA sequencing (data not shown).

### *Effects of pcDNA3-SCITK expression on HBV-infected cells*

The expressive efficiency of TK protein by 2.2.15 cells transfected with pcDNA$_3$-SCITK was confirmed in our previous investigations (data to be published). In the present study, the effects of pcDNA$_3$-SCITK transfection on the expression of HBsAg by 2.2.15 cells was observed. The results showed that HBsAg in the supernatant of transfected 2.2.15 cell culture was significantly decreased on the 6$^{th}$ day post-transfection as compared with that of the mock group ($P$<0.05, Table 1).

**Table 1** HBsAg expression in supernants of cell culture by experimental cells

| Groups | OD Value | | |
|---|---|---|---|
| | 1$^{st}$ day | 3$^{rd}$ day | 6$^{th}$ day |
| Mock group | 0.32±0.13 | 0.61±0.23 | 0.61±0.25 |
| Experimental group | 0.31±0.11 | 0.51±0.22 | 0.41±0.16[a] |

[a]$P$<0.05 *vs* Blank control.

The lethiferous effect of pcDNA$_3$-SCITK expression on 2.2.15 cells was first noted on the 3$^{rd}$ day after transfection and aggravated on the 6$^{th}$ day posttransfection, in which the majority of transfected 2.2.15 cells were observed shrunken, round in shape and even dead (Figure 3). With assessment by the trypan blue exclusion test, the survival cell ratio on the 6$^{th}$ day posttransfection was 95 % in the negative control and only 11 % in the experimental group.

## DISCUSSION

Chronic hepatitis B infection remains a major public health problem worldwide. Hepatitis B virus belongs to the family of hepadnaviruses that replicate their DNA genome via a reverse transcription pathway. The chronicity of infection in infected hepatocytes is maintained by the persistence of the viral covalently closed circular DNA. Traditionally, the strategy to combat chronic HBV infection depends mainly on the stimulation of specific antiviral immune response or on the inhibition of viral replication, or on both. However, it has been found that prolonged administration of therapeutic agents is often associated with the production of resistant mutants and serious side effects, as well as a control of viral replication rather than eradication. To further search for effective therapeutic modalities to deal with the chronic infection of HBV is therefore urgently needed to design a brand-new strategy to clear the causative virus.

Hepatitis C virus, a member of the Flaviviridae family and an enveloped virus with a single-stranded 9.6 kb RNA genome of positive polarity[29], utilizes a cap-independent mechanism to initiate translation on its genomic RNAs. This process could involve an internal ribosome entry sites (IRES) located in the 5' nontranslated RNA (5'-NTR), and both canonical and noncanonical translation initiation factors[30-32]. The former could be constructed in some of the bicistronic systems with a higher efficiency in the initiation of protein translation and has been used to construct recombinant vectors[33-36]. It has been recently found that HCV-C protein, another functional modulator localized both in cytoplasm and in perinuclear regions, could interact with IRES and down-regulate IRES-directed translation[37-42], which has been considered to be involved in the establishment or maintenance of the virus persistence[40-43]. Based on these observations, we hypothesized that a self-modulating mechanism by the interaction of HCV-C protein with IRES element was possible to be employed in gene therapy for the eradication of chronic HBV infection.

In the present study, we constructed a recombinant plasmid pcDNA3-SCITK by cloning HCV-C gene and IRES element, partial antisense sequence of HBV-S gene, and TK suicide gene into the eukaryotic expression vector pcDNA3, in which the expression of TK gene was driven in turn by human cytomegalovirus (CMV) promoter, HBV-anti-S and HCV core-IRES sequences. The prominent feature of this plasmid was the temperate expression of effector gene in transfected cells by a self-modulating mechanism. When HBV in the infected cells began to replicate, the transcribed mRNA of HBV-S gene could be combined with its counterpart produced by HBV-antisense-S gene in the recombinant plasmid to form a mRNA dimmer prior to the initiation codon AUG of HCV-C gene, which brought about a decreased expression of HCV-C protein. The consequently reduced HCV core protein-IRES combination could enhance the IRES element to promote the expression of TK suicide gene and further to cause a lethiferous effect on HBV infected cells. In contrast, the expression of effector gene TK was inhibited in non-infected cells because of the normal level of HCV-C protein expression and its normal combination with HCV-IRES element, which would not cause any damage to the healthy cells.

In order to identify the anti-HBV efficiency of pcDNA3-SCITK, 2.2.15 cells that carry the full HBV genome and can stably express series of HBV antigen were transfected with the plasmid. The results showed that HBsAg level in the supernatant of transfected 2.2.15 cell culture was significantly decreased on the 6$^{th}$ day post transfection as compared with that of the mock cells (Table 1). The lethiferous effect of pcDNA$_3$-SCITK expression on 2.2.15 cells was noted initially on the 3$^{rd}$ day after transfection and aggravated on the 6$^{th}$ day posttransfection, in which the majority of transfected 2.2.15 cells were observed shrunken, round in shape and even dead. However, the negative control HepG2 cells grew well (Figure 3). With assessment by the trypan blue exclusion test, the survival cell ratio on the 6$^{th}$ day post transfection was 95 % in the negative control and only 11 % in the experimental group. All these observations confirmed that plasmid pcDNA3-SCITK could exert a lethiferous effect on HBV infected cells via a self-modulating mechanism, which might also be served as a potential therapeutic strategy for the liver cancers derived from chronic HBV infection.

The limitation of the present study is the neglect of anti-HBV infection by preventing the pathogen from invading target cells or by interrupting the virus replication at the beginning of the disease. How to apply our present therapeutic strategy in combination with other treatment protocols to the eradication of chronic HBV infection is to be further investigated.

## REFERENCES


1 **Xu R,** Cai K, Zheng D, Ma H, Xu S, Fan ST. Molecular therapeutics of HBV. *Curr Gene Ther* 2003; **3:** 341-355

2 **Morrissey DV,** Lee PA, Johnson DA, Overly SL, McSwiggen JA, Beigelman L, Mokler VR, Maloney L, Vargeese C, Bowman K, O'Brien JT, Shaffer CS, Conrad A, Schmid P, Morrey JD, Macejak DG, Pavco PA, Blatt LM. Characterization of nuclease-resistant ribozymes directed against hepatitis B virus RNA. *J Viral Hepat* 2002; **9:** 411-418

3 **Gumina G,** Song GY, Chu CK. Advances in antiviral agents for hepatitis B virus. *Antivir Chem Chemother* 2001; **12**(Suppl 1): 93-117

4 **Feng Y,** Kong YY, Wang Y, Qi GR. Intracellular inhibition of the replication of hepatitis B virus by hammerhead ribozymes. *J Gastroenterol Hepatol* 2001; **16:** 1125-1130

5 **Staschke KA,** Colacino JM. Drug discovery and development of antiviral agents for the treatment of chronic hepatitis B virus infection. *Prog Drug Res* 2001; **Spec No:** 111-183

6 **Harrison GL,** Murray-McIntosh R, Penny D. Hepatitis B virus genotypes: a South Pacific perspective. *Pac Health Dialog* 2001; **8:** 188-192
7 **Zoulim F.** Therapy of chronic hepatitis B virus infection: inhibition of the viral polymerase and other antiviral strategies. *Antiviral Res* 1999; **44:** 1-30
8 **Merle P,** Trepo C, Zoulim F. Current management strategies for hepatitis B in the elderly. *Drugs Aging* 2001; **18:** 725-735
9 **Bernardi M,** Biselli M, Gramenzi A. Chronic hepatitis B. Recent advances in diagnosis and treatment. *Recenti Prog Med* 2002; **93:** 397-402
10 **Fang JN,** Jin CJ, Cui LH, Quan ZY, Choi BY, Ki M, Park HB. A comparative study on serologic profiles of virus hepatitis B. *World J Gastroenterol* 2001; **7:** 107-110
11 **Rabe C,** Pilz T, Klostermann C, Berna M, Schild HH, Sauerbruch T, Caselmann WH. Clinical characteristics and outcome of a cohort of 101 patients with hepatocellular carcinoma. *World J Gastroenterol* 2001; **7:** 208-215
12 **Roussos A,** Goritsas C, Pappas T, Spanaki M, Papadaki P, Ferti A. Prevalence of hepatitis B and C markers among refugees in Athens. *World J Gastroenterol* 2003; **9:** 993-995
13 **Liaw YF.** Therapy of chronic hepatitis B: current challenges and opportunities. *J Viral Hepat* 2002; **9:** 393-399
14 **Chin R,** Locarnini S. Treatment of chronic hepatitis B: current challenges and future directions**.** *Rev Med Virol* 2003; **13:** 255-272
15 **Karayiannis P.** Hepatitis B virus: old, new and future approaches to antiviral treatment. *J Antimicrob Chemother* 2003; **51:** 761-785
16 **Raj V.** Treatment of hepatitis B. *Clin Cornerstone* 2001; **3:** 24-36
17 **Zoulim F,** Trepo C. New antiviral agents for the therapy of chronic hepatitis B virus infection. *Intervirology* 1999; **42:** 125-144
18 **Klein C,** Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, Kubicka S, Manns MP, Trautwein C. Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. *Gastroenterology* 2003; **125:** 9-18
19 **Wo JE,** Wu XL, Zhu HH, Zhou LF, Yao HP, Chen LW. DNAzymes in vitro inhibit the expression of hepatitis B virus genes. *Zhejiang Daxue Xuebao Yixueban* 2003; **32:** 112-115
20 **Tung FYT,** Bowen SW. Targeted inhibition of hepatitis B virus gene expression: a gene therapy approach. *Front Biosci* 1998; **3:** a11-a15
21 **Guha C,** Shah SJ, Ghosh SS, Lee SW, Roy-Chowdhury N, Roy-Chowdhury J. Molecular therapies for viral hepatitis. *Bio Drugs* 2003; **17:** 81-91
22 **Wu C,** Zeng Z, Wang Q. Experimental study of inhibition of hepatitis B by dual-target antisense RNA. *Zhonghua Yixue Zazhi* 2001; **81:** 605-608
23 **Robaczewska M,** Guerret S, Remy JS, Chemin I, Offensperger WB, Chevallier M, Behr JP, Podhajska AJ, Blum HE, Trepo C, Cova L. Inhibition of hepadnaviral replication by polyethylenimine-based intravenous delivery of antisense phosphodiester oligodeoxynucleotides to the liver. *Gene Ther* 2001; **8:** 874-881
24 **Feng Y,** Kong YY, Wang Y, Qi GR. Inhibition of hepatitis B virus by hammerhead ribozyme targeted to the poly (A) signal sequence in cultured cells. *Biol Chem* 2001; **382:** 655-660
25 **Zu Putlitz J,** Wieland S, Blum HE, Wands JR. Antisense RNA complementary to hepatitis B virus specifically inhibits viral replication. *Gastroenterology* 1998; **115:** 702-713
26 **Yang YS,** Watson WJ, Tucker PW, Capra JD. Construction of recombinant DNA by exonuclease recession. *Nucleic Acids Res* 1993; **21:** 1889-1893
27 **Horton RM.** *In vitro* recombination and mutagenesis of DNA. SOEing together tailor-made genes. *Methods Mol Biol* 1997; **67:** 141-149
28 **Horton RM.** PCR-mediated recombination and mutagenesis. SOEing together tailor-made genes. *Mol Biotechnol* 1995; **3:** 93-99
29 **Bartenschlager R,** Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000; **81:** 1631-1648
30 **Reynolds JE,** Kaminski A, Kettinen HJ, Grace K, Clarke BE, Carroll AR, Rowlands DJ, Jackson RJ. Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J* 1995; **14:** 6010-6020
31 **Morgan RA,** Couture L, Elroy-Stein O, Ragheb J, Moss B, Anderson WF. Retroviral vectors containing putative internal ribosome entry sites: development of a polycistronic gene transfer system and applications to human gene therapy. *Nucleic Acids Res* 1992; **25:** 1293-1299
32 **Lu HH,** Wimmer E. Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. *Proc Natl Acad Sci* 1996; **93:** 1412-1417
33 **Urabe M,** Hasumi Y, Ogasawara Y, Matsushita T, Kamoshita N, Nomoto A, Colosi P, Kurtzman GJ, Tobita K, Ozawa K. A novel dicistronic AAV vector using a short IRES segment derived from hepatitis C virus genome. *Gene* 1997; **200:** 157-162
34 **Kruger M,** Beger C, Li QX, Welch PJ, Tritz R, Leavitt M, Barber JR, Wong-Staal F. Identification of eIF2Bgamma and eIF2gamma as cofactors of hepatitis C virus internal ribosome entry site-mediated translation using a functional genomics approach. *Proc Natl Acad Sci U S A* 2000; **97:** 8566-8571
35 **Zhang H,** Hanecak R, Brown-Driver V, Azad R, Conklin B, Fox MC, Anderson KP. Antisense oligonucleotide inhibition of hepatitis C virus (HCV) gene expression in livers of mice infected with an HCV-vaccinia virus recombinant. *Antimicrob Agents Chemother* 1999; **43:** 347-353
36 **Liang XS,** Lian JQ, Zhou YX, Nie QH, Hao CQ. A small yeast RNA inhibits HCV IRES mediated translation and inhibits replication of poliovirus *in vivo*. *World J Gastroenterol* 2003; **9:** 1008-1013
37 **Santolini E,** Migliaccio G, La Monica N. Biosynthesis of biochemical properties of the hepatitis C virus core protein. *J Virol* 1994; **68:** 3631-3641
38 **Yasui K,** Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M. The native form and maturation process of hepatitis C virus core protein. *J Virol* 1998; **72:** 6048-6055
39 **Urabe M,** Hasumi Y, Ogasawara Y, Matsushita T, Kamoshita N, Nomoto A, Colosi P, Kurtzman GJ, Tobita K, Ozawa K. A novel discistronic AAV vector using a short IRES segment derived from hepatitis c virus genome. *Gene* 1997; **200:** 157-162
40 **Shimoike T,** Mimori S, Tani H, Matsuura Y, Miyamura T**.** Interaction of hepatitis c virus core protein with viral sense RNA and suppression of its translation. *J Virol* 1999; **73:** 9718-9725
41 **Zhang J,** Yamada O, Yoshida H, Iwai T, Araki H. Autogenous translational inhibition of core protein: implication for switch from translation to RNA replication in hepatitis C virus. *Virology* 2002; **293:** 141-150
42 **Li D,** Takyar ST, Lott WB, Gowans EJ. Amino acids 1-20 of the hepatitis C virus (HCV) core protein specifically inhibit HCV IRES-dependent translation in HepG2 cells, and inhibit both HCV IRES- and cap-dependent translation in HuH7 and CV-1 cells. *J Gen Virol* 2003; **84:** 815-825
43 **Yao ZQ,** Ray S, Eisen-Vandervelde A, Waggoner S, Hahn YS. Hepatitis C virus: immunosuppression by complement regulatory pathway. *Viral Immunol* 2001; **14:** 277-295


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2221-2225
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

# Association between HLA class II gene and susceptibility or resistance to chronic hepatitis B

Ye-Gui Jiang, Yu-Ming Wang, Tong-Hua Liu, Jun Liu

**Ye-Gui Jiang, Yu-Ming Wang, Jun Liu,** Institute of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, China
**Tong-Hua Liu,** Department of Pharmacy, Xinqiao Hospital, Third Military Medical University, Chongqing 400038, China
**Correspondence to:** Dr. Ye-Gui Jiang, Institute of Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, China. jiangyegui@yahoo.com.cn
**Telephone:** +86-23-68754141 **Fax:** +86-23-68754479
**Received:** 2003-05-10 **Accepted:** 2003-06-19

## Abstract

**AIM:** To investigate the association between the polymorphism of HLA-DRB1, -DQA1 and -DQB1 alleles and viral hepatitis B.

**METHODS:** HLA-DRB1, -DQA1 and -DQB1 alleles in 54 patients with chronic hepatitis B, 30 patients with acute hepatitis B and 106 normal control subjects were analyzed by using the polymerase chain reaction/sequence specific primer (PCR/SSP) technique.

**RESULTS:** The allele frequency of HLA-DRB1*0301 in the chronic hepatitis B group was markedly higher than that in the normal control group (17.31 % *vs* 5.67 %), there was a significant correlation between them ($\chi^2$=12.3068, *Pc*=0.0074, RR=4.15). The allele frequency of HLA-DQA1*0501 in the chronic hepatitis B group was significantly higher than that in the normal control group (25.96 % *vs* 13.68 %), there was a significant correlation between them ($\chi^2$=9.2002, *Pc*=0.0157, RR=2.87). The allele frequency of HLA-DQB1*0301 in the chronic hepatitis B group was notably higher than that in the normal control group (35.58 % *vs* 18.87 %), there was a significant correlation between them ($\chi^2$=15.5938, *Pc*=0.0075, RR=4.07). The allele frequency of HLA-DRB1*1101/1104 in the chronic hepatitis B group was obviously lower than that in the normal control group (0.96 % *vs* 13.33 %), there was a significant correlation between them ($\chi^2$=11.9206, *Pc*=0.0145, RR=18.55). The allele frequency of HLA-DQA1*0301 in the chronic hepatitis B group was remarkably lower than that in the normal control group (14.42 % *vs* 30 %), there was a significant correlation between them ($\chi^2$=8.7396, *Pc*=0.0167, RR=0.35).

**CONCLUSION:** HLA-DRB1*0301, HLA-DQA1*0501 and HLA-DQB1*0301 are closely related with susceptibility to chronic hepatitis B, and HLA-DRB1*1101/1104 and HLA-DQA1*0301 are closely related with resistance to chronic hepatitis B. These findings suggest that host HLA class II gene is an important factor determining the outcome of HBV infection.

Jiang YG, Wang YM, Liu TH, Liu J. Association between HLA class II gene and susceptibility or resistance to chronic hepatitis B. *World J Gastroenterol* 2003; 9(10): 2221-2225
http://www.wjgnet.com/1007-9327/9/2221.asp

## INTRODUCTION

The progression of hepatitis B virus (HBV) infection may be influenced by a number of factors including the viral genotype and the level of viremia, but these factors alone do not account for the variability in outcome. There is an increasing awareness that host factors are involved. A great deal of evidences suggest that both cellular and humoral immune responses are required for viral clearance[1-3]. Polymorphisms of human leukocyte antigen (HLA) influence immune responses. Variability in immune response is often associated with HLA polymorphism. HLA genotype of an individual may influence the progression of HBV infection. Patients who have successfully recovered from acute hepatitis B develop strong HLA classes I and II restricted T cell response, whereas these responses are weak or absent in patients with chronic hepatitis B[4, 5]. In the present study, we have analyzed the polymorphism of HLA-DRB1, -DQA1 and -DQB1 alleles in patients with chronic and acute hepatitis B and healthy controls using the polymerase chain reaction with sequence specific primers (PCR/SSP). This study aimed at investigating whether these alleles might be associated with susceptibility or resistance to chronic hepatitis B.

## MATERIALS AND METHODS

### *Subjects*

Fifty-two patients (43 males, 9 females, mean age: 33.46 years) with chronic hepatitis B and 30 patients (24 males, 6 females, mean age: 33.25 years) with acute hepatitis B, and 106 healthy blood donors (88 males, 18 females, mean age: 31.27 years) were included in this study. All the patients were from the Institute of Infectious Diseases, Southwest Hospital of Third Military Medical University. The diagnosis of all the cases was made according to the criteria established on the Viral Hepatitis Conference held in 2000. All the patients and controls were Chinese Han people without relatives from Chongqing. The subjects were divided into chronic hepatitis B group, acute hepatitis B group and healthy control group.

### *Primer synthesis and reagents*

The polymorphisms of HLA-DRB1, -DQA1 and -DQB1 alleles were assessed by PCR/SSP technique. HLA-DRB1, -DQA1 and -DQB1 loci of specific PCR primers were designed by Olerup *et al*[6, 7], and synthesized by Shanghai Branch, Canadian Sangon Company. The primers amplifying human growth hormone gene (5' -primer: 5' -GCC TTC CCA ACC ATT CCC TTA-3' , 3' -primer: 5' -TCA CGG ATT TCT GTT GTG TTTC-3' ) were synthesized by Shanghai Branch, Canadian Sangon Company. Taq DNA polyenzyme and dNTP were purchased from Shanghai Branch, Canadian Sangon Company, pBR322/ Hand III marker and the ReadyPCR™ whole blood genomic DNA purification system were provided by Sino-American Biotechnology Company.

### *Methods*

**DNA extraction** Genomic DNA was extracted from peripheral blood by using the Ready PCR™ whole blood genomic DNA purification system.

### *PCR amplification*

A total amount of 25 μl PCR reaction solution contained 8 pmoles of each sequence specific primer (3.2 μl), 0.8 pmoles of each internal control primer (0.32 μl), 80-100 ng of genomic DNA (2 μl), 2.5 μl of 10×buffer, 25 mmol/L of $MgCl_2$ (2.5 μl), 10 mmol/L of dNTP (1 μl), 5 unit/μl of Taq polymerase (0.5 μl) and 13 μl of demonized $H_2O$. The PCR cycling parameters of HLA-DRB1 alleles were as follows: predenaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 50 s, annealing at 65 ℃ for 1 min, extension at 72 ℃ for 1 min, repetition for 30 cycles and final extension at 72 ℃ for 5 min. The PCR cycling parameters of HLA-DQA1 and -DQB1 alleles were as follows: predenaturation at 94 ℃ for 4 min, denaturation at 94 ℃ for 1 min, annealing at 65 ℃ for 1 min, extension at 72 ℃ for 1 min, repetition for 30 cycles and final extension at 72 ℃ for 2 min. In each PCR reaction a primer pair was included to amplify the human growth hormone gene, which functioned as an internal positive amplification control and gave rise to a 429 base pair fragment.

**Detection of PCR products** PCR products were loaded in 2 % agars gel containing 0.5 μg/ml of ethidium bromide, electrophoresed for 20 min at 15 V/cm, examined under ultraviolet light. The individual alleles were assigned for the specific pattern of appropriately sized bands.

### *Statistical analysis*

Allele frequencies of HLA-DRB1, -DQA1 and -DQB1 were calculated by direct count. AF for the study group was compared with that for the control group using Chi-square ($\chi^2$) test. The Fisher' s exact test was used when $\chi^2$ value exceeded 3.84, the *P* values were corrected for the number of alleles (corrected *P*=*Pc*). Relative risk frequencies (RR) were calculated according to Wolf formula.

## RESULTS

### *HLA-DRB1 alleles in patients with chronic and acute hepatitis B and healthy controls*

The distribution of HLA-DRB1 alleles is shown in Table 1. The allele frequencies of HLA-DRB1*0301 in the chronic hepatitis B group (17.31 %) were markedly higher than those in the normal control group (5.67 %), there was a significant correlation between them ($\chi^2$=12.3068, *Pc*=0.0074, RR=4.15). The allele frequencies of HLA-DRB1*1101/1104 in the chronic hepatitis B group (0.96 %) were significantly lower than those in the acute hepatitis B group (13.33 %), with significant correlation between them ($\chi^2$=11.9206, *Pc*=0.0145, RR=18.55). The data of electrophoresis of HLA-DRB1 alleles amplification are shown in Figure 1.

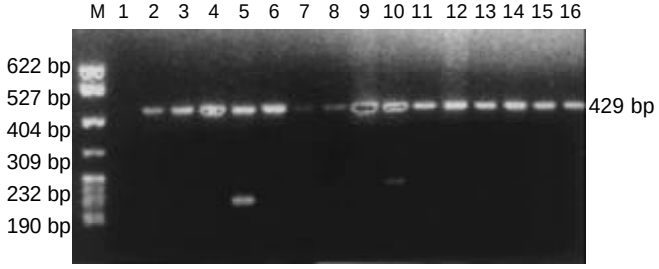



**Figure 1** Electrophoresis of HLA-DRB1 alleles amplification by PCR/SSP. M: pBR322DNA/MSP I marker, 1: negative control, 2: 0101/0103, 3: 0301, 4: 0401/0411, 5: 0701/0702, 6: 0801/0804, 7: 0901, 8: 1001, 9: 1101/1104, 10: 1201/1202, 11: 1301/1302, 12: 1303/1304, 13: 1401,1404, 14: 1402,1403, 15: 1501/1502, 16: 1601/1602.

**Table 1** Allele frequency of HLA-DRB1 in patients with chronic and acute hepatitis B and normal heathy individuals

| HLA-DRB1 allele | Normal control (*n*=106) | | Chronic hepatitis B (*n*=52) | | Acute hepatitis B (*n*=30) | |
|---|---|---|---|---|---|---|
| | PN | AF | PN | AF | PN | AF |
| 0101/0103 | 1 | 0.47 | 1 | 0.96 | 1 | 1.67 |
| 0301* | 12 | 5.66 | 18 | 17.31 | 6 | 10.00 |
| 0401/0411 | 24 | 11.32 | 13 | 12.50 | 7 | 11.67 |
| 0701/0702 | 11 | 5.19 | 8 | 7.69 | 4 | 6.67 |
| 0801/0804 | 9 | 4.25 | 6 | 5.77 | 3 | 5.00 |
| 0901 | 32 | 15.09 | 16 | 15.39 | 8 | 13.33 |
| 1001 | 2 | 0.94 | 2 | 1.92 | 1 | 1.67 |
| 1101/1104** | 13 | 6.13 | 1 | 0.96 | 8 | 13.33 |
| 1201/1202 | 34 | 16.04 | 15 | 14.42 | 8 | 13.33 |
| 1301/1302 | 4 | 1.89 | 1 | 0.96 | 1 | 1.67 |
| 1303/1304 | 1 | 0.47 | 1 | 0.96 | 1 | 1.67 |
| 1401,1404 | 14 | 6.60 | 6 | 5.77 | 4 | 6.67 |
| 1402,1403 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| 1501/1502 | 34 | 16.04 | 11 | 10.58 | 5 | 8.33 |
| 1601/1602 | 13 | 6.13 | 2 | 1.92 | 1 | 1.67 |
| Blank | 8 | 3.77 | 3 | 2.89 | 2 | 3.33 |

PN: positive number, AF: allele frequency. *$\chi^2$=12.3068, *Pc*=0.0074, RR=4.15. **$\chi^2$=11.9206, *Pc*=0.0145, RR=18.55.

### *HLA-DQA1 alleles in patients with chronic and acute hepatitis B and healthy controls*

The distribution of HLA-DQA1 alleles is shown in Table 2. The allele frequencies of HLA-DQA1*0501 in the chronic hepatitis B group (25.96 %) were markedly higher than those in the normal control group (13.68 %), there was a significant correlation between them ($\chi^2$=9.2002, *Pc*=0.0157, RR=2.87). The allele frequencies of HLA-DQA1*0301 in the chronic hepatitis B group (14.42 %) was significantly lower than those in the acute hepatitis B group (30 %), there was a significant correlation between them ($\chi^2$=7.6781, *Pc*=0.0388, RR=3.70). The data of electrophoresis of HLA-DQA1 alleles amplification are shown in Figure 2.

**Table 2** Allele frequency of HLA-DQA1 in patients with chronic and acute hepatitis B and normal heathy individuals

| HLA-DQA1 allele | Normal control (*n*=106) | | Chronic hepatitis B (*n*=52) | | Acute hepatitis B (*n*=30) | |
|---|---|---|---|---|---|---|
| | PN | AF | PN | AF | PN | AF |
| 0101 | 17 | 8.02 | 9 | 8.65 | 4 | 6.67 |
| 0102 | 45 | 21.23 | 22 | 21.15 | 12 | 20.00 |
| 0103 | 9 | 4.25 | 5 | 4.81 | 2 | 3.33 |
| 0104 | 3 | 1.42 | 1 | 0.96 | 1 | 1.67 |
| 0201 | 7 | 3.30 | 3 | 2.88 | 1 | 1.67 |
| 0301* | 57 | 26.89 | 15 | 14.42 | 18 | 30.00 |
| 0302 | 1 | 0.47 | 0 | 0.00 | 0 | 0.00 |
| 0401 | 2 | 0.49 | 1 | 0.96 | 1 | 1.67 |
| 0501** | 29 | 13.68 | 27 | 25.96 | 10 | 16.67 |
| 0601 | 23 | 10.85 | 12 | 11.54 | 6 | 10.00 |
| Blank | 19 | 8.96 | 9 | 8.65 | 5 | 8.33 |

PN: positive number, AF: allele frequency. *$\chi^2$=7.6781, *Pc*=0.0388, RR=3.70. **$\chi^2$=9.2002, *Pc*=0.0157, RR=2.87.

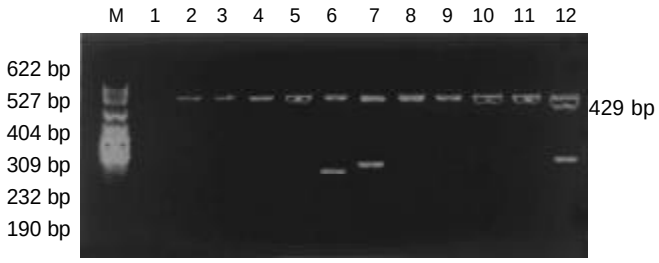



**Figure 2** Electrophoresis of HLA-DQA1 alleles amplification by PCR/SSP. M: pBR322DNA/MSPImarker, 1: negative control, 2: 0101/0104, 3: 0101/0102/0104, 4: 0102/0103, 5: 0103, 6: 0201, 7: 0301, 8: 0302, 9: 0401, 10: 0501, 11: 0601, 12: A (when the amplification product was -DQA1*0104, "A" was negative. When the amplification product was non-DQA1*0104, "A"was positive.).

### *HLA-DQA1 alleles in patients with chronic and acute hepatitis B and healthy controls*

The distribution of HLA-DQB1 alleles is shown in Table 3. The allele frequencies of HLA-DQB1*0301 allele in the chronic hepatitis B group (35.58 %) were markedly higher than those in the normal control group (18.87 %), there was a significant correlation between them ($\chi^2$=15.5938, *Pc*=0.0075, RR=4.07). The data of electrophoresis of HLA-DQB1 alleles amplification are shown in Figure 3.

**Table 3** Allele frequency of HLA-DQB1 in patients with chronic and acute hepatitis B and normal heathy individuals

| HLA-DQB1 allele | Normal control (*n*=106) | | Chronic hepatitis B (*n*=52) | | Acute hepatitis B (*n*=30) | |
|---|---|---|---|---|---|---|
| | PN | AF | PN | AF | PN | AF |
| 0201 | 23 | 10.85 | 10 | 9.62 | 6 | 10.00 |
| 0301* | 40 | 18.87 | 37 | 35.58 | 16 | 26.67 |
| 0302 | 14 | 6.61 | 6 | 5.77 | 3 | 5.00 |
| 0303 | 35 | 16.51 | 15 | 14.42 | 10 | 16.67 |
| 0401 | 11 | 5.19 | 5 | 4.81 | 3 | 5.00 |
| 0402 | 2 | 0.94 | 1 | 0.96 | 1 | 1.67 |
| 0501 | 9 | 4.25 | 3 | 2.88 | 2 | 3.33 |
| 0502 | 20 | 9.43 | 7 | 6.73 | 3 | 5.00 |
| 0503 | 6 | 2.83 | 2 | 1.92 | 1 | 1.67 |
| 0601 | 20 | 9.43 | 7 | 6.73 | 7 | 11.67 |
| 0602 | 12 | 5.66 | 4 | 3.85 | 3 | 5.00 |
| 0603 | 5 | 2.36 | 2 | 1.92 | 1 | 1.67 |
| 0604 | 7 | 3.30 | 2 | 1.92 | 2 | 3.33 |
| Blank | 8 | 3.77 | 3 | 2.89 | 2 | 3.33 |

PN: positive number, AF: allele frequency. $\chi^2$=15.5938, *Pc*=0.0075, RR=4.07.

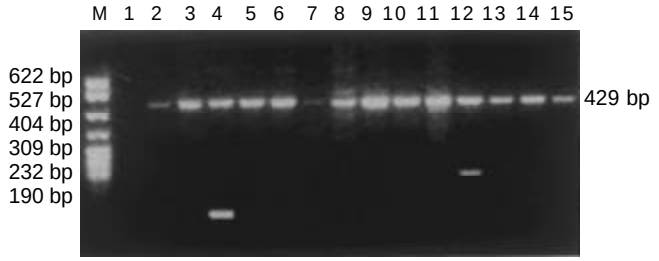



**Figure 3** Electrophoresis of HLA-DQB1 alleles amplification by PCR/SSP. M: pBR322DNA/MSP I marker, 1: negative control, 2: 0201, 3: 0201/0302, 4: 0301, 5: 0302/0303, 6: 0303, 7: 0401, 8: 0402, 9: 0501, 10: 0502, 11: 0503, 12: 0601, 13: 0602, 14: 0603, 15: 0604.

## DISCUSSION

Host and viral factors undoubtedly influence the clinical expression and behavior of chronic hepatitis B. Attempts to explain the clinical expression and the behavior of chronic hepatitis B by viral factors have shown the importance of viral genotypes and viraemia level for the clinical presentation. However, there remain large inconsistencies, and it is very likely that immune response to hepatitis B virus (HBV) of the host can modify disease outcome[8-10]. HLA is a critical genetic factor that determines individual variations of immune response. The ternary structure of HLA molecules and their roles in the control of immune response have been clearly elucidated. There are many reports about statistical associations between HLA and diseases. HLA gene contributes to the host response against HBV[11-28]. Individuals with different HLA types may differ in susceptibility or resistance to disease[29-35], and associations between HLA polymorphism and susceptibility or resistance to diseases have been identified.

Researches on the correlation between HLA and hepatitis B have been performed for many years. Traditional serological method was used in some investigations, but it has become obsolete and inaccurate. To have a better understanding of the disease, correlation between hepatitis B and HLA should be further studied using nucleotide-typing techniques. Therefore, in the present study, we examined the HLA-DRB1, -DQA1 and -DQB1 alleles by PCR/SSP technique in patients with hepatitis B in an attempt to investigate the association between the polymerase of HLA class II gene and hepatitis B. Fourteen HLA-DRB1 alleles, ten HLA-DQA1 alleles and thirteen HLA-DQB1 alleles were detected. The allele frequencies of HLA-DRB1, -DQA1 and -DQB1 in healthy individuals tallied with genetic characteristics of the Han people in southern region of China.

A previous study showed that the allele frequencies of HLA-B8, DR3, A30, DQA1*0501 in patients with chronic hepatitis B were markedly increased, suggesting that these alleles are associated with chronic hepatitis B[36]. Thio *et al*[37] found that HBV persistence was significantly associated with two class II alleles, DQA1*0501 (OR=2.6) and DQB1 *0301 (OR=3.9), the two-locus haplotype consisted of these same two alleles (OR=3) and the three-locus haplotype consisted of DQA1 *0501, DQB1 *0301 and DRB1 *1102 (OR=10.7). The study by Shen *et al* suggested that the susceptibility to chronic hepatitis B was strongly associated with HLA-DRB1*10 allele in northern Chinese patients[38]. In the present study, we found that the allele frequencies of HLA-DRB1*0301, -DQA1*0501 and -DQB1*0301 in the chronic hepatitis B group were markedly higher than those in the normal control group, there was a significant correlation between them (Tables 1, 2 and 3). These findings suggest that HLA-DRB1*0301, -DQA1*0501 and -DQB1*0301 are closely associated with the susceptibility to chronic hepatitis B, and may be the susceptible gene.

Cotrina *et al*[39] analyzed the HLA-DRB1 genotype in a series of patients with chronic hepatitis B and acute hepatitis B, which further confirmed that HLA-DRB1*1301 and -DRB1*1302 alleles were associated with the clearance of HBV infection and protected people against chronic hepatitis B. Diepolder *et al*[40] found that a strong virus-specific $CD4^+$ and $CD8^+$ T lymphocyte response to hepatitis B virus was associated with viral clearance, patients with acute hepatitis B carrying HLA-DR13 had a more vigorous $CD4^+$ T cell response to HBV core than patients not carrying HLA-DR13, suggesting that HLA-DR13 is associated with a self-limited course of HBV infection, and the beneficial effect of HLA-DR13 alleles on the outcome of HBV infection could be explained by a more vigorous HBV core-specific $CD4^+$ T cell response, which might be either due to a more proficient antigen presentation by HLA-

DR13 molecules themselves or due to a linked polymorphism in a neighboring immunoregulatory gene. In the present study, we found that the allele frequencies of HLA-DRB1*1101/1104 and HLA-DQA1*0301 in the chronic hepatitis B group were markedly lower than those in the acute hepatitis B group, there was a significant correlation between them (Tables 1 and 2). These findings suggest that HLA-DRB1*1101/1104 and -DQA1*0301 are closely associated with the resistance to chronic hepatitis B, and may be the resistant gene.

The results of the present study suggest that HLA-DRB1*0301, -DQA1*0501 and -DQB1*0301 may be the susceptible gene, and HLA-DRB1*1101/1104 and -DQA1*0301 may be the resistant genes to chronic hepatitis B, and that host HLA class II gene is an important factor determining the outcome of HBV infection, which will give some new clues to the study of pathogenesis of chronic hepatitis B.

## REFERENCES


1 **Takayama T**, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet* 2000; **356**: 802-807

2 **Chiari R**, Hames G, Stroobant V, Texier C, Maillere B, Boon T, Coulie PG. Identification of a tumor-specific shared antigen derived from an Eph receptor and presented to CD4 T cells on HLA class II molecules. *Cancer Res* 2000; **60**: 4855-4863

3 **Feinmesser M**, Sulkes A, Morgenstern S, Sulkes J, Stern S, Okon E. HLA-DR and beta 2 microglobulin expression in medullary and atypical medullary carcinoma of the breast: histopathologically similar but biologically distinct entities. *J Clin Pathol* 2000; **53**: 286-291

4 **Zhang SL**, Liu M, Zhu J, Chai NL. Predominant Th-2 immune response and chronic hepatitis B virus infection. *Shijie Huaren Xiaohua Zazh* 1999; **7**: 513-515

5 **Chen WN**, Oon CJ. Mutation "hot spot" in HLA class I- restricted T cell epitope on hepatitis B surface antigen in chronic carriers and hepatocellular carcinoma. *Biochem Biophys Commun* 1999; **262**: 757-761

6 **Olerup O**, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; **39**: 225-235

7 **Olerup O**, Aldener A, Fogdell A. HLA-DQB1 and DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993; **41**: 119-134

8 **Jiang YG**, Li QF, Mao Q, WangYM. Primary human fetal hepatocytes with HBV infection *in vitro*. *Shijie huaren Xiaohua Zazhi* 2000; **8**: 403-405

9 **Sing G**, Butterworth L, Chen X, Bryant A, Cooksley G. Composition of peripheral blood lymphocyte populations during different stages of chronic infection with hepatitis B virus. *J Viral Hepat* 1998; **5**: 83-93

10 **Cao T**, Meuleman P, Desombere I, Sallberg M, Leroux-Roels G. *In vivo* inhibition of anti-hepatitis B virus core antigen (HBcAg) immunoglobulin G production by HBcAg-specific CD4(+) Th1-type T-cell clones in a hu-PBL-NOD / SCID mouse modle. *J Virol* 2001; **75**: 11449-11456

11 **Du YP**, Deng CS, Lu DY, Huang MF, Guo SF, Hou W. The relation between HLA-DQA1 genes and genetic susceptibility to duodenal ulcer in Wuhan Hans. *World J Gastroenterol* 2000; **6**: 107-110

12 **Ding HL**, Cheng H, Fu ZZ, Deng QL, Yan T. The relationship of lmp2 and DR3 genes with susceptibility to type I diabetes mellitus in south China Han population. *World J Gastroenterol* 2000; **6**: 111-114

13 **Lin J**, Deng CS, Sun J, Zheng XG, Huang X, Zhou Y, Xiong P, Wang YP. HLA-DRB1 allele polymorphisms in genetic susceptibility to esophageal carcinoma. *World J Gastroenterol* 2003; **9**: 412-416

14 **Pu J**, Yang XS, Zhang YL, Pan LJ, Zhou DY. Expression of HLA-DR in epithelie around lymphofollicle of human gastrointestinal mucosa. *Shijie huaren Xiaohua Zazhi* 2000; **8**: 706-707

15 **Zhai SH**, Liu JB, Zhu P, Wang YH. CD54, CD80, CD86 and HLA-ABC expressions in liver cirrhosis and hepatocarcinoma. *Shijiehuaren Xiaohua Zazhi* 2000; **8**: 292-295

16 **Qu S**, Li QF, Deng YZ, Zhang JM, Zhang J. Cloning and expression of HLA-B7 gene. *World J Gastroenterol* 1999; **5**: 345-348

17 **Asti M**, Martinetti M, Zavaglia C, Cuccia MC, Gusberti L, Tinelli C, Cividini A, Bruno S, Salvaneschi L, Ideo G, Mondelli MU, Silini EM. Human leukocyte antigen class II and III alleles and severity of hepatitis C virus-related chronic liver disease. *Hepatology* 1999; **29**: 1272-1279

18 **Barrett S**, Ryan E, Crowe J. Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. *J Hepatol* 1999; **30**: 979-983

19 **Lechmann M**, Schneider EM, Giers G, Kaiser R, Dumoulin FL, Sauerbruch T, Spengler U. Increased frequency of the HLA-DR15 (B1*15011) allele in German patients with self-limited hepatitis C virus infection. *Eur J Clin Invest* 1999; **29**: 337-343

20 **Mangia A**, Gentile R, Cascavilla I, Margaglione M, Villani MR, Stella F, Modola G, Agostiano V, Gaudiano C, Andriulli A. HLA class II favors clearance of HCV infection and progression of the chronic liver damage. *J Hepatol* 1999; **30**: 984-989

21 **Chang KM**, Gruener NH, Southwood S, Sidney J, Pape GR, Chisari FV, Sette A. Identification of HLA-A3 and –B7-restricted CTL response to hepatitis C virus in patients with acute and chronic hepatitis C. *J Immunol* 1999; **162**: 1156-1164

22 **Aaltonen L**, Partanen J, Auvinen E, Rihkanen H, Vaheri A. HLA-DQ alleles and human papillomavirus DNA in adult-onset laryngeal papillomatosis. *J Infect Dis* 1999; **179**: 682-685

23 **Harcourt G**, Hellier S, Bunce M, Satsangi J, Collier J, Chapman R, Phillips R, Klenerman P. Effect of HLA class II genotype on T helper lymphocyte responses and viral control in hepatitis C virus infection. *J Viral Hepat* 2001; **8**: 174-179

24 **Zhou HC**, Xu DZ, Wang XP, Zhang JX, Huang Y, Yan YP, Zhu Y, Jin BQ. Identification of the epitopes on HCV core protein recognized by HLA-A2 restricted cytotoxic T lymphocytes. *World J Gastroenterol* 2001; **7**: 583-586

25 **Ma X**, Qiu DK. Relationship between autoimmune hepatitis and HLA-DR4 and DRbeta allelic sequences in the third hypervariable region in Chinese. *World J Gastroenterol* 2001; **7**: 718-721

26 **Godkin A**, Jeanguet N, Thursz M, Openshaw P, Thomas H. Characterization of novel HLA-DR11-restricted HCV epitopes reveals both qualitative and quantitative differences in HCV-specific CD4+ T cell responses in chronically infected and non-viremic patients. *Eur J Immunol* 2001; **31**: 1438-1446

27 **Bosi I**, Ancora G, Mantovani W, Miniero R, Verucchi G, Attard L, Venturi V, Papa I, Sandri F, Dallacasa P, Salvioli GP. HLA DR13 and HCV vertical infection. *Pediatr Res* 2002; **51**: 746-749

28 **Hue S**, Cacoub P, Renou C, Halfon P, Thibault V, Charlotte F, Picon M, Rifflet H, Piette JC, Pol S, Caillat-Zucman S. Human leukocyte antigen class II alleles may contribute to the severity of hepatitis C virus-related liver disease. *J Infect Dis* 2002; **186**: 106-109

29 **Mc Dermott AB**, Cohen SB, Zuckerman JN, Madrigal JA. Human leukocyte antigens influence the immune response to a pre-S / S hepatitis B vaccine. *Vaccine* 1999; **17**: 330-339

30 **McDermott AB**, Madrigal JA, Sabin CA, Zuckerman JN, Cohen SB. The influence of host factors and immunogenetics on lymphocyte responses to Hepagene vaccination. *Vaccine* 1999; **17**: 1329-1337

31 **Wang FS**, Xing LH, Liu MX, Zhu CL, Liu HG, Wang HF, Lei ZY. Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection. *World J Gastroenterol* 2001; **7**: 537-541

32 **Sobao Y**, Sugi K, Tomiyama H, Saito S, Fujiyama S, Morimoto M, Hasuike S, Tsubouchi H, Tanaka K, Takiguch M. Identification of hepatitis B virus-specific CTL epitopes presented by HLA-A*2402, the most common HLA class I allele in East Asia. *J Hepatol* 2001; **34**: 922-929

33 **Thimme R**, Chang KM, Pemberton J, Sette A, Chisari FV. De-

generate immunogenicity of an HLA-A2-restricted hepatitis B virus nucleocapsid cytotoxic T-lymphocyte epitope that is also presented by HLA-B51. *J Virol* 2001; **75**: 3984-3987

34 **Pellegris G**, Ravagnani F, Notti P, Fissi S, Lombardo C. B and C hepatitis viruses, HLA-DQ1 and -DR3 alleles and autoimmunity in patients with hepatocellular carcinoma. *J Hepatol* 2002; **36**: 521-526

35 **Desombere I**, Gijbels Y, Verwulgen A, Leroux-Roels G. Characterization of the T cell recognition of hepatitis B surface antigen (HBsAg) by good and poor responders to hepatitis B vaccines. *Clin Exp Immunol* 2000; **122**: 390-399

36 **Chen DF**, Kliem V, Endres W, Brunkhorst R, Tillmann HL, Koch KM, Manns MP, Stangel W. Relationship between human leukocyte antigen determinants and courses of hepatitis B virus infection in Caucasian patients with end-stage renal disease. *Scand J Gastroenterol* 1996; **31**: 1211-1215

37 **Thio CL**, Carrington M, Marti D, O'Brien SJ, Vlahov D, Nelson KE, Astemborski J, Thomas DL. Class II HLA alleles and hepatitis B virus persistence in African Americans. *J Infect Dis* 1999; **179**: 1004-1006

38 **Shen JJ**, Ji Y, Gu XL, Huang RJ, Sun YP. The association of HLA-DRB1*10 with chronic hepatitis B in Chinese patients. *Zhonghua Weishengwuxue He Mianyixue Zazhi* 1999; **19**: 58-59

39 **Cotrina M**, Buti M, Jardi R, Rodriguez-Frias F, Campins M, Esteban R, Guardia J. Study of HLA-II antigens in chronic hepatitis C and B and in acute hepatitis B. *Gastroenterol Hepatol* 1997; **20**: 115-118

40 **Diepolder HM**, Jung MC, Keller E, Schraut W, Gerlach JT, Gruner N, Zachoval R, Hoffmann RM, Schirren CA, Scholz S, Pape GR. A vigorous virus-specific CD4+ T cell response may contribute to the association of HLA-DR13 with viral clearance in hepatitis B. *Clin Exp Immunol* 1998; **113**: 244-251

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2226-2231
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

# No requirement of HCV 5'NCR for HCV-like particles assembly in insect cells

Wei Zhao, Guo-Yang Liao, Yan-Jun Jiang, Shu-De Jiang

**Wei Zhao, Guo-Yang Liao, Yan-Jun Jiang, Shu-De Jiang,** Laboratory of Vaccine Research, Institute of Medical Biology, Chinese Academy of Medical Sciences, Peking Union Medical College, Kunming 650118, Yunnan Province, China
**Correspondence to:** Shu-De Jiang, Laboratory of Vaccine Research, Institute of Medical Biology, Chinese Academy of Medical Sciences. 379 Jiaoling Road, Kunming 650118, Yunnan Province, China. jsd2000@163.net
**Telephone:** +86-871-8334330 **Fax:** +86-871-8334483
**Received:** 2003-08-05 **Accepted:** 2003-09-10

## Abstract

**AIM:** To express all three HCV structural proteins in the presence or absence of HCV 5'NCR to investigate the requirement of 5'NCR for the assembly of HCV-like particles in insect cells.

**METHODS:** HCV structural protein encoding sequences CE1E2 and 5'NCR-CE1E2 were amplified with PCR. Recombinant baculovirus were constructed with recombinant DNA techniques. HCV structural proteins expressed in insect cells were analyzed by immunofluorescence and SDS-PAGE. Immunoprecipitation experiment of insect cell lysates with anti-E2 monoclonal antibody (MAb) was carried out and the immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting with anti-C, anti-E2 MAbs and HCV positive serum. The virus-like particles in insect cells were visualized by electron microscopy (EM). The HCV-like particles were purified by sucrose gradient centrifugation and identified by EM and immune aggregation EM.

**RESULTS:** The recombinant baculovirus reBV/CE1E2 containing HCV C, E1, E2 genes and reBV/CS containing the same structural protein genes plus 5'NCR were constructed. The insect cells infected with either reBV/CE1E2 or reBV/CS expressed HCV C, E1 and E2 proteins with a molecular weight of 20 kD, 35 kD and 66 kD respectively. The results of immunoprecipitation and the immunoblotting revealed the coimmunoprecipitation of C, E1, and E2 proteins, indicating the interaction of HCV structural proteins expressed in insect cells. Electron microscopy of insect cells infected with reBV/CE1E2 or reBV/CS demonstrated spherical particles (40 to 60 nm in diameter) similar to the HCV virions from sera or hepatic tissues of HCV infected humans. The HCV-like particles were partially purified by sucrose gradient centrifugation, and the purified VLPs showed immuno-reactivity with anti-HCV antibodies.

**CONCLUSION:** HCV 5'NCR is not required for the assembly of HCV-like particles in insect cells, HCV core and envelope proteins are sufficient for viral particle formation.

Zhao W, Liao GY, Jiang YJ, Jiang SD. No requirement of HCV 5'NCR for HCV-like particles assembly in insect cells. *World J Gastroenterol* 2003; 9(10): 2226-2231
http://www.wjgnet.com/1007-9327/9/2226.asp

## INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of posttransfusion and sporadic non-A, non-B hepatitis. It is estimated that 170 million people worldwide are infected with HCV[1], more than 75 % of infected individuals develop a chronic infection, frequently with severe long-term pathologies such as cirrhosis and hepatocellular carcinoma[2]. Neither an effective treatment for chronic HCV infection nor a vaccine to prevent HCV infection is available at the present time.

HCV belongs to the genus *hepacivirus* of the *flaviviridae* family. Its genome is a 9.6-kb single-stranded RNA of positive polarity with a 5' noncoding region (5' NCR) that functions as an internal ribosome entry site, a single long open reading frame encoding a polyprotein of approximately 3 000 amino acids (aa) and a 3' NCR. This polyprotein is posttranslationally cleaved by host cell peptidases to yield structural proteins and by viral proteases, which generate nonstructural proteins. The three structural proteins, namely core (C) and envelope glycoproteins E1 and E2, are located within the amino-terminal region of the polyprotein. The nonstructural proteins (NS) 2 to 5B reside within the carboxyl-terminal part. By analogy with other *flaviviruses*, HCV virion is presumed to consist of a nucleocapsid or core protein and a viral genome coated by a lipid envelope containing glycoproteins E1 and E2. The study of HCV has been hampered by the low level of viral particles in infected individuals, the inability to propagate efficiently the virus in cultured cells, and the lack of a convenient animal model. Due to these obstacles, neither the structure of the virus nor the prerequisites for its assembly have been clearly defined. Synthesis of virus like particles (VLPs) in eukaryotic cells has opened up new possibilities for defining the structural requirements for viral particle assembly under natural intracellular conditions. In this way, by manipulating the sequence of the different proteins involved, it will be possible to define experimentally the essential morphogenic interactions required for the assembly process.

The assembly of VLPs has been reported for a number of viruses[3,4] with the most successful example of human *papillomavirus* (HPV). Baumert *et al*. have reported the recombinant *baculovirus* containing entire structural protein encoding sequences plus part of 5' NCR led to the expression and assembly of HCV-like particles in insect cells[5].

In the present study we reported the expression of HCV structural proteins in the presence or absence of HCV 5' NCR to investigate the requirement of 5' NCR for the assembly of HCV-like particles in insect cells.

## MATERIALS AND METHODS

### *Cloning of cDNAs encoding HCV structural proteins*

HCV cDNA was isolated from a HCV patient from Hebei Province, China, as previously described[6], and used as the amplifying template. cDNA fragments encoding HCV structural proteins were generated by PCR with the following primers: P1: 5'-ACAGATCTACCATGAGCACGAATCCTAAACC-3', P2: 5'-ACAGATCTACTCCACCATAGATCACTCCCC-3', P3: 5'-ATCAAGCTTACGCGTCTGCTAGTAGAAGGA-3',

P1 and P3, P2 and P3 were for CE1E2 and 5' NCR-CE1E2 respectively. A *Bgl*II site was introduced in P1 and P2 primers separately, a stop codon and a *Hin*dIII site were introduced in P3 primer. The correct sequences of CE1E2 and 5' NCR-CE1E2 were confirmed by DNA sequencing.

### *Baculovirus constructs and insect cell cultures*

For the construction of recombinant *baculoviruses*, a Bac-to-Bac *baculovirus* expression system (Gibco-BRL/Life Technologies) was applied. The *Bgl*II-*Hin*dIII digestion products of PCR fragments were subcloned into *Bam*HI-*Hin*dIII site (multiple cloning site) of *baculovirus* donor plasmid pFastBacI. After identification by restriction digestion and PCR, each of the recombinant plasmids was used to transform DH10Bac. Through Tn7 transposon-mediated site-specific transposition foreign gene expression cassette was integrated into a *baculovirus* shuttle vector (bacmid). The size of inserts was confirmed by PCR with the pUC/M13 amplification primers, which were directed at sequences on either side of the mini-*att*Tn7 site in the bacmid. The recombinant bacmids were used for transfection of Sf9 cells (*Spodoptera frugiperda*). Recombinant *baculoviruses* were harvested thereafter and purified by plaque screening. The recombinant *baculoviruses* were verified by PCR with CE1E2 and 5' NCR-CE1E2 gene specific primers and amplified by subsequent rounds of Sf9 cell infection until a final titer of $5\times10^7$ PFU/ml was achieved. Detailed methods for *baculovirus* manipulation were referred to the instruction manual. Sf9 insect cells were maintained in spinner or monolayer cultures at 27 ℃ in Grace' s medium (Gibco-BRL/Life Technologies) supplemented with 10 % fetal bovine serum.

### *Protein expression assay*

For all protein expression experiments, Sf9 cells in mid-log growth in monolayer cultures were infected with a multiplicity of infection (MOI) of 10. Infection of insect cells with non-recombinant *baculovirus* served as a negative control in all experiments. The expression of HCV structural proteins was analyzed at 72 h postinfection by immunofluorescence and SDS-PAGE. For immunofluorescence, cells were fixed with -20 ℃ acetone for 5 min. The primary antibody used was anti-HCV serum, and the secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody. Detailed methods were referred to Ausubel[7]. For SDS-PAGE, the cells were lysed by ultrasonic, the cell lysate was briefly centrifuged to remove cell debris and nuclei, and the supernatant was subjected to electrophoresis on a SDS-12 % polyacrylamide gel. Detailed methods were referred to Sambrook[8].

### *Immunoprecipitation and immunoblotting of HCV proteins*

The Immunoprecipitation starter packthe (Amersham Pharmacia Biotech) was used for immunoprecipitation analysis, details of the experimental procedures were referred to the manufacturer' s protocols. Briefly, at 96 h postinfection, cells were lysed with RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 0.1 % Nonidet P-40 (NP-40), 5 mM EDTA (pH 7.5), 0.5 % sodium deoxycholate (DOC), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell lysate was cleared of cell debris and nuclei by low-speed centrifugation (15 min at 15 000×g and 4 ℃) and immunoprecipitated with anti-E2 monoclonal antibody (MAb). The immunoprecipitated proteins were analyzed by electrophoresis on a 12 % polyacrylamide gel. After gel transfer to polyvinylidene difluoride membranes, the blots were probed with anti-C, anti-E2 MAb, or anti-HCV serum followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse (for anti-C and anti-E2 MAb) or anti-human (for serum) IgG antibody. Detailed methods for immunoblotting were referred to Sambrook[8].

### *Electron microscopy (EM) of VLPs in insect cells*

A flask (25 $cm^2$) of infected cells (MOI=10, at 96 h postinfection) was harvested, washed in PBS and centrifuged at 1 000 r/min for 5 min. The cell pellets were fixed with 3.8 % glutaraldehyde in PBS for 24 h at 4 ℃. After fixation, the cells were washed three times in PBS (8 h each time) at 4 ℃ and post-fixed with 1 % osmium tetroxide for 2 h at 4 ℃. The samples were then dehydrated with acetone of a series of graded concentration, and embedded in 1:1 acetone-Epon 812 for 30 min at room temperature, further embedded in Epon 812 overnight at 37 ℃, and then polymerized for 24 h at 60 ℃. Ultrathin sections were cut with LKBIII ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, washed, dried and finally examined under transmission electron microscope.

### *Purification of VLPs*

Cells were harvested at 96 h postinfection, All purification steps were carried out on ice. Insect cells infected with reBV/CS or reBV/CE1E2 (approximately $5\times10^7$ cells, grown in suspension) were lysed in 50 mM Tris, 50 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 0.1 % NP-40. Sonication of the lysate was performed. The lysate was homogenized and subjected to low-speed centrifugation (15 min at 4 ℃ and 15 000×g), and the supernatant was pelleted over a 30 % (wt/vol) sucrose (in 20 mM Tris, 150 mM NaCl [pH 7.4]) cushion (6 h at 4 ℃ and 150 000×g). The pellet was resuspended in 50 mM Tris, 100 mM NaCl (pH 7.4), homogenized, and subjected to a second sucrose gradient centrifugation. The resuspended pellet was layered onto a 20 % to 60 % (wt/wt) sucrose (in 50 mM Tris, 100 mM NaCl [pH 7.4]) gradient and centrifuged for 22 h at 4 ℃ and 150 000×g. Fractions (0.5 ml) were collected from the top and analyzed by immunoblotting.

### *Electron microscopy and immune aggregation electron microscopy of purified VLPs*

After the sucrose gradient centrifugation, VLPs were diluted in PBS (1:10), centrifuged for 2 h at 4 ℃ and 150 000×g, the pellet was resuspended in PBS. Anti-HCV serum (diluted 1:200 in PBS) was homogenized, incubated at 37 ℃ for 1 h, then stored at 4 ℃ overnight. Samples were centrifuged again for 1h at 4 ℃ and 150 000×g, the pellet was resuspended in PBS. Samples (3 ul) were absorbed on the surface of carbon-coated 300 mesh copper grids, and stained negatively with 2 % phosphotungustic acid for EM examination.

## RESULTS

### *Construction and identification of recombinant baculoviruses*

The fragment CE1E2 consisting of HCV C, E1, E2 encoding sequences and the fragment 5' NCR-CE1E2 containing the same structural protein genes plus 5' NCR were produced by PCR, with the size of 2 578 bp and 2 238 bp respectively (Figure 1). The correct sequences of CE1E2 and 5' NCR-CE1E2 were confirmed by DNA sequencing.

5' NCR-CE1E2 and CE1E2 were separately subcloned into *baculovirus* donor plasmid pFastBacI. The resulting recombinant plasmids pFB1-CS and pFB1-CE1E2 were verified (Figure 2) and used to transform DH10Bac. Through Tn7 transposon-mediated site-specific transposition foreign gene expression cassette was integrated into a *baculovirus* shuttle vector (bacmid). The result of PCR with M13/pUC forward and reverse primers indicted the correct insertion of 5' NCR-CE1E2 and CE1E2 fragments into bacmids (Figure 3),

and the resulting recombinant bacmids were named as bacmid/CS and bacmid/CE1E2 respectively. The non-recombinant bacmid was named as bacmid/0.

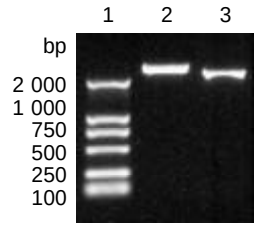



**Figure 1** PCR products of HCV structural protein genes. Lane1: DNA molecular weight marker (DL-2000), lane2: 5' NCR-CE1E2, lane3: CE1E2.

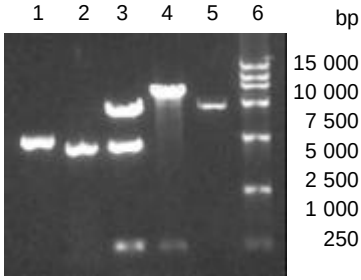



**Figure 2** Confirmation of recombinant *baculovirus* donor plasmids. Lane1: PCR of pFB1-CS, lane 2: PCR of pFB1-CE1E2, lane 3: pFB1-CS digested by *Xho*I / *Hin*dIII, lane 4: pFB1-CE1E2 digested by *Xho*I / *Hin*dIII, lane 5: pFastBacI digested by *Bam*HI / *Hin*dIII, lane 6: DL-15000 marker.

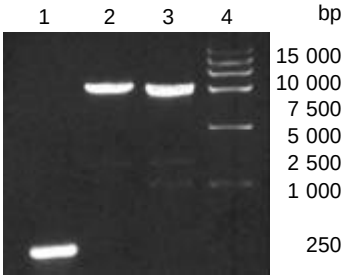



**Figure 3** Confirmation of recombinant bacmids by PCR. Lane 1: bacmid / 0 control, lane 2: bacmid / CS, lane 3: bacimd / CE1E2, lane 4: DL-15000 marker.

Bacmid/CS and bacmid/CE1E2 were separately used to transfect Sf9 cells to generate recombinant *baculoviruses*, yielding reBV/CS and reBV/CE1E2, respectively. Bacmid/0 was used to generate non-recombinant *baculovirus* control, BV/Bac.

A large number of *baculoviruses* could be observed in nuclei of the Sf9 cells transfected with bacmid DNAs (Figure 4).

The recombinant *baculoviruses* reBV/CS and reBV/CE1E2 were confirmed by PCR with 5' NCR-CE1E2 and CE1E2 specific primers (Figure 5).

### *Immunofluorescence of HCV proteins*

Either the recombinant *baculovirus* reBV/CS or reBV/CE1E2 directed the production of HCV structural proteins in insect cells, as demonstrated by immunofluorescence analysis of infected insect cells with anti-HCV antibodies (Figure 6). Immuno-staining was observed in the cells infected with recombinant *baculovirus* (Figures 6A and B). The anti-HCV antibodies used in this study did not display any cross-reactivity against insect cell or *baculovirus* proteins (Figure 6C).

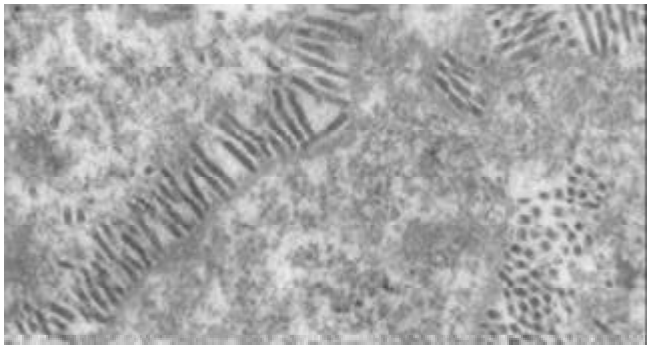


**Figure 4** *Baculoviruses* in nuclei of Sf9 cells transfected with recombinant bacmid DNA (×25 000).

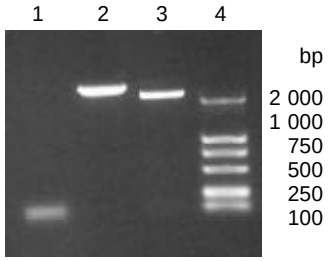



**Figure 5** Confirmation of recombinant *baculoviruses* by PCR. Lane 1: BV / Bac control, lane 2: reBV / CS, lane 3: reBV / CE1E2, lane 4: DL-2000 marker.

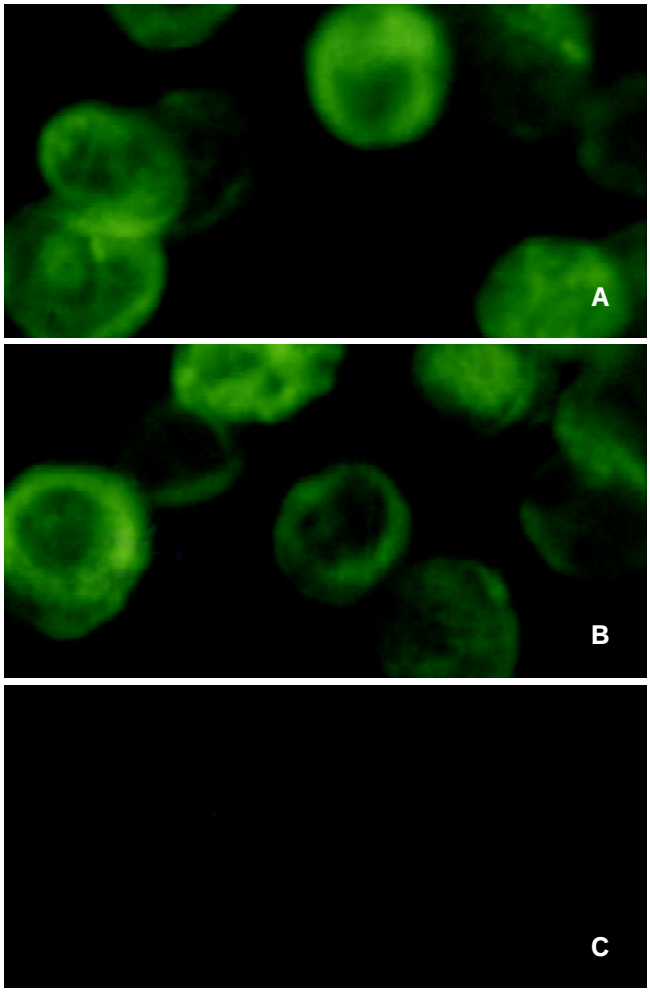



**Figure 6** Immunofluorescence analysis of expression of HCV structural proteins in Sf9 cells. Sf9 cells infected with A: reBV / CS, B: reBV / CE1E2, C: control BV / Bac.

### *HCV structural proteins expressed in insect cells*

SDS-PAGE analysis of lysates from Sf-9 cells infected with either reBV/CS or reBV/CE1E2 demonstrated 3 novel bands of the size of expected HCV E2, E1 and C proteins which were 66 kD, 35 kD and 20 kD respectively, which were not present in cells infected with non-recombinant *baculovirus* BV/Bac (Figure 7).

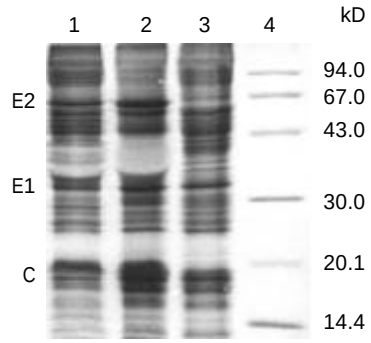



**Figure 7** Identification of HCV structural proteins expressed in Sf9 cells by SDS-PAGE. Lanes 1-2: cells infected with reBV/CS and reBV/CE1E2 respectively, lane 3: control cells infected with BV/Bac, lane 4: molecular weight marker.

### *Coimmunoprecipitation of C and E1 with E2 protein*

To further investigate the association of C, E1, and E2 proteins expressed in insect cells, cell extracts were immunoprecipitated with anti-E2 monoclonal antibody (MAb), and the precipitated proteins were separately probed with anti-C, anti-E2 MAb, or anti-HCV serum.

As shown in Figure 8, E2 protein was precipitated by anti-E2 MAb (Figure 8A), C protein could be detected in the immunoprecipitation complex when probed with anti-C MAb (Figure 8B), while C and E1 proteins could be detected together with E2 protein when probed with anti-HCV serum (Figure 8C). These results revealed the coimmunoprecipitation of C, E1 and E2 proteins.

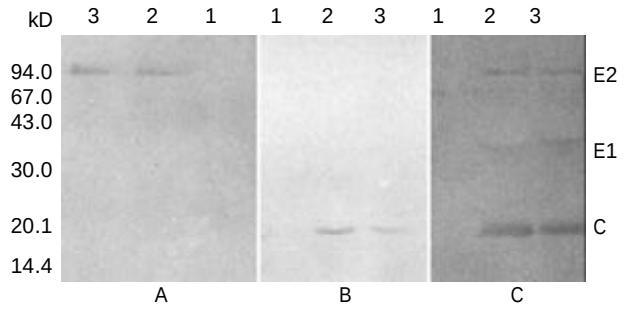



**Figure 8** Immunoblotting of anti-E2 antibody immunoprecipitated proteins with anti-E2 mAb (A), anti-C mAb (B) and anti-HCV serum from HCV-infected patients (C). Lane 1: control BV/Bac, lane 2: reBV/CE1E2, lane 3: reBV/CS.

### *Electron microscopy of infected Sf9 cells*

Transmission electron microscopy of the cells infected with either reBV/CS or reBV/CE1E2 revealed abundant virus-like particles in cytoplasm (Figures 9A and B). These particles (indicated by arrow "➡"), 40 to 60 nm in diameter, were polymorphic in appearance, many of them had unevenly distributed electron-dense structures suggestive of possible nucleocapsids. No such structures were observed in the control cells infected with BV/Bac (Figure 9C) in spite of the existence of abundant *baculoviruses* in the nuclei (indicated by arrow "➡").

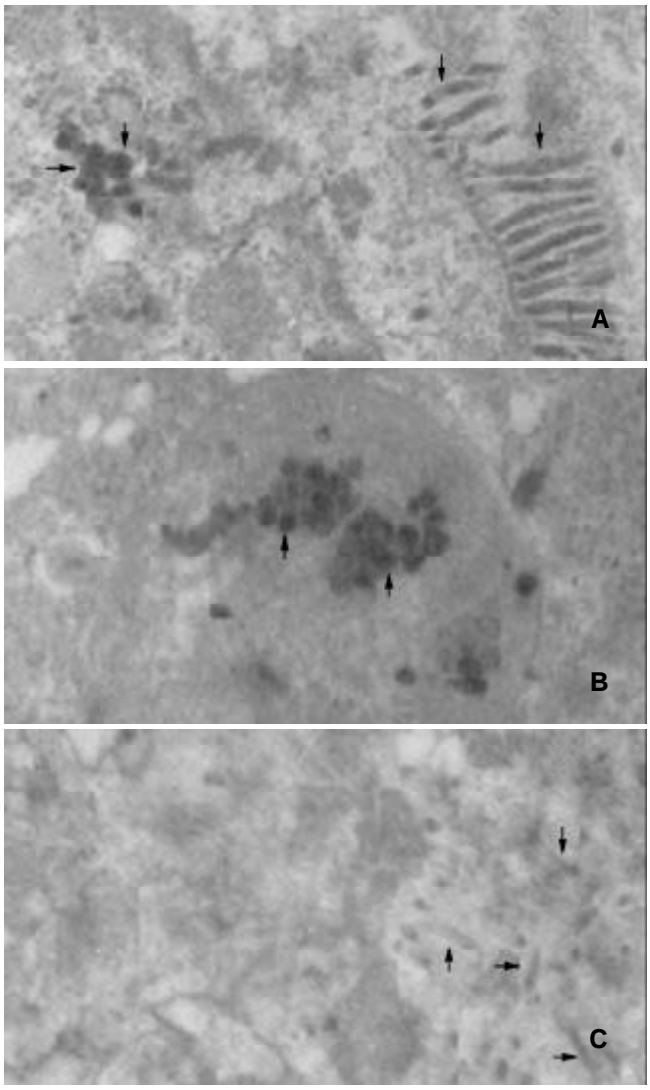



**Figure 9** Electron micrographs of *baculovirus*-infected Sf9 cells (×25 000). Sf9 cells infected with A: reBV/CS, B: reBV/CE1E2, C: control BV/Bac.

### *Purification and identification of VLPs*

VLPs were purified from large-scale cell cultures. The band corresponding to VLPs in sucrose gradient centrifugation was indicated in Figure 10. Portions of the VLPs were examined by EM which showed separate spherical particles similar to those seen in Sf9 cells infected with recombinant baculoviruses (Figures 11A and B), other portions of VLPs were examined by immune aggregation EM which revealed aggregated VLPs by anti-HCV serum (Figures 11C and D), indicating the immuno-reactivity of VLPs with anti-HCV antibodies.

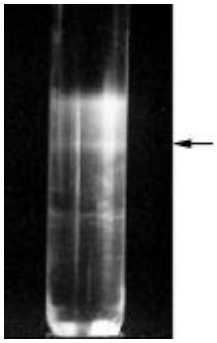


**Figure 10** VLP band (indicated by arrow) in sucrose gradient centrifugation.

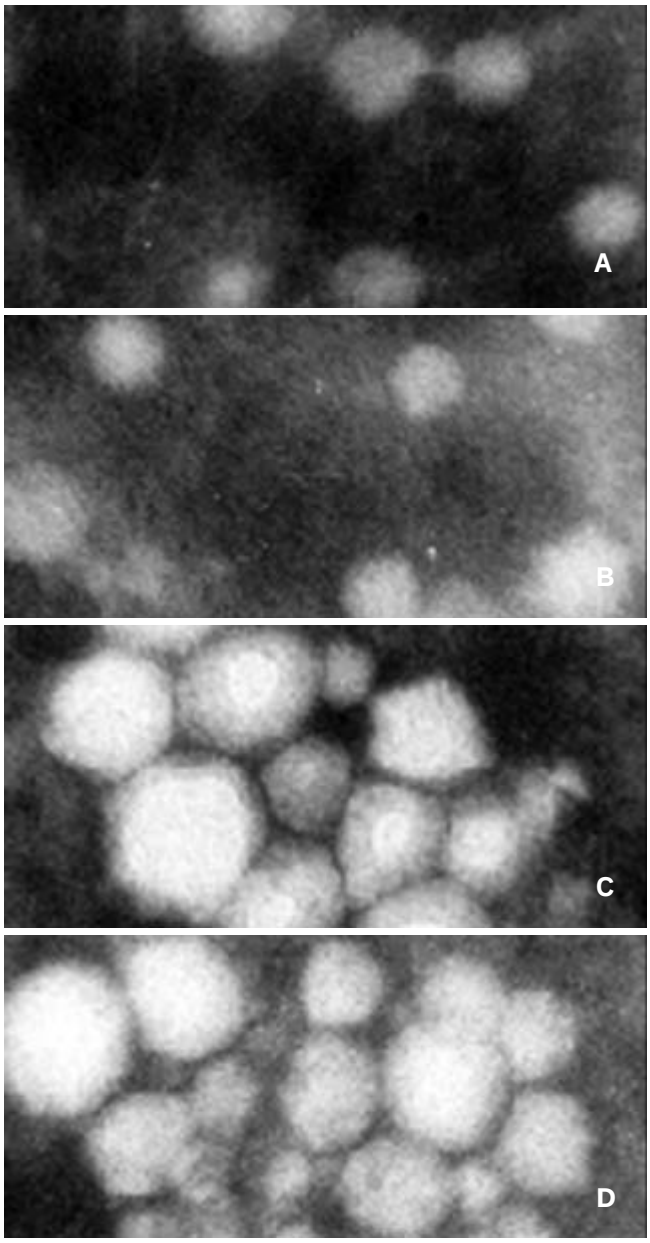



**Figure 11** Electron microscopy and immune aggregation electron microscopy analysis of VLPs (negative staining). A and B (×100 000): VLPs isolated from Sf9 cells infected with reBV/CS and reBV/CE1E2, respectively. C and D (×150 000): Immune aggregation of VLPs from A and B with anti-HCV serum, respectively.

## DISCUSSION

In this study, either in the presence or absence of HCV 5' NCR, the recombinant *baculovirus* (reBV/CS or reBV/CE1E2) efficiently expressed all the three HCV structural proteins, indicating the efficient cleavage of the polyproteins into individual proteins. The sizes of C, E1 and E2 proteins were 20 kD, 35 kD and 66 kD respectively, similar to those of HCV structural proteins expressed in mammalian cells[9], suggesting similar posttranslational processing of HCV structural proteins in insect and mammalian expression systems.

The interactions of HCV structural proteins expressed in insect cells were investigated by means of immunoprecipitation and immunoblotting. In addition to E2 protein, C and E1 proteins were also present in the immunoprecipitation complex (Figures 8B and C), indicating that C and E1 proteins were precipitated together with E2 protein by anti-E2 MAb. The result of immunoblotting of the immunoprecipitated proteins with anti-E2 MAb (Figure 8A) showed that the coimmunoprecipitation of the three structural proteins was not precipitated because there existed cross-reactivity between E2 MAb and C or E1 protein. So, we could conclude that the coimmunoprecipitation of C, E1 and E2 proteins was due to the association of the three structural proteins. These results extended the findings of previous studies which demonstrated interactions of E1 and E2 proteins[10] as well as C and E1 proteins[11], and provided immunological and biochemical evidences for the assembly of HCV structural proteins into VLPs in insect cells.

Detail ultrastructural features of HCV virions remain elusive since direct visualization of virus particles from infected serum and tissues has proven to be difficult. Filtration studies have estimated the virion particle size was 30 to 60 nm in diameter[12], Shimizu *et al*. detected VLPs with a diameter of approximately 50 nm in cytoplasm of the liver cells obtained during the acute phase of hepatitis C from a chimpanzee[13]. Kaito *et al*. reported visualization of 55 to 65 nm VLPs by immunogold electron microscopy with antibodies to HCV envelope protein[14]. Takahashi *et al*. observed by EM 55 nm VLPs in a gradient fraction when human plasma containing HCV was separated by potassium bromide density gradient centrifugation[15].

In our study, abundant spherical particles with the size of 40-60 nm in diameter were observed in the cytoplasm of Sf9 cells infected with either reBV/CS or reBV/CE1E2. Such particles could not be found in the cells infected with nonrecombinant *baculovirus* although the existence of abundant *baculoviruses* in the nuclei, indicating that VLPs in the infected cells were not correlated with the propagation of *baculovirus* in insect cells, but were resulted from the expression of HCV structural proteins in insect cells. Furthermore, VLPs isolated from recombinant *baculovirus* infected cells showed immuno-reactivity with anti-HCV antibodies, indicating that VLPs were derived from HCV proteins. Our data suggest that the morphology of HCV-like particles synthesized in insect cells is similar to the features described for HCV virions isolated from HCV-infected humans and chimpanzees. These results provided the morphological evidence for the assembly of HCV structural proteins into HCV-like particles.

In conclusion, either in the presence or absence of 5' NCR, the recombinant *baculovirus* containing HCV structural protein encoding sequences can direct the expression of correctly processed individual HCV structural proteins, which assemble into VLPs similar to HCV particles from sera or hepatic tissues of HCV infected humans or chimpanzees. HCV core and envelope proteins are sufficient for viral particle formation, 5' NCR is not required for the assembly of VLPs in insect cells.

## REFERENCES

1 **Cohen J.** The scientific Challenge of hepatitis C. *Science* 1999; **285**: 26-30
2 **Shimotohno K.** Hepatitis C virus and its pathogenesis. *Semin Cancer Biol* 2000; **10**: 233-240
3 **Schiller JT,** Lowy DR. Papillomavirus-like particle based vaccines: cervical cancer and beyond. *Expert Opin Biol Ther* 2001; **1**: 571-581
4 **Latham T,** Galarza JM. Formation of wild-type and chimeric influenza virus-like particles following simultaneous expression of only four structural proteins. *J Virol* 2001; **75**: 6154-6165
5 **Baumert TF,** Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* 1998; **72:** 3827-3836
6 **Bi SL,** Bai XH, Cong ME, Tian HW, Sun DG, Margolis HS, Liu CB. Primary Structure and Variation of Chinese Hepatitis C Virus Genome. *Bingdu Xuebao* 1993; **9**: 114-127

7 **Ausubel FM,** Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Short Protocols in Molecular Biology. 3rd ed. *John Wiley Sons, Inc* 1995: 23-29

8 **Sambrook J,** Fritsch EF, Maniatis T. Molecular cloning, A Laboratory Manual. 2nd ed. *Cold Spring Harbor Laboratory Press* 1989: 620-665

9 **Moradpour D,** Wakita T, Wands JR, Blum HE. Tightly regulated expression of the entire hepatitis C virus structural region in continuous human cell lines. *Biochem Biophys Res Commun* 1998; **246**: 920-924

10 **Patel J,** Patel AH, McLauchlan J. Covalent interactions are not required to permit or stabilize the non-covalent association of hepatitis C virus glycoproteins E1 and E2. *J Gen Virol* 1999; **80** (Pt 7): 1681-1690

11 **Ma HC,** Ke CH, Hsieh TY, Lo SY. The first hydrophobic domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein. *J Gen Virol* 2002; **83**(Pt 7): 3085-3092

12 **He LF,** Alling D, Popkin T, Shapiro M, Alter HJ, Purcell RH. Determining the size of non-A, non-B hepatitis virus by filtration. *J Infect Dis* 1987; **156**: 636-640

13 **Shimizu YK,** Feinstone SM, Kohara M, Purcell RH, Yoshikura H. Hepatitis C virus: detection of intracellular virus particles by electron microscopy. *Hepatology* 1996; **23**: 205-209

14 **Kaito M,** Watanabe S, Tsukiyama-Kohara K, Yamaguchi K, Kobayashi Y, Konishi M, Yokoi M, Ishida S, Suzuki S, Kohara M. Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* 1994; **75**(Pt 7): 1755-1760

15 **Takahashi K,** Kishimoto S, Yoshizawa H, Okamoto H, Yoshikawa A, Mishiro S. p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts. *Virology* 1992; **191**: 431-434

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2232-2235
World Journal of Gastroenterology
 ISSN 1007-9327



# Interobserver variation in histopathological assessment of *Helicobacter pylori* gastritis

Ozlem Aydin, Reyhan Egilmez, Tuba Karabacak, Arzu Kanik

**Ozlem Aydin, Reyhan Egilmez, Tuba Karabacak, Arzu Kanik,** Department of Pathology and Biostatistics, Medical School, Mersin University, Mersin-Icel, Turkey
**Correspondence to:** Ozlem Aydin, M.D. Department of Pathology, Medical School, Mersin University, 33079 Mersin- Icel, Turkey. ozlemaydin66@hotmail.com
**Telephone:** +90-324-3374300/1192 **Fax:** +90-324-3374305
**Received:** 2003-07-04 **Accepted:** 2003-08-19

## Abstract

**AIM:** Because the presence or absence of *H pylori* infection has important implications for therapeutic decisions based on histological assessment, the reproducibility of Sydney system is important. The study was designed to test the reproducibility of features of *Helicobacter pylori* gastritis, using the updated Sydney classification.

**METHODS:** Gastric biopsies of 40 randomly selected cases of *H pylori* gastritis were scored semiquantitatively by three pathologists. Variables analysed included chronic inflammation, inflammatory activity, atrophy, intestinal metaplasia, *H pylori*, surface epithelial damage. κ values below 0.5 represented poor, those between 0.5 and 0.75 good and values over 0.75 excellent interobserver agreement.

**RESULTS:** The best interobserver agreement (κ=0.62) was present for intestinal metaplasia. The agreement was the poorest for evaluating atrophy (κ=0.31).

**CONCLUSION:** Although the results of this study were in accordance with some previous studies, an excellent agreement could not be reached for any features of *H pylori* gastritis. This low degree of concordance is assumed to be due to the personal evaluation differences in grading the features, the lack of standardized diagnostic criteria, and the ignorance to reach a consensus about the methods to be used in grading the features of *H pylori* gastritis before initiating the study.

Aydin O, Egilmez R, Karabacak T, Kanik A. Interobserver variation in histopathological assessment of *Helicobacter pylori* gastritis. *World J Gastroenterol* 2003; 9(10): 2232-2235
http://www.wjgnet.com/1007-9327/9/2232.asp

## INTRODUCTION

Although gastritis was first interpreted to be due to aging and lifelong exposure to various insults, it is now clear that the most common cause of this inflammatory condition is infection with *H pylori*[1]. It has been shown[2] that this organism is strongly associated with chronic active gastritis as well as gastric adenocarcinoma and MALToma.

The Sydney system for grading and classifying chronic gastritis was devised to provide a standardized approach to the histologic interpretation of gastric biopsies in 1990[3,4], and it was later updated in 1994[5,6]. Although it was reported that the Sydney systems' weakness was that it was used in complex descriptions rather than true diagnosis[7]. After the updating of the Sydney classification, several studies on interobserver variation for the assessment of *H pylori* gastritis have been reported[6,8-11]. The evaluation of interobserver agreement by using kappa (κ)-statistics has been accepted by pathologists for several years[9].

Although the histologic examination of gastric biopsy specimens is accepted as the gold standard[12,13] for the diagnosis of *H pylori* gastritis, it has not been demonstrated that histopathologic assessment is both accurate and reproducible[9].

The study was designed to test the reproducibility of the features of *H pylori* gastritis, using the updated Sydney classification by κ-statistics.

## MATERIALS AND METHODS

Three pathologists participated in the study. One was a professor with primary interest in gastrointestinal pathology. The second was a 4th-year assistant professor in pathology. An other was an 18th-month pathology resident. The slides were examined independently, and also incombination with any clinical information by each of the pathologists.

### *Histologic evaluation*

From 130 cases diagnosed as *H pylori* gastritis in our department (Department of Pathology, Medical School, Mersin University.) in a period of 17 months, 40 [22 (55.0 %) female, 18 (45.0 %) male] were randomly selected for study, their age ranged from 23 to 72 years, with a mean of 47.2. The specimens were excluded from the study because they were insufficient in mucosal thickness for proper assessment of atrophy and without surface epithelium before the selection. Slides were coded using a computer generated list of random numbers.

Biopsy samples from the antrum and body were formalin-fixed and paraffin-embedded and cut into 2-3 μm sections which were stained using hematoxylin and eosin (H&E), and alcien blue/PAS for intestinal metaplasia. Five H&E sections were examined for each case. The biopsies were scored semiquantitatively by three pathologists according to the updated Sydney classification[14].

The updated Sydney system has a scale of 0-3 for scoring the features of chronic gastritis[14]. In order to improve assessment of minor degrees of alteration, a detailed histopathological classification was used, which also provides numerical data for statistical analysis[15]. At first, each variable was divided into seven subcategories, resulting in a score on a scale of 0-6. But the κ values could not be calculated using this classification. The 6 subcategories (excluding 0, none) were then amalgamated by pairs (none, 0; mild, 1-2; moderate, 3-4; severe, 5-6), but the calculation of the κ values was again impossible for the majority of variations using this classification, and the calculated values were found to be low. So, we came to a conclusion that the agreement between pathologists could be improved when a different amalgamated 3-point scale classification was used for each variable (Table 1).

**Table 1** Histologic features evaluated on each slide and score scale

| Histologic features | Score | Grading |
|---|---|---|
| Chronic inflammation | 0, none | 0, none |
| | 1, <10 cic*/HPF** | 1-2-3, mild |
| | 2, >10 cic/HPF | 4-5-6, moderate to marked |
| | 3, some areas with dense cic | |
| | 4, diffuse infiltration with dens cic | |
| | 5, nearly whole mucosa contains a dense cic | |
| | 6, entire mucosa contains a dense cic infiltrate | |
| Inflammatory activity | 0, none | 0, none |
| | 1, only one crypt involved/ biopsy | 1-2, mild |
| | 2, two crypts involved/ biopsy | 3-4-5-6, moderate to marked |
| | 3, many crypts (<25%) involved/ biopsy | |
| | 4, 25-50% of crypts involved/ biopsy | |
| | 5, >50% of crypts involved/ biopsy | |
| | 6, all crypts involved | |
| Atrophy | 0, none | 0, none |
| | 1, foci where a few gastric glands are lost or replaced by ie• | 1-2, mild |
| | 2, small areas in which gastric glands have disappeared or been replaced by ie | 3-4-5-6, moderate to marked |
| | 3, <25% of gastric glands lost or replaced by ie | |
| | 4, 25-50% of gastric glands lost or replaced by ie | |
| | 5, >50% of gastric glands lost or replaced by ie | |
| | 6, only a few small areas of gastric glands remaining | |
| Intestinal metaplasia | 0, none | 0, none |
| | 1, only one crypt replaced by ie | 1-2-3, mild |
| | 2, one focal area (1-4 crypts) in one of two biopsies | 4-5-6, moderate to marked |
| | 3, two separate foci | |
| | 4, multipl foci in one or both biopsies | |
| | 5,>50% of gastric epithelium diffusely replaced by ie | |
| | 6, only a few small area of gastric epithelium are not replaced by ie | |
| *H pylori* | 0, none | 0, none |
| | 1, *H pylori* found only in one place | 1-2-3-4, mild |
| | 2, only a few *H pylori* found | 5-6, moderate to marked |
| | 3, scattered *H pylori* found in separate areas/foci | |
| | 4, numerous *H pylori* in separate areas/foci | |
| | 5, nearly complete gastric surface covered by a layer of *H pylori* | |
| | 6, continuous gastric surface coverage by a thick layer of *H pylori* | |
| Surface epithelial damage | 0, none | 0, none |
| | 1, slight | 1-2-3-4, mild |
| | 2, mild deg♦ in the top of the epithelial cells | 5-6, moderate to marked |
| | 3, moderate deg with disorientation of the epithelial lining | |
| | 4, indistinct cell borders at the surface of the epithelium | |
| | 5, flattened epithelial cells with severe deg and enlarged nuclei | |
| | 6, flattened to erosive epithelium of the entire surface | |

*: Chronic inflammatory cells, **: High power field, •: Intestinal type epithelium, ♦: Degeneration.

**Table 2** Kappa values and their 95 % confidence intervals between three pathologists for *H pylori* gastritis

| Variable | Pairwise analysis between pathologists | | | | | |
|---|---|---|---|---|---|---|
| | 1:2 | | 1:3 | | 2:3 | |
| | Kappa | 95 % CI | Kappa | 95 % CI | Kappa | 95 % CI |
| Chronic inflammation | 0.49[a] | 0.13-0.85 | -0.34 | NS | 0.14 | NS |
| Inflammatory activity | 0.44[a] | 0.13-0.71 | -0.13 | NS | -0.27 | NS |
| Atrophy | 0.31[a] | 0.83-0.56 | 0.03 | NS | 0.14 | NS |
| Intestinal metaplasia | 0.51[a] | 0.25-0.85 | 0.52[a] | 0.20-0.80 | 0.62[a] | 0.40-0.85 |
| *H pylori* | 0.40[a] | 0.10-0.71 | 0.38[a] | 0.06-0.71 | 0.56[a] | 0.28-0.84 |
| Surface epithelial damage | -0.01 | NS | - | - | - | - |

NS: Non-significant, 95 % CI: 95 % confidence interval, -: Kappa statistics could not be done because data table had less than two rows or columns; [a]$P<0.05$.

### *Statistical analysis*

Interobserver agreement was analysed with the use of κ statistics (BMDP software: Cork, Ireland). The benchmarks suggested by Svanholm *et al*[16] were accepted. Values below 0.5 represented poor, those between 0.5 and 0.75 good and values over 0.75 excellent interobserver agreement. Only values greater than 0.5 were considered good enough for diagnostic reliability. Confidence interval was calculated for only statistically significant values.

## RESULTS

κ values and their 95 % confidence intervals between three pathologists for *H pylori* gastritis are shown in Table 2. On

blinded review of the coded slides the best interobserver agreement (κ=0.62, CI: 0.40-0.85) was present for intestinal metaplasia. The good agreement was reached in the assessment of the grade of *H pylori*, with κ value of 0.56 (CI: 0.28-0.84). The interobserver agreement was the poorest for evaluating atrophy (κ=0.31, CI: 0.13-0.56). Following atrophy, the two variables with poor agreement were chronic inflammation (κ=0.49, CI: 0.13-0.85) and inflammatory activity (κ=0.44, CI: 0.13-0.71).

There was an agreement among the three observers for only evaluating intestinal metaplasia and the grade of *H pylori*. There was no interobserver agreement among the three pathologists for the assessment of surface epithelial damage. An excellent agreement could not be reached in any features of *H pylori* gastritis in our study.

## DISCUSSION

Correct and reliable histological diagnosis of *H pylori* gastritis has a great influence on clinical practice as an indicator for therapy. Reliability in assessing intestinal metaplasia and atrophy in histological specimens was especially important because these changes were associated with an increased risk of gastric cancer[12,17-19]. Andrew *et al*[8] and Tepes *et al*[12] held that histopathology was a reliable diagnostic method for *H pylori* gastritis based on their results.

The best interobserver agreement was reached for intestinal metaplasia. The κ values were 0.51-0.62 (CI: 0.40-0.85). As in our study, others have also shown a good agreement for scoring intestinal metaplasia, with κ values varying from 0.54 (CI: 0.31-0.77) in the study by Tepes *et al*[12] to 0.73 in the study by Andrew *et al*[8]. However, our κ values were lower than those reported by Fiocca *et al*[20]; (κ=0.75-0.92). Although, the H&E stain has been the standard basis for recognition of intestinal metaplasia[21], we based our observations on the alcian blue/PAS in addition to H&E because of ease to identify the goblet cells.

In the present study, the grading of *H pylori* reached good reproducibility, with κ value of 0.56 (CI: 0.28-0.84). This result was consistent with the study of Fiocca *et al*[20] (κ=0.62), Andrew *et al*[8] (κ=0.74) and Tepes *et al*[12] (κ=0.43), but lower than the value reported by El-Zimaity *et al*[9] (κ=0.90). Our results have also confirmed that H&E was an adequate stain for the detection of *H pylori*. There was no need for an additional staining like Warthin-Starry to identify the organism.

The lack of explicit criteria for the diagnosis of normal gastric mucosa when mononuclear cells were present, made grading difficult[12]. Therefore, the κ value for assessment of the degree of chronic inflammation (κ=0.49, CI:0.13-0.85) using semiquantitative scoring was lower than that for intestinal metaplasia and for the grading of *H pylori* in the present study. Tepes *et al*[12], also found a κ value for chronic inflammation ranged from 0.39 to 0.53. Our result is also in accordance with those of Fiocca *et al*[20], who reported κ values ranging from 0.49 to 0.82 and Andrew *et al*[8] who reported κ value of 0.58.

The interobserver agreement was poor with κ value of 0.44 (CI: 0.13-0.71) for scoring neutrophil infiltration in gastric mucosa. This result was consistent with those of Tepes *et al*[12] (κ=0.28-0.41) and Andrew *et al*[8] (κ=0.69). But the interobserver agreements of the studies of El-Zimaity *et al*[9] (κ=0.80) and Fiocca *et al*[20] (κ=0.58-0.77) were better than ours. Inflammatory activity and *H pylori* infection were present together and when only neutrophils were discovered in the tissue specimen the pathologists should intensively search for some residual *H pylori*[22].

Recently, it has been shown in several studies that even experienced gastrointestinal pathologists had poor interobserver agreement over the assessment of gastric atrophy of *H pylori* gastritis[6,8-11]. In the present study, the interobserver agreement for the grade of atrophy was lower than that for the other gastritis features. As in our study (κ=0.31, CI:0.13-0.56), others have also shown the lowest agreement for scoring atrophy, with κ values varying from 0.42 in the study of Fiocca *et al*[20] to 0.51 in the study of Andrew *et al*[8]. Tepes *et al*[12] also found the lowest interobserver agreement for atrophy (κ=0.17-0.57). Although El-Zimaity *et al*[9] also found the poorest agreement for atrophy, with κ value ranged from 0.08 to 0.29, the agreement in our study for the evaluation of atrophy was still better.

Among the similar previous studies, the surface epithelial damage in *H pylori* gastritis has been evaluated in only the study of Chen *et al*[15]. They reached good to excellent reproducibility in grading this feature, with weighed κ values of 0.6 and 0.73. But there was no interobserver agreement between the three pathologists for the assessment of surface epithelial damage in our study. Although the Sydney classification has been used routinely, the surface epithelial damage in *H pylori* gastritis have not been evaluated in our department until the present study was designed. It is suggested that the reason of this disagreement may be the lack of our experience in evaluating epithelial damage.

The results of this study suggest that assessment of many histopathologic features of *H pylori* infection have a low degree of concordance. Interobserver variation has been rather high in this study as in some other studies[9,12,23]. This may be due to the discrepancies in the semiquantitative evaluation of the features of *H pylori* gastritis, or due to the observations of the pathologists. Essentially, a perfect agreement by pathologists was practically impossible because pathology results were based on subjective interpretation of different features and classification, and numerous studies on the reproducibility of histopathologic data have reached similar conclusion. Pathologists could usually agree in the presence or absence of a particular histological characteristic, but were seldom consistent when they estimated its degree[24-27].

In the present study, the best interobserver agreement was reached between the assistant professor and the pathology resident, suggesting that the scale of the score is more important than experiences.

Because of the level of agreement in the presence or absence of *H pylori* infection had important implications for therapeutic decisions based on histological assessment[8], reproducibility of Sydney system is important. The updated Sydney system for scoring *H pylori* gastritis is useful and reproducible, but it needs to be improved in the criteria for grading the histologic features[15]. The lack of standardized diagnostic criteria is likely to have contributed significantly to the poor interobserver agreement found in certain features such as atrophy[9] as in our study. More exact criteria will probably further improve the interobserver agreement in assessing the histologic features, but some interobserver variability will probably persist because of the subjectivity that has been part of all semiquantitative grading systems[12]. The point that where cases were reviewed and numerical parameters were established was the best strategy to improve diagnostic concordance between pathologists[28].

Although, the results of this study were in accordance with some previous studies, an excellent agreement could not be reached for any features of *H pylori* gastritis. In conclusion, this unexpectedly low degree of concordance is assumed to be due to the personal evaluation differences in grading the features, and the lack of the standardized diagnostic criteria, as well as the ignorance to reach a consensus about the methods to be used in grading the features of *H pylori* gastritis before initiating the study.

## REFERENCES

1 **Soll AH**. Gastritis and *Helicobacter pylori*. In: Goldman L,

Bennett JC, eds. Cecil Textbook of Medicine, 21st ed. Philadelphia: *Saunders* 2000: 643-767
2 **Peterson WL**, Graham DY. *Helicobacter pylori*. In: Feldman M, Scharschmidt BF, Sleisenger MH, eds. Sleisenger&Fordtran's Gastrointestinal and Liver Disease. Pathophysiology (Diagnosis) Management, 6th ed. Philadelphia: *Saunders* 1998: 604-620
3 **Misiewice JJ**, Tytgat GNJ, Goodwin CS. The Sydney system: a new classification of gastritis. *J Hepatol Gastroenterol* 1991; **6**: 209-222
4 **Owen DA**. The Stomach. In: Sternberg SS, ed. Diagnostic Surgical Pathology. 3rd ed. Philadelphia: *Lippincott Williams Wilkins* 1999: 1311-1349
5 **Genta RM**, Dixon MF. The Sydney system revisited: the Houston international gastritis workshop. *Am J Gastroenterol* 1995; **90**: 1039-1041
6 **Genta RM**. *Helicobacter pylori*, inflammation, mucosal damage, and apoptosis: pathogenesis and definition of gastric atrophy. *Gastroenterology* 1997; **113**(Suppl): 551-555
7 **Fenoglio-Preiser CM**, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB, Rilke FO. The Nonneoplastic Stomach. In: Fenoglio-Preiser CM, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB, Rilke FO, eds. Gastrointestinal Pathology An Atlas and Text, 2nd ed. Philadelphia: *Lippincott-Raven* 1999: 153-237
8 **Andrew A**, Wyatt JI, Dixon MF. Observer variation in the assessment of chronic gastritis according to the Sydney system. *Histopathol* 1994; **25**: 317-322
9 **el-Zimaity HM**, Graham DY, al-Assi MT, Malaty H, Karttunen TJ, Graham DP, Huberman RM, Genta RM. Interobserver variation in the histopathological assessment of *Helicobacter pylori* gastritis. *Hum Pathol* 1996; **27**: 35-41
10 **Alhomsi MF**, Adeyemi EO. Grading *Helicobacter pylori* gastritis in dyspeptic patients. *Comp Immunol Microbiol Infect Dis* 1996; **19**: 147-154
11 **van Grieken NC**, Weiss MM, Meijer GA, Bloemena E, Lindeman J, Offerhaus GJ, Meuwissen SG, Baak JP, Kuipers EJ. Rapid quantitative assessment of gastric corpus atrophy in tissue sections. *J Clin Pathol* 2001; **54**: 63-69
12 **Tepes B**, Ferlan-Marolt V, Jutersek A, Kaucic B, Zaletel-Kragelj L. Interobserver agreement in the assessment of gastritis reversibility after *Helicobacter pylori* eradication. *Histopathology* 1999; **34**: 124-133
13 **Genta RM**. Pathology of *Helicobacter pylori* infection. In: Weinstein RS, ed. Advances in Pathology an Laboratory Medicine. *St. Louise, Mosby* 1994: 443-465
14 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis, the updated Sydney system. *Am J Surg Pathol* 1996; **20**: 1161-1181
15 **Chen XY**, Hulst RWM, Bruno MJ, van der Ende A, Xiao SD, Tytgat GN, Ten Kate FJ. Interobserver variation in the histopathological scoring of *Helicobacter pylori* related gastritis. *J Clin Pathol* 1999;**52**: 612-615
16 **Svanholm H**, Starklint H, Gundersen HJ, Fabricius J, Bartebo H, Olsen S. Reproducibility of histomorphologic diagnoses with special reference to kappa statistics. *APMIS* 1989; **97**: 689-698
17 **Correa P**. Human gastric carcinogenesis: a multistep and multifactorial process. First american cancer society award lecture on cancer epidemiology and prevention. *Cancer Res* 1992; **52**: 6735-6740
18 **Sipponen P**. Gastric cancer-a long term consequence of *Helicobacter pylori* infection? *Scand J Gastroenterol Suppl* 1994; **201**: 24-27
19 **Meining A**, Stolte M. Close correlation of intestinal metaplasia and corpus gastritis in patients infected with *Helicobacter pylori*. *Z Gastroenterol* 2002; **40**: 557-560
20 **Fiocca R**, Villani L, Cornaggia M. Interobserver variation in the assessment of *H pylori* gastritis [abstract]. *Gut* 1996; **Suppl 2**: A104-105
21 **Segura DI**, Montero C. Histochemical characterization of different types of intestinal metaplasia in gastric mucosa. *Cancer* 1983; **52**: 498-503
22 **Genta RM**, Lew GM, Graham DY. Changes in the gastric mucosa following eradication of *Helicobacter pylori*. *Mod Pathol* 1993; **6**: 281-289
23 **Landis JR**, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**: 159-174
24 **Riddell RH**, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC, Sommers SC, Yardley JH. Displasia in inflammatory bowel disease: Standardized classification with provisional clinical application. *Hum Pathol* 1983; **14**: 931-968
25 **Reid BJ**, Haggitt RC, Rubin CE, Roth G, Surawicz CM, Van Belle G, Lewin K, Weinstein WM, Antonioli DA, Goldman H, Mac Donaid W, Owen D. Observer variation in the diagnosis of dysplasia in Barrett's esophagus. *Hum Pathol* 1988; **19**: 166-178
26 **Dawson A**, Ibrahim NB, Gibbs AR. Observer variation in the histopathological classification of thymoma: Correlation with prognosis. *J Clin Pathol* 1994; **47**: 519-523
27 **Sorensen JB**, Hirsch FR, Gazdar A, Olsen JE. Interobserver variability in histopathologic subtyping and grading of pulmonary adenocarcinoma. *Cancer* 1993; **71**: 2971-2976
28 **Guarner J**, Herrera-Goepfert R, Mohar A, Sanchez L, Halperin D, Ley C, Parsonnet J. Interobserver variability in application of the revised Sydney classification for gastritis. *Hum Pathol* 1999; **30**: 1431-1434

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2236-2239
World Journal of Gastroenterology
 ISSN 1007-9327



# Gastric polypoid lesions: Analysis of 150 endoscopic polypectomy specimens from 91 patients

Rasim Gencosmanoglu, Ebru Sen-Oran, Ozlem Kurtkaya-Yapicier, Erol Avsar, Aydin Sav, Nurdan Tozun

**Rasim Gencosmanoglu, Ebru Sen-Oran,** the Unit of Surgery, Institute of Gastroenterology, School of Medicine, Marmara University, Istanbul, Turkey
**Ozlem Kurtkaya-Yapicier, Aydin Sav,** Department of Pathology, School of Medicine, Marmara University, Istanbul, Turkey
**Erol Avsar, Nurdan Tozun,** the Unit of Gastroenterology, Institute of Gastroenterology, School of Medicine, Marmara University, Istanbul, Turkey
**Correspondence to:** Rasim Gencosmanoglu, M.D., Unit of Surgery, Institute of Gastroenterology, Marmara University,Basibuyuk, Maltepe, PK:53, TR-81532, Istanbul, Turkey. rgencosmanoglu@marmara.edu.tr
**Telephone:** +90-216-383-3057 **Fax:** +90-216-399-9912
**Received:** 2003-06-16 **Accepted:** 2003-08-02

## Abstract

**AIM:** To analyze gastric polypoid lesions in our patient-population with respect to histopathologic features and demographic, clinical, and endoscopic characteristics of patients.

**METHODS:** Clinical records and histopathologic reports of patients with gastric polypoid lesions were analyzed retrospectively. All lesions had been totally removed by either endoscopic polypectomy or hot biopsy forceps. The histopathologic slides were re-evaluated by the same histopathologist.

**RESULTS:** One-hundred and fifty gastric polypoid lesions were identified in 91 patients. There were 53 (58 %) women and 38 (42 %) men with a median age of 53 (range, 31 to 82) years. The most frequent presenting symptom was dyspepsia that was observed in 35 (38.5 %) patients. Symptoms were mostly related to various associated gastric abnormalities such as chronic gastritis or *H pylori* infection rather than polypoid lesion itself. Polypoid lesions were commonly located in the antrum followed by cardia. Out of 150 lesions, 80 (53 %) had the largest dimensions less than or equal to 5 mm and only 7 were pedunculated. The frequencies of hyperplastic polyps, foveolar hyperplasia, and fundic gland polyps were 46 %, 18 %, and 14 % respectively. We also detected gastritis varioliformis in 12 specimens, lymphoid follicles in 9, 4 adenomatous polyps in 4, polypoid lesions with edematous mucosa in 4, inflammatory polyps in 3, and carcinoid tumor in 1. Adenomatous changes were observed within two hyperplastic polyps and low grade dysplasia in one adenoma. Histopathologic evaluation of the surrounding gastric mucosa demonstrated chronic gastritis in 72 (79 %) patients and *H pylori* infection in 45 (49 %).

**CONCLUSION:** Hyperplastic polyps are the most frequently encountered subtype of gastric polypoid lesions. They are usually associated with chronic gastritis or *H pylori* gastritis. Contrary to the previous belief, they may harbour adenomatous changes or dysplastic foci. Therefore, endoscopic polypectomy seems as a safe and fast procedure for both diagnosis and treatment of gastric polypoid lesions at the same session. In addition, edematous mucosa may appear misleadingly as a polypoid lesion in some instances and it can be ruled out only by histopathologic examination.

Gencosmanoglu R, Sen-Oran E, Kurtkaya-Yapicier O, Avsar E, Sav A, Tozun N. Gastric polypoid lesions: Analysis of 150 endoscopic polypectomy specimens from 91 patients. *World J Gastroenterol* 2003; 9(10): 2236-2239
http://www.wjgnet.com/1007-9327/9/2236.asp

## INTRODUCTION

The frequency of gastric polyps is gradually increasing due to widespread use of endoscopic examinations[1,2]. Any discrete lesion protruding into the lumen of gastrointestinal (GI) tract appeared at endoscopy is called as "polypoid lesion"[3]. However, a polyp is defined as a proliferative or neoplastic lesion of gastrointestinal mucosal layer[3]. Although endoscopic appearances of some polyps may be diagnostic, the term "polyp" should not be used for every discrete protrusions identified at endoscopy unless histopathologically confirmed[4,5]. According to the classification suggested by Oberhuber and Stolte[4], gastric polypoid lesions are divided into five distinct groups: non-neoplastic polyps such as hyperplastic polyp, hamartomatous polyps such as Peutz-Jeghers polyps, heterotopic tissue polyps such as heterotopic pancreas, neoplastic polyps such as adenoma, and reactive polypoid lesions such as foveolar hyperplasia. In their series, hyperplastic polyps and fundic gland polyps are the two most common subtypes of gastric polypoid lesions[4].

In this study, the demographic, clinical, and endoscopic characteristics of patients with gastric polypoid lesions were presented. The histopathologic features of these lesions were also reported and their malignancy potential is discussed.

## MATERIALS AND METHODS

Patients with gastric polypoid lesions detected by upper gastrointestinal endoscopy at the Institute of gastroenterology, Marmara University, between January 1998 and December 2002 were analyzed retrospectively. All gastroscopies were performed under sedation with intravenous midazolam and topical pharyngeal anesthesia with 10 % lidocaine. The location, shape, size, and surface appearance of each polypoid lesion were assessed. Polypoid lesions were totally removed either by endoscopic polypectomy with an attempt or by hot biopsy forceps with multiple attempts according to their size. In addition, multiple biopsies were collected from both antrum and incisura angularis for *Helicobacter pylori* (*H pylori*) urease test and from surrounding gastric mucosa to detect any associated histopathologic changes such as chronic gastritis, *H pylori* infection, or intestinal metaplasia. Endoscopic records were screened for any complication resulted from polypectomy.

An experienced pathologist (O. K-Y.) re-evaluated all the tissue samples to confirm the histopathologic subclassification of gastric polypoid lesions in our series according to the classification suggested by Oberhuber and Stolte[4].

## RESULTS

### *Demographic features*

One hundred and fifty gastric polypoid lesions were identified in 91 consecutive patients from 2 630 (3.4 %) upper GI endoscopies. There were 53 (58 %) women and 38 (42 %) men with slightly female predominance (1.4:1). The ages of patients ranged from 31 to 82 years with a median of 53.

### *Clinical features*

The most frequent presenting symptom was dyspepsia (38.5 %) followed by epigastric pain (27.5 %), and anemia (11 %). Symptoms were usually related to the associated gastric abnormalities such as gastritis rather than polypoid lesion itself. Exceptionally, there was an antral hyperplastic polyp causing intermittent gastric outlet obstruction in one case in which the problem was solved with its removal by endoscopic polypectomy. The patient was symptom-free in the 8 months of follow-up.

### *Endoscopic features*

Characteristics of the gastric polypoid lesions are shown in Table 1. Out of 150 gastric polypoid lesions, 61 (40.6 %) were located in the antrum followed by 40 (26.6 %) in cardia, 34 (22.6 %) in corpus, and 13 (8.6 %) in fundus. In two patients who had previously undergone partial gastrectomy, the lesions were detected around anastomoses (1.2 %). Only 7 out of 150 (4.6 %) lesions were pedunculated (Figure 1A), while the remainder was sessile.

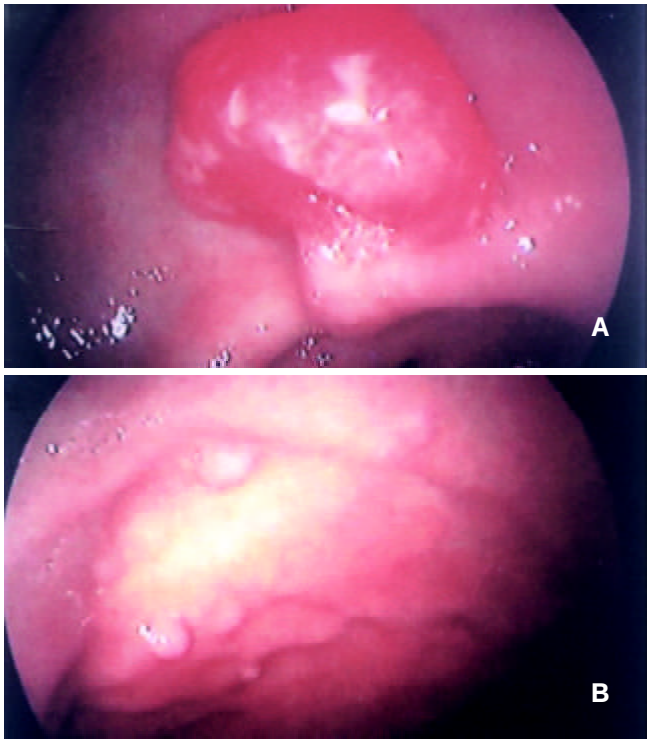



**Figure 1** Endoscopic views of pedunculated gastric polyp (A) and multiple gastric polyps (B).

Eighty (53 %) gastric polypoid lesions with the largest dimensions less than or equal to 5 mm were totally removed by hot biopsy forceps. In the remainder, the largest dimension ranged from 6 to 30 mm and all lesions but one were excised by snare polypectomy. One lesion was initially evaluated by forceps biopsy because it was suspected to be a submucosal lesion. After a definite histopathologic diagnosis of carcinoid tumor was established in this patient, it was totally removed by snaring. Among 17 (11 %) polypoid lesions with surface erosion, 5 were larger than 10 mm. Multiple lesions were detected in 29 (32 %) patients (Figure 1B). No complication developed following the endoscopic procedure in any patient.

**Table 1** Characteristics of gastric polypoid lesions

| Characteristics | Number* (%) |
|---|---|
| Location | |
| Antrum | 61 (40.7%) |
| Cardia | 40 (26.7%) |
| Corpus | 34 (22.7%) |
| Fundus | 13 (8.7%) |
| Peri-anastomotic | 2 (1.2%) |
| Shape | |
| Sessile | 143 (95.3%) |
| Pedunculated | 7 (4.7) |
| Size | |
| <5 mm | 80 (53.4%) |
| 5-10 mm | 51 (34.0%) |
| 10-20 mm | 17 (11.4%) |
| 20-30 mm | 2 (1.2%) |
| Histologic subtypes | |
| Hyperplastic polyp | 69 (46.0%) |
| Foveolar hyperplasia | 27 (18.0%) |
| Fundic gland polyp | 21 (14.0%) |
| Gastritis varioliformis | 12 (8.0%) |
| Lymphoid follicles | 9 (6.0%) |
| Adenoma | 4 (2.7%) |
| Edematous mucosa | 4 (2.7%) |
| Inflammatory polyp | 3 (2.0%) |
| Carcinoid tumor | 1 (0.6%) |

*Number of lesions.

### *Histopathologic features of the lesions*

The most frequently encountered histopathologic subtype of gastric polypoid lesions was hyperplastic polyp (Figure 2A) which was diagnosed in 46 % of the lesions, followed by foveolar hyperplasia (Figure 2B), fundic gland polyp (Figure 2C), and gastritis varioliformis (Table 2). Three of 43 patients with hyperplastic polyp had adenomas (Figure 2D) as well. Interestingly, the lesions ranging from 4 to 15 mm in diameter were observed as polypoid in 4 patients at endoscopy, but no pathologic changes except an edematous mucosa were determined in samples of endoscopic polypectomy. On the other hand, adenomatous changes were noted within two hyperplastic polyps. Out of 150 lesions, low grade dysplasia was found in one adenoma.

**Table 2** Histopathologic features of surrounding gastric mucosa in patients with gastric polyp

| Surrounding gastric mucosa | Type of Polyp | | | |
|---|---|---|---|---|
| | Hyperplastic | Fundic gland | Adenomatous | Inflammatory |
| Normal | 10 | 3 | 1 | - |
| Chronic gastritis | 33 | 1 | 3 | 3 |
| *H pylori* gastritis | 19 | 1 | 1 | 1 |
| Intestinal metaplasia | 12 | - | 3 | 1 |

Evaluation of the surrounding gastric mucosa revealed that 72 (79 %) patients had chronic gastritis, 45 (49 %) associated *H pylori* infection, and 33 (36 %) intestinal metaplasia, while the remainder (21 %) had normal gastric mucosa (Table 2).

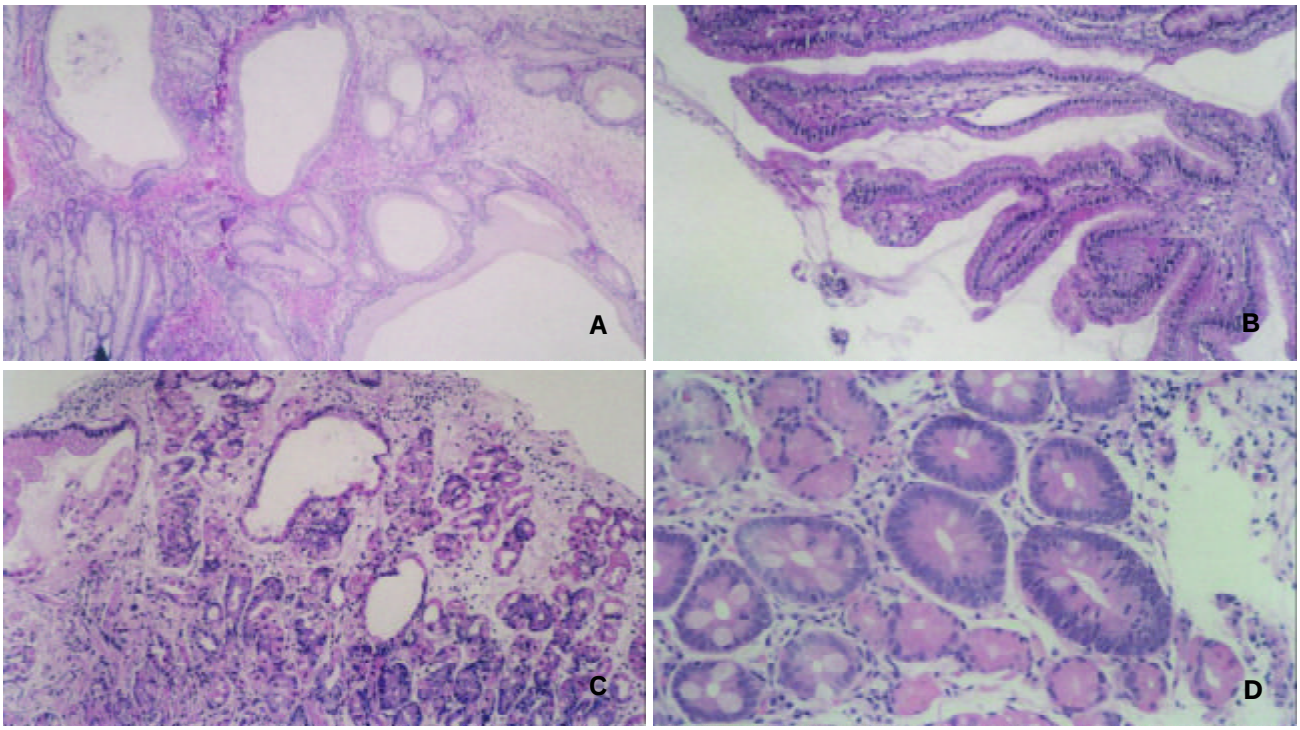



**Figure 2** Microscopic pictures of gastric hyperplastic polyp, H&E, X40 (A), foveolar hyperplasia, H&E, X100 (B), fundic gland polyp, H&E, X100 (C), and tubular adenoma,H&E, X200 (D).

The average size of hyperplastic polyps was 8 mm in diameter. However, there was quite a large, hyperplastic polyp 30 mm in diameter, in one patient, and it caused gastric outlet obstruction by plugging the pyloric channel because of its antral location. They were preferentially located in the antrum (42 %) and out of 69 hyperplastic polyps, 5 (7 %) were pedunculated. Only fourteen patients (32 %) had multiple hyperplastic polyps, while all the fundic gland polyps were multiple. Coexistent gastric abnormalities such as chronic gastritis, *H pylori* infection, and intestinal metaplasia were determined in 33 (77 %), 19 (44 %), and 12 (28 %) patients, respectively.

## DISCUSSION

The incidence of gastric polyps was 2 % in 2 630 patients who underwent upper GI endoscopic examination at our institution. Our rate was very similar to previously reported frequencies of 2-3 %[4]. However, the overall incidence of gastric polypoid lesions in our patient-population was 3.4 %. Gastric polyps were usually seen as small and sessile polypoid lesions at endoscopy in patients over 40 years old[6]. Although a vast majority of gastric polyps were asymptomatic, they might cause epigastric pain, gastrointestinal bleeding, and symptoms of gastric outlet obstruction[6-8]. The complication risk was generally related to polyp size. We detected only two polyps larger than 2 cm in diameter in our series. A relationship between the presenting symptom and the polyp itself was observed only in one lesion as detailed above. Our results support the fact that underlying gastric abnormalities are likely to be responsible factors for presenting symptoms in patients with gastric polyps.

While polypectomy was performed either endoscopically or surgically in the treatment of symptomatic gastric polyps, there were still no standardized guidelines concerning the management of asymptomatic ones[5,9]. Many endoscopists first took forceps biopsies from gastric polypoid lesions. After obtaining a definite histopathologic diagnosis, they performed polypectomy to these lesions[5]. However, forceps biopsies carried the risk of missing the neoplastic foci within polyp, since only a small portion of the lesion was sampled in this technique[10]. Seifert and Elster[11] compared histopathologic results of biopsy materials and polypectomy specimens of same lesions and showed a remarkable discrepancy between them in 70 % of cases. With this observation, the authors recommended to remove all gastric polyps larger than 5 mm in diameter by an experienced endoscopist[10,12]. In our study, all lesions except one, which was likely to be a submucosal lesion, were totally removed by either snare or hot biopsy forceps without any complication. Endoscopic polypectomy seems as a safe and fast procedure for both diagnosis and treatment of gastric polypoid lesions at the same session.

Some authors reported hyperplastic polyps or fundic gland polyps as the most frequently encountered subtypes of gastric polyps[13-16]. These lesions have previously been accepted as completely benign. However, some current studies showed that they might include adenomatous changes or dysplastic areas[4,7,13,17]. The reported frequencies of dysplastic foci within hyperplastic polyps showed a wide range (4-22 %)[18,19]. Hizawa[20] and Zea-Iriarte[21] found the incidence of malignancy in hyperplastic polyps was 2 % and 1.8 %, respectively. Since hyperplastic polyps were usually associated with chronic gastritis, particularly autoimmune gastritis and *H pylori* gastritis, patients with hyperplastic polyp had an increased risk of synchronous or metachronous adenocarcinomas elsewhere in the stomach[2,4,22]. In a series including 21 patients with hyperplastic polyps, chronic atrophic gastritis was found in all of them and *H pylori* gastritis in 16[16]. On the other hand, the rate of gastric cancer development in nonpolypoid mucosa was reported as 3.3 % in another study of 2 036 hyperplastic polyps[23]. In our series, the presence of chronic gastritis and *H pylori* gastritis was demonstrated in 76 % and in 45 % of hyperplastic polyps, respectively. In addition, two of 69 hyperplastic polyps contained adenomatous changes. These results support the suggestion of removing polypoid lesions instead of taking biopsy from them if feasible, when they are observed at endoscopy.

Gastric adenomas, similar to hyperplastic polyps, were usually detected in pathologically abnormal gastric mucosa

such as gastritis or intestinal metaplasia[13,16]. The malignant potential of gastric adenomas was related to their histologic features, size, shape, and surface appearance[24]. While malignancy risk of adenomas smaller than 2 cm in diameter ranged from 1 % to 5 %, it was higher than 50 % in larger ones[8]. The risk of gastric cancer in other parts of gastric mucosa in patients with adenoma varied between 8 % and 59 %[4]. Contrarily, fundic gland polyps were almost always associated with normal gastric mucosa and sometimes with long-term use of omeprazole[4,13]. They are divided into two different forms: sporadic form and syndromic form, which are associated with familial adenomatous polyposis (FAP) and attenuated variants. In a series including 319 fundic gland polyps from the John Hopkins Hospital, the FAP-associated polyps tended to include low grade dysplasia more than the sporadic ones (25 % *versus* 1 %)[25]. Furthermore, patients with fundic gland polyp might have an increased risk of harbouring a colorectal adenoma or carcinoma[4,23]. Nevertheless, prospective studies are necessary to ascertain whether fundic gland polyp is a sign for colorectal tumours or not. In our study, 75 % of patients with adenoma had chronic gastritis and intestinal metaplasia, whilst gastritis was detected solely in one patient with fundic gland polyps.

Foveolar hyperplasia was the second most common type of gastric polypoid lesions in our series. There is still a debate on whether foveolar hyperplasia is a precursor of hyperplastic polyps[4,23]. The most important distinction between the two lesions was based on the presence of neoplastic foci. Orlowska and associates[26] analyzed 751 hyperplastic lesions and did not find carcinoma in any of foveolar hyperplasia either by primer biopsy or at follow-up. It is wise to separate foveolar hyperplasia and hyperplastic polyp from each other as two distinct subtypes of foveolar hyperplastic lesions, since foveolar hyperplasia has no malignant potential.

We determined that four lesions with polypoid appearance at endoscopy were actually areas of edematous mucosa by histopathological examination. This observation showed that a lesion protruding into the gastric lumen might appear as polypoid lesion even it was not a true polyp. In order to prevent diagnostic confusion, the term "polyp" should be used after achieving exact histopathologic diagnosis as well-supported by the above instance.

In summary, hyperplastic polyp is the most frequently encountered subtype of gastric polypoid lesions. It is usually associated with chronic gastritis or *H pylori* gastritis. Hyperplastic polyps have been described as harmless lesions until recently. However, risk of their malignant transformation has been emphasized in the current literature. For this reason, endoscopic polypectomy seems to be a sensible diagnostic and therapeutic procedure of hyperplastic polyps when feasible. Finally, endoscopic surveillance in these patients may be recommended to exclude both possibility of recurrence and cancer development elsewhere in stomach.

## ACKNOWLEDGEMENTS

The authors thank Dr. Ilgin Ozden for contributing to the redaction of this article.

## REFERENCES

1 **Silverstein FE,** Tytgat GNJ. Stomach II: Tumors and polyps. In: Silverstein FE, Tytgat GNJ, eds. Gastrointestinal Endoscopy. 3rd ed. *London*: *Mosby* 1997: 147-180
2 **Dent TL,** Kukora JS, Buinewicz BR. Endoscopic screening and surveillance for gastrointestinal malignancy. *Surg Clin North Am* 1989; **69**: 1205-1225
3 **Crawford JM**. The gastrointestinal tract. In: Cotran RS, Kumar V, Robbins SL, eds. Pathologic Basis of Disease. 6th ed. *Philadelphia: W. B. Saunders Company* 1999: 775-843
4 **Oberhuber G**, Stolte M. Gastric polyps: an update of their pathology and biological significance. *Virchows Arch* 2000; **437**: 581-590
5 **Lau CF**, Hui PK, Mak KL, Wong AM, Yee KS, Loo CK, Lam KM. Gastric polypoid lesions-illustrative cases and literature review. *Am J Gastroenterol* 1998; **93**: 2559-2564
6 **Sebastian MW**. Benign tumors of stomach. In: Sabiston DC, ed. The biological basis of modern surgical practise. 15th ed. Philadelphia: *W.B. Saunders Company* 1997: 868-875
7 **Dean PG,** Davis PM, Nascimento AG, Farley DR. Hyperplastic gastric polyp causing progressive gastric outlet obstruction. *Mayo Clin Proc* 1998; **73**: 964-967
8 **Kumar A**, Quick CR, Carr-Locke DL. Prolapsing gastric polyp, an unusual cause of gastric outlet obstruction: a review of the pathology and management of gastric polyps. *Endoscopy* 1996;**28**: 452-455
9 **Kudis V,** Siegel E, Schilling D, Nusse T, Bohrer MH, Riemann JF. The hyperplastic gastric polyp-a praecancerosis. *Z Gastroenterol* 2002; **40**: 295-298
10 **Muehldorfer SM,** Stolte M, Martus P, Hahn EG, Ell C. Diagnostic accuracy of forceps biopsy versus polypectomy for gastric polyps: a prospective multicentre study. *Gut* 2002; **50**: 465-470
11 **Seifert E,** Elster K. Gastric polypectomy. *Am J Gastroenterol* 1975; **63**: 451-456
12 **Ginsberg GG**, Al-Kawas FH, Fleischer DE, Reilly HF, Benjamin SB. Gastric polyps: relationship of size and histology to cancer risk. *Am J Gastroenterol* 1996; **91**: 714-717
13 **Debongnie JC**. Gastric polyps. *Acta Gastroenterol Belg* 1999; **62**: 187-189
14 **Stolte M,** Sticht T, Eidt S, Ebert D, Finkenzeller G. Frequency, location, and age and sex distribution of various types of gastric polyp. *Endoscopy* 1994; **26**: 659-665
15 **Papa A**, Cammarota G, Tursi A, Montalto M, Cuoco L, Certo M, Fedeli G, Gasbarrini G. Histologic types and surveillance of gastric polyps: a seven year clinico-pathological study. *Hepatogastroenterology* 1998; **45**: 579-582
16 **Ljubicic N**, Kujundzic M, Roic G, Banic M, Cupic H, Doko M, Zovak M. Benign epithelial gastric polyps-frequency, location, and age and sex distribution. *Coll Antropol* 2002; **26**: 55-60
17 **Declich P,** Ambrosiani L, Grassini R, Tavani E, Bellone S, Bortoli A, Gozzini C, Prada A. Fundic gland polyps: a still elusive entity on the eve of the year 2000. *Pol J Pathol* 2000; **51**: 3-8
18 **Daibo M,** Itabashi M, Hirota T. Malignant trasformation of gastric hyperplastic polyps. *Am J Gastroenterol* 1990; **85**: 327-328
19 **Davaris P,** Petraki K, Archimandritis A, Haritopoulos N, Papacharalampous N. Mucosal hyperplastic polyps of the stomach. Do they have any potential to malignancy? *Pathol Res Pract* 1986; **181**: 385-389
20 **Hizawa K**, Fuchigami T, Iida M, Aoyagi K, Iwashita A, Daimaru Y, Fujishima M. Possible neoplastic transformation within gastric hyperplastic polyp. Application of endoscopic polypectomy. *Surg Endosc* 1995; **9**: 714-718
21 **Zea-Iriarte WL,** Sekine I, Itsuno M, Makiyama K, Naito S, Nakayama T, Nishisawa-Takano JE, Hattori T. Carcinoma in gastric hyperplastic polyps: a phenotypic study. *Dig Dis Sci* 1996; **41**: 377-386
22 **Abraham SC**, Singh VK, Yardley JH, Wu TT. Hyperplastic polyps of the stomach: associations with histologic patterns of gastritis and gastric atrophy. *Am J Surg Pathol* 2001; **25**: 500-507
23 **Stolte M.** Clinical consequences of the endoscopic diagnosis of gastric polyps. *Endoscopy* 1995; **27**: 32-37
24 **Park DI,** Rhee PL, Kim JE, Hyun JG, Kim YH, Son HJ, Kim JJ, Paik SW, Rhee JC, Choi KW, Oh YL. Risk factors suggesting malignant transformation of gastric adenoma: univariate and multivariate analysis. *Endoscopy* 2001; **33**: 501-506
25 **Wu TT**, Kornachi S, Rashid A, Yardley JH, Hamilton SR. Dysplasia and dysregulation of proliferation in foveolar and surface epithelia of fundic gland polyps from patients with familial adenomatous polyposis. *Am J Surg Pathol* 1998; **22**: 293-298
26 **Orlowska J,** Jarosz D, Pachlewski J, Butruk E. Malignant transformation of benign epithelial gastric polyps. *Am J Gastroenterol* 1995; **90**: 2152-2159

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2240-2250
World Journal of Gastroenterology
 ISSN 1007-9327



# Construction of expression systems for *flaA* and *flaB* genes of *Helicobacter pylori* and determination of immunoreactivity and antigenicity of recombinant proteins

Jie Yan, Shao-Hui Liang, Ya-Fei Mao, Li-Wei Li, Shu-Ping Li

**Jie Yan, Ya-Fei Mao, Li-Wei Li, Shu-Ping Li,** Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejiang University, Hangzhou 310031, Zhejiang Province, China
**Shao-Hui Liang,** Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejiang University, Hangzhou 310031, Zhejiang Province, China and Department of Parasitology, Wenzhou Medical College, Wenzhou 325027, Zhejiang Province, China
**Supported by** the Excellent Young Teacher Fund of Chinese Education Ministry and the General Research Plan of the Science and Technology Department of Zhejiang Province, No. 001110438
**Correspondence to:** Jie Yan, Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejiang University, 353 Yan an Road, Hangzhou 310031, Zhejiang Province, China. yanchen@mail.hz.zj.cn
**Telephone:** +86-571-87217385 **Fax:** +86-571-87217044
**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To clone flagellin genes A (*flaA*) and B (*flaB*) from a clinical strain of *Helicobacter pylori* (*H pylori*) and to construct prokaryotic expression systems of the genes and identify immunity of the fusion proteins.

**METHODS:** The *flaA* and *flaB* genes from a clinical *H pylori* isolate Y06 were amplified by high fidelity PCR. The nucleotide sequences of target DNA amplification fragments from the two genes were sequenced after T-A cloning. The recombinant expression vector *pET32a* inserted with *flaA* and *flaB* genes was constructed, respectively. The expressions of FlaA and FlaB fusion proteins in *E. coli* BL21DE3 induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations were examined by SDS-PAGE. Western blot using commercial antibodies against whole cell of *H pylori* and immunodiffusion assay using self-prepared rabbit antiserum against FlaA (rFlaA) or FlaB (rFlaB) recombinant proteins were applied to the determination of the fusion proteins immunity. ELISA was used to detect the antibodies against rFlaA and rFlaB in sera of 125 *H pylori* infected patients and to examine rFlaA and rFlaB expression in 98 clinical isolates of *H pylori*, respectively.

**RESULTS:** In comparison with the reported corresponding sequences, the nucleotide sequence homologies of the cloned *flaA* and *flaB* genes were from 96.28-97.13 % and 96.31-97.73 %, and their putative amino acid sequence homologies were 99.61-99.80 % and 99.41-100 % for the two genes, respectively. The output of rFlaA and rFlaB expressed by *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 systems was as high as 40-50 % of the total bacterial proteins. Both rFlaA and rFlaB were able to combine with the commercial antibodies against whole cell of *H pylori* and to induce rabbits to produce specific antibodies with the same 1:2 immunodiffusion titers after the animals were immunized with the two recombinant proteins. Ninety-eight and zero point 4 and 92.80 % of the serum samples from 125 patients infected with *H pylori* were positive for rFlaA and rFlaB antibodies, respectively. One hundred percent and 98.98 % of the 98 tested isolates of *H pylori* were detectable for rFlaA and rFlaB epitopes, respectively.

**CONCLUSION:** Two prokaryotic expression systems with high efficiency of *H pylori flaA* and *flaB* genes were successfully established. The expressed rFlaA and rFlaB showed satisfactory immunoreactivity and antigenicity. High frequencies of FlaA and FlaB expression in different *H pylori* clinical strains and the general existence of specific antibodies against FlaA and FlaB in *H pylori* infected patients strongly indicate that FlaA and FlaB are excellent antigen candidates for developing *H pylori* vaccine.

Yan J, Liang SH, Mao YF, Li LW, Li SP. Construction of expression systems for *flaA* and *flaB* genes of *Helicobacter pylori* and determination of immunoreactivity and antigenicity of recombinant proteins. *World J Gastroenterol* 2003; 9(10): 2240-2250
http://www.wjgnet.com/1007-9327/9/2240.asp

## INTRODUCTION

In China, chronic gastritis and peptic ulceration are two most common gastric diseases, and gastric cancer is one of the malignant tumors with high mortalities and morbidities[1-34]. *Helicobacter pylori* (*H pylori*), a microaerophilic, spiral and Gram-negative bacterium, is considered as a human-specific gastric pathogen that colonizes the stomach of at least half of the world population[35]. Most infected individuals are asymptomatic. However, in some subjects, the infection causes acute, chronic gastritis and peptic ulceration, and plays important roles in the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin' s lymphoma[36-43]. This microorganism has been categorized as class I carcinogen by the World Health Organization[44], and direct evidence of carcinogenesis has been recently demonstrated in animal models[45, 46]. Immunization against the bacterium represents a cost-effective strategy to prevent *H pylori*-associated peptic ulcer diseases and to reduce the incidence of global gastric cancer[47]. Selection of antigenic targets is critical in design of *H pylori* vaccine. So far, no vaccine preventing *H pylori* infection has been commercially available. The majority of studies attempting to produce a vaccine have focused on urease enzyme, heat shock protein, and vacuolating cytotoxin[35, 48-50], but rarely on *H pylori* flagellin. *H pylori* flagellin is composed of two subunits, named as FlaA with 53KDa and FlaB with 54 KDa respectively. The flagellin plays a main role in motility and is necessary for colonization or persistence of *H pylori* infection[51]. The motility of *H pylori* is a virulent factor in the pathogenesis of gastric mucosal injury[52]. The data mentioned

above indicate that FlaA as well as FlaB may be used as antigen candidates for *H pylori* vaccine. Therefore, in this study, two prokaryotic vectors responsible for expressing recombinant FlaA (rFlaA) and FlaB (rFlaB) were constructed. Immunoreactivity and antigenicity of rFlaA and rFlaB were further examined. Furthermore, these two recombinant proteins were used for detecting specific antibodies in sera from *H pylori* infected patients, and rabbit anti-rFlaA and anti-rFlaB sera were prepared for examining the corresponding epitopes of *H pylori* clinical isolates. The results of this study may contribute to the development of *H pylori* vaccines.

## MATERIALS AND METHODS

### *Materials*

A clinical strain of *H pylori* was used in this study, which was provisionally named Y06, and well-characterized by the Department of Medical Microbiology and Parasitology, College of Medical Sciences, Zhejing University. A plasmid *pET32a* (Novagen) and an *E. coli* strain BL21DE3 (Novagen) were used as the expression vector and host cell, respectively. Primers for PCR amplification, the Pfu-Taq high fidelity PCR kit and restriction endonucleases were purchased from BioAsia (Shanghai, China). The T-A cloning kit and sequencing service were provided by BBST (Shanghai, China). Rabbit antiserum against the whole cell of *H pylori*, HRP-labeling sheep antisera against rabbit IgG and against human IgG were purchased from DAKO and Jackson ImmunoResearch, respectively. Agents used in isolation and identification of *H pylori* were purchased from Sigma and bioMérieux. Gastric biopsy specimens with positive *H pylori* isolation from 126 patients (86 males and 40 females, age range: from 6-78 years old, mean age: 40.5 years old) referred for gastroduodenoscopic examination in four different hospitals in Hangzhou were collected during the period between December 2001 and June 2002. Each of the patients gave a written informed content for this study. Of the 126 patients, 68 had chronic gastritis (CG, 48 superficial, 10 active and 10 atrophic), and the other 58 had peptic ulcer disease (PUD, 12 gastric ulcer, 40 duodenal ulcer and 6 gastric and duodenal ulcer). None of the patients had taken nonsteroidal anti-inflammatory drugs, antacids and antibiotics during the two weeks before seeking medical advice. At the same time, serum specimens were also collected from these patients.

### *Methods*

**Isolation and identification of *H pylori*** Each gastric biopsy specimen was homogenized with a tissue grinder and then inoculated on Columbia agar plates supplemented with 8.0 % (V/V) sheep blood, 0.5 % (W/V) cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2 500 U/L cefsoludin and 2.5 mg/L amphotericin B. The plates were incubated at 37 ℃ under microaerobic conditions (5 % $O_2$, 10 % $CO_2$ and 85 % $N_2$) for 3 to 5 days. A bacterial isolate was identified as *H pylori* according to typical Gram staining morphology, biochemical tests positive for urease and oxidase, and agglutination with the commercial rabbit antibody against whole cell of the microbe. All of *H pylori* isolates were stored at -70 ℃ for ELISA.

**Preparation of DNA template** Genomic DNA of *H pylori* strain Y06 was extracted by the conventional phenol-chloroform method and DNase-free RNase treatment[52]. The obtained DNA was dissolved in TE buffer, and its concentration and purity were determined by ultraviolet spectrophotometry[52].

**Polymerase chain reaction** Oligonucleotide primers were designed to amplify the whole sequence of *flaA* and *flaB* genes from *H pylori* strain Y06 based on the published corresponding genomic sequences[53-56]. The sequence of *flaA* sense primer with an endonuclease site of *Eco*RV was 5' -CCG GATATCATGGCTTTTCAGGTCAA-3' . The sequence of *flaA* antisense primer with an endonuclease site of *Xho*I was 5' -CCGCTCGAGAAACTAAGTTAAAAGCC-3' . The sequence of *flaB* sense primer with an endonuclease site of *Eco*RI was 5' -CCGGAATTCATGAGTTTTAGGATAAA-3' . The sequence of *flaB* antisense primer with an endonuclease site of *Xho*I was 5' -CCGCTCGAGCTGTTATTGTAAAA GCC-3' . The total volume per PCR was 100 μl containing 2.5 mol· $L^{-1}$ each dNTP, 500 nmol· $L^{-1}$ each of the two primers, 15 mol· $L^{-1}$ $MgCl_2$, 3.0 U Pfu-Taq polymerase, 100 ng DNA template and 1×PCR buffer (pH8.8). The parameters for PCR were at 94 ℃ for 5 min, ×1; at 94 ℃ for 30 s, at 52 ℃ for 30 s, at 72 ℃ for 90 s, ×10; at 94 ℃ for 30 s, at 52 ℃ for 30 s, at 72 ℃ for 100 s (10 s addition for the each of the following cycles), ×15; then at 72 ℃ for 10 min, ×1. The results of PCR were observed under UV light after electrophoresis in 15 g· $L^{-1}$ agarose pre-stained with ethidium bromide. The expected sizes of target amplification fragments were 1 530 bp for *flaA* gene and 1 542 bp for *flaB* gene.

**Cloning and sequencing** The target amplification DNA fragments from *flaA* and *flaB* genes were respectively cloned into *pUCm*-T vectors (*pUCm*-T-*flaA* and *pUCm*-T-*flaB*) by using the T-A cloning kit according to the manufacturer' s instructions. The recombinant plasmids were amplified in an *E. coli* strain DH5α and then extracted by the Sambrook' s method[57]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragments. Three strains of *E. coli* DH5α containing *pUCm*-T-*flaA*, *pUCm*-T-*flaB* and expression vector *pET32a* were amplified in BL medium, and the three plasmids were extracted, respectively[57]. These plasmids were digested with *Eco*RV and *Xho*I, *Eco*RI and *Xho*I, respectively. The *flaA* target fragment and *pET32a*, and the *flaB* fragment and *pET32a* were recovered and then ligased. The recombinant expression vectors *pET32a-flaA* and *pET32a-flaB* were respectively transformed into *E.coli* BL21DE3, and the expression systems were named as *pET32a-flaA*-BL21DE3 and pET32a-*flaB*-BL21DE3. The target fragments of *flaA* and *flaB* genes inserted in *pET32a* plasmid were sequenced again.

**Expression and identification of fusion proteins** *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 were rotatively cultured in LB medium at 37 ℃ induced by isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol· $L^{-1}$. The supernatant and precipitate were separated through centrifugation after the bacterial pallet was ultrasonically broken (300V, 5 s×3). The molecular weight and output of rFlaA and rFlaB were examined by SDS-PAGE. The two recombinant proteins were collected by Ni-NTA affinity chromatography. The commercial rabbit antiserum against whole cell of *H pylori* and HRP-labeling sheep antiserum against rabbit IgG were used as the first and second antibodies to determine the immunoreactivity of rFlaA and rFlaB by Western blot. Rabbits were immunized with rFlaA and rFlaB, respectively, for preparation of antisera. Immunodiffusion assay was applied to the determination of the antigenicity of rFlaA and rFlaB.

**ELISA** The specific antibodies against FlaA and FlaB in sera of the 126 patients infected with *H pylori* were detected by ELISA, by using rFlaA and rFlaB as antigens at the coated concentration of 20 μg/ml and a patient serum sample (1:400 dilution) as the first antibody and HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody. The result of ELISA for a patient' s serum sample was considered as positive if the optical density at 490 nm ($OD_{490}$) was over the mean plus 3 SD of five negative serum samples[58]. FlaA and FlaB expression in clinical isolates of *H pylori* was detected by ELISA using the ultrasonic supernatant of each *H pylori*

isolate (50 μg/ml) as a coated antigen, the self-prepared rabbit antisera against rFlaA and rFlaB (1:800 dilution in both) as the first antibody and HRP-labeling sheep antibody against rabbit IgG (1:3 000 dilution) as the second antibody. The result of ELISA for a *H pylori* ultrasonic supernatant sample was considered as positive if its $OD_{490}$ value was over the mean plus 3 SD of five ultrasonic supernatant samples at the same protein concentration of *E. coli* ATCC 25922[58].

**Date analysis** The nucleotide sequences of the cloned *flaA* and *flaB* genes were compared for homology with the 3 published *flaA* gene sequences (NC000915, NC000921, X60746)[53-55] and the 4 published *flaB* gene sequences (NC000915, NC000921, L08907, AF479024)[53,54,56,59] by using a molecular biological analysis software.

## RESULTS

### PCR

Target fragments of *flaA* and *flaB* genes with expected sizes amplified from DNA template of *H pylori* stain Y06 are shown in Figure 1.

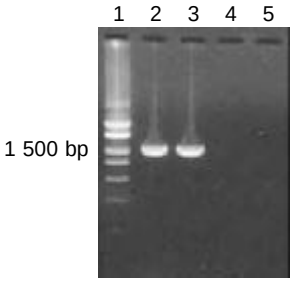



**Figure 1** Target fragments of *flaA* and *flaB* genes amplified from *H pylori* strain Y06 DNA.

### Nucleotide sequence analysis

The nucleotide sequences of *flaA* gene in *pUCm*-T-*flaA* and *pET32a-flaA* were completely the same and so as for *flaB* gene. The homologies of nucleotide and putative amino acid sequences of the cloned *flaA* gene compared with the published *flaA* sequences[53-55] were from 96.28 % to 97.13 % and from 99.61 % to 99.80 %, respectively (Figures 2 and 3). The homologies of nucleotide and putative amino acid sequences of the cloned *flaB* gene were 96.31-97.73 % and 99.41-100 %, compared with the published *flaB* sequences (Figures 4 and 5)[53,54,56,59].

### Expression of target fusion proteins

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol· $L^{-1}$ efficiently induced the expression of rFlaA and rFlaB in *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 systems. The products of rFlaA and rFlaB were mainly presented in ultrasonic precipitates, and the output was 40-50 % of the total bacterial proteins (Figures 6 and 7).

### Immunoreactivity and antigenicity of rFlaA and rFlaB

The commercial rabbit antibodies against the whole cell of *H pylori* combined with rFlaA and rFlaB as confirmed by Western blot (Figures 8 and 9). Both the titer of immunodiffusion assay between rFlaA and its rabbit antiserum, rFlaB and its rabbit antiserum was 1:2.

### ELISA

Since the mean ±SD of $OD_{490}$ values of the five negative serum samples were 0.338±0.036 for rFlaA and 0.102±0.051 for rFlaB in the detection of specific antibodies in patients' sera, the positive reference value was 0.446 for FlaA and 0.255 for FlaB. According to the reference values, 98.4 % (123/125, one serum sample was contaminated) of the tested patients' serum samples were positive for antibodies against rFlaA with an $OD_{490}$ value range of 0.52-1.76, and 92.8 % (116/125) were positive for antibodies against rFlaB with an $OD_{490}$ value range of 0.26-1.50. Since the mean ±SD of $OD_{490}$ of the five negative bacterial controls was 0.200±0.046 for FlaA and 0.170±0.044 for FlaB in the detection of clinical *H pylori* isolates, the positive reference value was 0.338 for FlaA and 0.302 for FlaB. According to the reference values, 100 % (98/98) of the tested *H pylori* isolates were detectable for the epitope of rFlaA with an $OD_{490}$ value range of 0.36-2.01 and 99 % (97/98) of the isolates were detectable for the epitope of rFlaB with an $OD_{490}$ value range of 0.31-1.78.

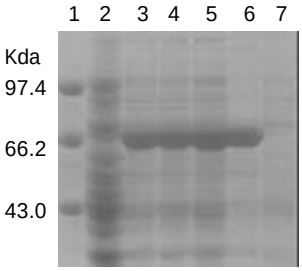



**Figure 6** Expression of rFlaA induced by IPTG at different concentrations.

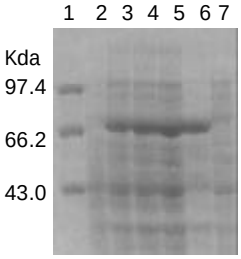



**Figure 7** Expression of rFlaB induced by IPTG at different concentrations.

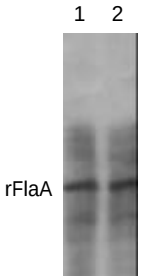



**Figure 8** Western blot result of rabbit antibodies against whole cell of *H pylori* and rFlaA.

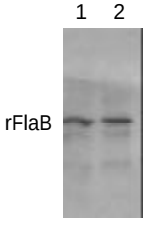



**Figure 9** Western blot result of rabbit antibodies against whole cell of *H pylori* and rFlaB.

```
(1)1    ATGGCTTTTCAGGTCAATACAAATATCAATGCGATGAATGCGCATGTGCAATCCGCACTC
(2)1    ............................................................
(3)1    ...........................................................A....
(4)1    ............................................................

(1)61   ACTCAAAATGCGCTTAAAACTTCATTGGAGAGATTGAGTTCAGGTTTAAGGATTAATAAA
(2)61   ........C.....................C...........................
(3)61   ............................................................
(4)61   ........C..A..................C.....................C......

(1)121  GCGGCTGATGATGCATCAGGCATGACGGTGGCAGATTCTTTGCGTTCACAAGCGAGCAGT
(2)121  ...........C.....................G...........................
(3)121  ...........C.....................G...........................
(4)121  ...........C.....................G.............G............

(1)181  TTGGGTCAAGCGATTGCCAACACGAATGACGGCATGGGGATTATCCAAGTTGCGGATAAG
(2)181  ................................................G............
(3)181  ................................................G............
(4)181  ................................................G............

(1)241  GCTATGGATGAGCAGTTAAAAATCTTAGACACCGTTAAGGTTAAAGCGACTCAAGCGGCT
(2)241  ............................................................
(3)241  ............................................................
(4)241  ..............A.............................................

(1)301  CAAGACGGGCAAACTACGGAATCTCGTAAAGCGATTCAATCTGACATCGTTCGTTTGATT
(2)301  .....T...........A.....................................
(3)301  .....T.....................................................
(4)301  .....T.....................................................

(1)361  CAAGGTTTAGATAATATCGGTAACACGACTACTTATAACGGGCAAGCGTTATTGTCTGGT
(2)361  ...........C...........T.....................................
(3)361  ...........C...........T.....................................
(4)361  ........G.................A..G...............................

(1)421  CAATTCACTAACAAAGAATTCCAAGTAGGGGCTTATTCTAACCAAAGCATTAAGGCTTCT
(2)421  ........C.....................................................A......
(3)421  ................................................T............
(4)421  ............................................................

(1)481  ATCGGCTCTACCACTTCGGATAAAATCGGTCAGGTTCGTATCGCTACAGGCGCGTTAATC
(2)481  ..................C.................................T.........
(3)481  ..................C.....................................
(4)481  ..................C.....................................

(1)541  ACGGCTTCTGGGGATATTAGCTTGACTTTTAAACAAGTGGATGGCGTGAATGATGTAACT
(2)541  ..C........A.....................................
(3)541  ...........................................T................
(4)541  ............................................................

(1)601  TTAGAGAGCGTAAAAGTTTCTAGTTCAGCAGGCACGGGGATCGGTGTGTTAGCGGAAGTG
(2)601  ..................................A.....T..C........A......
(3)601  ..................C.................A.....T..C........A......
(4)601  ..................................A........C........A......

(1)661  ATTAACAAAAATTCTAACCGAACAGGGGTTAAAGCTTATGCGAGCGTTATCACCACGAGC
(2)661  ........G..C.............C........C.....................
(3)661  ..C.....................................................
(4)661  ..C..T.....C.....................................

(1)721  GATGTGGCGGTCCAATCAGGAAGTTTGAGTAATTTAACTTTAAATGGGATCCATTTGGGT
(2)721  ..............G.....................C...........T........G
(3)721  ..............G.....................C.....................C
(4)721  ..............G.....................C...........T.........

(1)781  AATATCGCAGATATTAAGAAAAATGACTCAGACGGAAGGTTAGTCGCAGCGATCAATGCG
```

```
(2)781  ......................C............C.A.....................
(3)781  ..........................................................
(4)781  ......................C...................................

(1)841  GTTACTTCAGAAACCGGCGTGGAAGCTTATACGGATCAAAAAGGGCGCTTGAATTTGCGC
(2)841  ..........................................................
(3)841  ................T.........................................
(4)841  ..C............T.........................................

(1)901  AGTATAGATGGTCGTGGGATTGAAATCAAAACCGATAGCGTCAGTAATGGGCCTAGTGCT
(2)901  ....................................T.....................
(3)901  ..................................C.....................
(4)901  ..................................C..........C........C...

(1)961  TTAACGATGGTCAATGGCGGTCAGGATTTAACAAAAGGTTCTACTAACTATGGGAGGCTT
(2)961  ..........T........................C..........C..A......
(3)961  ..................................C..........C..A......
(4)961  ..................................C..........C..A......

(1)1021 TCTCTCACACGCTTAGACGCTAAAAGCATCAATGTCGTTTCGGCTTCTGATTCGCAACAT
(2)1021 ......................G.........................C.........
(3)1021 ..........A..........G..........A.....A........C..A..G...
(4)1021 ..........A..........G.........................C..A..G...

(1)1081 TTAGGTTTCACAGCGATTGGTTTTGGGGAATCTCAAGTGGCAGAAACCACGGTGAATTTG
(2)1081 .....C....................................................
(3)1081 ..........................................................
(4)1081 .....C.....G..............................................

(1)1141 CGCGATGTTACTGGGAATTTTAACGCTAATGTCAAATCAGCCAGTGGCGCGAACTATAAC
(2)1141 ..........C.................................T.............
(3)1141 ..........C..........................................
(4)1141 ..........................................................

(1)1201 GCCGTGATCGCTAGCGGTAACCAAAGCTTGGGATCTGGGGTTACAACCTTGAGAGGCGCG
(2)1201 .....C........T..C..T............................A.....T...
(3)1201 .....C........T..C..T............................A.........
(4)1201 ..................................................A.........

(1)1261 ATGGTGGTGATTGATATTGCGGAATCGGCGATGAAAATGTTGGATAAAGTCCGCTCTGAT
(2)1261 ..............C.....C..G..T.........................A......
(3)1261 ..................C..G..T..............................
(4)1261 ..............C.....C.....A..............................C

(1)1321 TTAGGTTCTGTGCAAAATCAAATGATTAGCACCGTGAATAACATCAGCATCACTCAAGTG
(2)1321 ..........................................................
(3)1321 ..........................................................
(4)1321 ..............................T...........................

(1)1381 AATGTTAAAGCGGCTGAGTCTCAAATCAGGGATGTGGATTTTGCTGAAGAGAGCGCGAAT
(2)1381 ................A.....................................
(3)1381 ................A.....................................
(4)1381 ................A.....................C...............

(1)1441 TTCAATAAAAACAATATTTTGGTGCAATCAGGCAGCTATGCGATGAGTCAAGCTAACACC
(2)1441 ..............C.......CA........T...................C......
(3)1441 ..............C.......C.............................C......
(4)1441 .....C........C.......C.............................C..T...

(1)1501 GTCCAACAAAATATCTTAAGGCTTTTAACTTAG
(2)1501 ..T..........................
(3)1501 ..T..........................
(4)1501 ..T..........................
```

**Figure 2** Homologies of nucleotide sequence of cloned *H pylori flaA* gene with reported sequences. (1): the sequencing result of *H pylori* strain Y06 *flaA* gene; (2)-(4): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. X60746, strain 898-1). Underlined areas indicate the positions of primer sequences.

```
(1)1   MAFQVNTNINAMNAHVQSALTQNALKTSLERLSSGLRINKAADDASGMTVADSLRSQASS
(2)1   ............................................................
(3)1   ............................................................
(4)1   .................E..........................................

(1)61  LGQAIANTNDGMGIIQVADKAMDEQLKILDTVKVKATQAAQDGQTTESRKAIQSDIVRLI
(2)61  ............................................................
(3)61  ............................................................
(4)61  ............................................................

(1)121 QGLDNIGNTTTYNGQALLSGQFTNKEFQVGAYSNQSIKASIGSTTSDKIGQVRIATGALI
(2)121 ............................................................
(3)121 ............................................................
(4)121 ............................................................

(1)181 TASGDISLTFKQVDGVNDVTLESVKVSSSAGTGIGVLAEVINKNSNRTGVKAYASVITTS
(2)181 ............................................................
(3)181 ............................................................
(4)181 ............................................................

(1)241 DVAVQSGSLSNLTLNGIHLGNIADIKKNDSDGRLVAAINAVTSETGVEAYTDQKGRLNLR
(2)241 ............................................................
(3)241 ............................................................
(4)241 ............................................................

(1)301 SIDGRGIEIKTDSVSNGPSALTMVNGGQDLTKGSTNYGRLSLTRLDAKSINVVSASDSQH
(2)301 ............................................................
(3)301 ............................................................
(4)301 ............................................................

(1)361 LGFTAIGFGESQVAETTVNLRDVTGNFNANVKSASGANYNAVIASGNQSLGSGVTTLRGA
(2)361 ............................................................
(3)361 ............................................................
(4)361 ............................................................

(1)421 MVVIDIAESAMKMLDKVRSDLGSVQNQMISTVNNISITQVNVKAAESQIRDVDFAEESAN
(2)421 ............................................................
(3)421 ............................................................
(4)421 ............................................................

(1)481 FNKNNILVQSGSYAMSQANTVQQNILRLLT      510aa
(2)481 .......A......................
(3)481 .......A......................
(4)481 .......A......................
```

**Figure 3** Homologies of putative amino acid sequence of *H pylori flaA* gene with reported sequences. (1): the sequencing result of cloned *H pylori* strain Y06 *flaA* gene; (2)-(4): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. X60746, strain 898-1).

```
(1)1    ATGAGTTTTAGGATAAATACCAATATCGCCGCTTTAACTTCTCATGCGGTAGGGGTTCAA
(2)1    ............................................................
(3)1    ...........................................................G........
(4)1    ............................................................
(5)1    ............................................................

(1)61   AACAACAGAGACCTTTCAAGCTCGCTTGAAAAGTTAAGCTCAGGGCTTAGGATCAATAAG
(2)61   ............................................................A
(3)61   ..........................................T.................A
(4)61   ............................................................A
(5)61   ............................................................A

(1)121  GCCGCTGACGATTCTAGTGGGATGGCGATCGCTGATAGCTTAAGGAGTCAAAGCGCGAAT
(2)121  ............................................................
(3)121  ............................................................
(4)121  ............................................................
(5)121  ............................................................
```

```
(1)181  TTAGGCCAGGCGATTCGCAACGCTAATGACGCTATTGGTATGGTTCAAACCGCTGATAAA
(2)181  ..G..T..A.....C........C.............................A......
(3)181  ..................................................................
(4)181  ..G..T..A.....C.....................................................
(5)181  ..G.....................................................................

(1)241  GCGATGGATGAGCAAATCAAAATCTTAGACACCATTAAAACCAAAGCCGTTCAAGCCGCT
(2)241  ..................................................................
(3)241  ..................................................................
(4)241  ..................................................................
(5)241  ..................................................................

(1)301  CAAGATGGGCAAACTTTAGAAAGCCGAAGAGCACTCCAGAGCGATATTCAAAGGTTGTTA
(2)301  ................................G.....................................
(3)301  ................................G.....................................
(4)301  ................................G.....................................
(5)301  ................................G.....................................

(1)361  GAAGAACTGGACAATATCGCTAACACCACAAGCTTTAACGGCCAACAAATGCTTTCAGGA
(2)361  ..................................................................
(3)361  ........A.....................................................
(4)361  ........A.....................................................
(5)361  ........A...........C.........................................

(1)421  AGTTTTTCTAACAAAGAATTTCAAATTGGCGCGTATTCTAACACCACGGTTAAAGCGTCT
(2)421  ..........................................G.....................
(3)421  ..................................................................
(4)421  ..................................................................
(5)421  ..........................................................G.......

(1)481  ATTGGCTCAACAAGCTCAGATAAGATTGGGCATGTGCGCATGGAAACCTCTTCTTTTAGC
(2)481  ...........G.....................................T.............
(3)481  ...........G..T..G.....A.......................T..A.........
(4)481  ...........G...........A..C...................T.............
(5)481  ...........G...........A.....................................

(1)541  GGTGAAGGCATGCTCGCTAGCGCGGCGG CGCAAAACTTGACTGAAGTGGGATTGAATTT
(2)541  ....C..................... A..............................
(3)541  ............................ ..............................
(4)541  ........................C.G...... ..........................
(5)541  ..C...................A.... ..............................

(1)600  CAAACAAGTCAATGGCGTGAACGATTATAAGATTGAAACCGTGCGCATTTCTACGAGCGC
(2)600  ......................T.....................................T..
(3)600  ......................T.....................................
(4)600  ......................T.....................................
(5)600  ......................T.................................A.....

(1)660  TGGCACTGGGATCGGAGCGTTAAGCGAAATCATCAATCGTTTTTCTAACACTTTAGGCGT
(2)660  ............T.....................................................
(3)660  C...........T..G.....................C...........C.......
(4)660  ......A.....T.....................................................
(5)660  ......A.....T..G...........G...........C...........C.......

(1)720  TAGGGCGTCTTATAATGTCATGGCTACCGGCGGCACTCCCGTGCAATCAGGAACTGTTAG
(2)720  ......T.....................................................
(3)720  ...A..A.................................T.............C..G..
(4)720  ..........................................A...............
(5)720  ......T........C.............T.....A..G.................G..

(1)780  GGAGCTTACCATTAATGGCGTAGAAATTGGGACCGTGAATGATGTGCATAAAAATGACGC
(2)780  ..................................................C.....
(3)780  A.............................A.....................C.....
(4)780  ..................................................C.....
(5)780  A..A..C.....................A.............G......C.....

(1)840  TGATGGGAGGTTGACTAATGCGATCAACTCCGTCAAAGACAGGACCGGCGTGGAAGCGAG
```

```
(2)840  .........A..........A.....................G..T..........
(3)840  ...C.................................TC.C.....T..........
(4)840  ...C................................................
(5)840  ...C..C..A..................................G.....A........

(1)900  CTTGGATATTCAAGGGCGCATTAATTTGCACTCCATTGACGGGCGTGCGATTTCTGTGCA
(2)900  ........................A.................C.............
(3)900  ....................................T.....C.............
(4)900  ....................................................C.............
(5)900  ....................................T.....C.............

(1)960  TGCAGCGAGCGCGAGCGGTCAGGTTTTTGGGGGAGGGAATTTTGCAGGGATTTCTGGGAC
(2)960  ............................................................
(3)960  ..........................................A.............
(4)960  .........T.................................A..............
(5)960  ..................C.........................................

(1)1020 ACAACATGCGGTTATTGGGCGCTTAACCTTGACCAGGACCGACGCTAGAGACATCATTGT
(2)1020 ...G.................................A.....................
(3)1020 ...G.............................T...................T..C..
(4)1020 ...G.....A.........................A.................T.....
(5)1020 ...G.........................A..T..A........C.....T........

(1)1080 GAGCGGTGTGAATTTTAGCCATGTGGGCTTTCATTCCGCTCAAGGGGTGGCAGAATACAC
(2)1080 ..............C.............................................
(3)1080 ............................................................
(4)1080 ............................................................
(5)1080 ............C.............................................

(1)1140 CGTGAATTTGAGAGCGGTTAGGGGCATTTTTGATGCGAATGTGGCTTCAGCAGCCGGAGC
(2)1140 ............................................................
(3)1140 ..........................................................G.........
(4)1140 ............................................................G..
(5)1140 ............G.............................................

(1)1200 GAACGCTAATGGCGCACAAGCGGAGACCAATTCTCAAGGTATAGGGGCTGGGGTAACAAG
(2)1200 ..............G.............................................
(3)1200 ..............G.............................A..C..A........
(4)1200 ....................T.................C....................
(5)1200 ..............G.............................................

(1)1260 CCTTAAAGGAGCGATGATTGTGATGGATATGGCGGACTCAGCGCGCACGCAATTAGACAA
(2)1260 .........G.....................A..T.................G.....
(3)1260 .........G.....................A..T.....................
(4)1260 .........G................C.........T.....................
(5)1260 .........G.........................T..T...................

(1)1320 GATCCGCTCGGATATGGGTTCGGTGCAAATGGAATTGGTTACAACCATTAATAATATTTC
(2)1320 ............................................................
(3)1320 ..........................................C........C........
(4)1320 ......A.....................................................
(5)1320 ..........................................C.................

(1)1380 TGTAACCCAAGTGAATGTTAAAGCGGCTGAATCTCAAATCAGAGATGTGGATTTTGCTGA
(2)1380 ............................................................
(3)1380 ......T.....................G.............C.............
(4)1380 ............................................................
(5)1380 ..............................................C.............

(1)1440 AGAAAGTGCGAACTTTTCTAAATACAATATTTTGGCGCAAAGCGGGAGTTTTGCTATGGC
(2)1440 ...G..C.....................................................
(3)1440 ...G..C.....................................................
(4)1440 ...G........................................................
(5)1440 ...G..C.....................................T...............

(1)1500 ACAAGCGAATGCGGTGCAACAAAATGTCTTAAGGCTTTTACAATAA
```

```
(2)1500 G.....................................
(3)1500 G.............................A.......
(4)1500 ..................G.....................
(5)1500 G.................G.....................
```

**Figure 4** Homologies of nucleotide sequence of cloned *H pylori flaB* gene with reported sequences. (1) the sequencing result of *H pylori* strain Y06 *flaB* gene; (2)-(5): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. L08907, strain 85P; No. AF479024, strain CH-CTX1). Underlined areas indicate the positions of primer sequences.

```
(1)1   MSFRINTNIAALTSHAVGVQNNRDLSSSLEKLSSGLRINKAADDSSGMAIADSLRSQSAN
(2)1   ............................................................
(3)1   ............................................................
(4)1   ............................................................
(5)1   ............................................................

(1)61  LGQAIRNANDAIGMVQTADKAMDEQIKILDTIKTKAVQAAQDGQTLESRRALQSDIQRLL
(2)61  ............................................................
(3)61  ............................................................
(4)61  ............................................................
(5)61  ............................................................

(1)121 EELDNIANTTSFNGQQMLSGSFSNKEFQIGAYSNTTVKASIGSTSSDKIGHVRMETSSFS
(2)121 ................................A.......................
(3)121 ............................................................
(4)121 ............................................................
(5)121 ............................................................

(1)181 GEGMLASAAAQNLTEVGLNFKQVNGVNDYKIETVRISTSAGTGIGALSEIINRFSNTLGV
(2)181 .A..........................................................
(3)181 ............................................................
(4)181 .........GA.................................................
(5)181 ............................................................

(1)241 RASYNVMATGGTPVQSGTVRELTINGVEIGTVNDVHKNDADGRLTNAINSVKDRTGVEAS
(2)241 ............................................................
(3)241 ............................................................
(4)241 ............................................................
(5)241 ................................R...........................

(1)301 LDIQGRINLHSIDGRAISVHAASASGQVFGGGNFAGISGTQHAVIGRLTLTRTDARDIIV
(2)301 ............................................................
(3)301 ............................................................
(4)301 ................................T...........................
(5)301 ............................................................

(1)361 SGVNFSHVGFHSAQGVAEYTVNLRAVRGIFDANVASAAGANANGAQAETNSQGIGAGVTS
(2)361 ............................................................
(3)361 ............................................................
(4)361 ............................................................
(5)361 ............................................................

(1)421 LKGAMIVMDMADSARTQLDKIRSDMGSVQMELVTTINNISVTQVNVKAAESQIRDVDFAE
(2)421 ............................................................
(3)421 ............................................................
(4)421 ............................................................
(5)421 ............................................................

(1)481 ESANFSKYNILAQSGSFAMAQANAVQQNVLRLLQ    514aa
(2)481 ..............................
(3)481 ..............................
(4)481 ..............................
(5)481 ..............................
```

**Figure 5** Homologies of putative amino acid sequences of *H pylori flaB* gene with reported sequences. (1): the sequencing result of *H pylori* strain Y06 *flaB* gene; (2)-(5): the reported sequences from GenBank (No. NC000915, strain 26695; No. NC_000921, strain J99; No. L08907, strain 85P; No. AF479024, strain CH-CTX1).

## DISCUSSION

In the present study, *H pylori flaA* and *flaB* genes were detected in genomic DNA of almost all *H pylori* isolates, and their nucleotide and amino acid sequences were considerably conserved[53,54]. The FlaA and FlaB expressed by *H pylori* rendered the organism strong motility in mucous environment, induced IL-8 secretion and facilitated inflammation in gastric tissue[51,52]. Furthermore, we observed that serum antibodies against FlaA and FlaB were present in approximate 98.4 % and 92.8 % of *H pylori* infected patients, respectively, the rates were significantly higher than those of heat shock protein (68 %) and vacuolating cytotoxin (68 %)[60]. These data indicate that *flaA* and *flaB* genes express their products in majority of *H pylori* strains and efficiently induce specific antibodies, implying a brilliant potential for developing *H pylori* vaccine.

The *flaA* gene from *H pylori* strain Y06, cloned in this study, showed high homologies of nucleotide and putative amino acid sequences compared with the published corresponding sequences (Figures 2 and 3)[53-55]. Similarly, the homologies of nucleotide and putative amino acid sequences of the cloned *flaB* gene from *H pylori* strain Y06 were quite high when compared with the published corresponding sequences (Figures 4 and 5)[53,54,56-59]. The high conservation of nucleotide and putative amino acid sequences found in the cloned *flaA* and *flaB* genes were probably due to their expression products just as the structural peptides of *H pylori*.

In the present study, SDS-PAGE demonstrated that the constructed expression systems *pET32a-flaA*-BL21DE3 and *pET32a-flaB*-BL21DE3 were able to efficiently produce the target recombinant proteins. However, rFlaA and rFlaB were mainly presented with the form of inclusion body even if they were induced by IPTG at a lower concentration. The high output of rFlaA and rFlaB (40-50 %) was beneficial to the production of a possible *H pylori* vaccine.

The rabbit antiserum against the whole cell of *H pylori* recognizes and combined with rFlaA and rFlaB as confirmed by Western blot, indicated that the two recombinant proteins had a relatively high immunoreactivity. The immunodiffusion assay performed in this study demonstrated that rFlaA and rFlaB could efficiently induce rabbit to produce specific antibodies with a higher titer, which indicated that these two recombinant proteins exhibited favorable antigenicity.

All tested *H pylori* isolates (98/98) expressed FlaA while 99.0 % (97/98) of the tested isolates expressed FlaB, as detected by ELISA. Of the *H pylori* infected patients, 98.4 % (123/125) and 92.8 % (116/125) were seropositive for the specific antibodies against rFlaA and against rFlaB, respectively. The universal existence of FlaA and FlaB in *H pylori* strains and the efficient induction of specific antibodies against FlaA and FlaB in patients were the strong favorable evidences for using these two recombinant proteins as the potential antigens in the development of *H pylori* vaccine.

In conclusion, FlaA and FlaB are excellent and ideal antigens that can be potentially used for the development of *H pylori* vaccine, and the expression systems of FlaA and FlaB with a high efficiency has been successfully constructed.

## ACKNOWLEDGEMENTS

We are grateful to the four hospitals in Hangzhou that provided gastric biopsies in this study, which help us to complete the research subject.

## REFERENCES

1 **Ye GA**, Zhang WD, Liu LM, Shi L, Xu ZM, Chen Y, Zhou DY. *Helicobacter pylori vacA* gene polymorphism and chronic gastrosis. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 593-595

2 **Lu SY**, Pan XZ, Peng XW, Shi ZL. Effect of Hp infection on gastric epithelial cell kinetics in stomach diseases. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 760-762

3 **Zhang Z**, Yuan Y, Gao H, Dong M, Wang L, Gong YH. Apoptosis, proliferation and p53 gene expression of *H pylori* associated gastric epithelial lesions. *World J Gastroenterol* 2001; **7**: 779-782

4 **Lu XL**, Qian KD, Tang XQ, Zhu YL, Du Q. Detection of *H pylori* DNA in gastric epithelial cells by in situ hybridization. *World J Gastroenterol* 2002; **8**: 305-307

5 **Yao YL**, Xu B, Song YG, Zhang WD. Overexpression of cyclin E in Mongolian gerbil with *Helicobacter pylori*-induced gastric precancerosis. *World J Gastroenterol* 2002; **8**: 60-63

6 **Guo DL**, Dong M, Wang L, Sun LP, Yuan Y. Expression of gastric cancer-associated MG7 antigen in gastric cancer, precancerous lesions and *H pylori*-associated gastric diseases. *World J Gastroenterol* 2002; **8**: 1009-1013

7 **Peng ZS**, Liang ZC, Liu MC, Ouyang NT. Studies on gastric epithelial cell proliferation and apoptosis in Hp associated gastric ulcer. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 218-219

8 **Hiyama T**, Haruma K, Kitadai Y, Miyamoto M, Tanaka S, Yoshihara M, Sumii K, Shimamoto F, Kajiyama G. B-cell monoclonality in *Helicobacter pylori*-associated chronic atrophic gastritis. *Virchows Arch* 2001; **483**: 232-237

9 **Harry XH**. Association between *Helicobacter pylori* and gastric cancer: current knowledge and future research. *World J Gastroenterol* 1998; **4**: 93-96

10 **Quan J**, Fan XG. Progress in experimental research of *Helicobacter pylori* infection and gastric carcinoma. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 1068-1069

11 **Liu HF**, Liu WW, Fang DC. Study of the relationship between apoptosis and proliferation in gastric carcinoma and its precancerous lesion. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 649-651

12 **Zhu ZH**, Xia ZS, He SG. The effects of ATRA and 5-Fu on telomerase activity and cell growth of gastric cancer cells *in vitro*. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 669-673

13 **Tu SP**, Zhong J, Tan JH, Jiang XH, Qiao MM, Wu YX, Jiang SH. Induction of apoptosis by arsenic trioxide and hydroxy camptothecin in gastric cancer cells *in vitro*. *World J Gastroenterol* 2000; **6**: 532-539

14 **Cai L**, Yu SZ, Zhang ZF. *Helicobacter pylori* infection and risk of gastric cancer in changle county, Fujian Province, China. *World J Gastroenterol* 2000; **6**: 374-376

15 **Yao XX**, Yin L, Zhang JY, Bai WY, Li YM, Sun ZC. Htert expression and cellular immunity in gastric cancer and precancerosis. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 508-512

16 **Xu AG**, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins *Bcl*-2, *p*53 and *C-myc* in the development of gastric cancer. *World J Gastroenterol* 2001; **7**: 403-406

17 **Wang X**, Lan M, Shi YQ, Lu J, Zhong YX, Wu HP, Zai HH, Ding J, Wu KC, Pan BR, Jin JP, Fan DM. Differential display of vincristine-resistance-related genes in gastric cancer SGC7901 cells. *World J Gastroenterol* 2002; **8**: 54-59

18 **Liu JR**, Li BX, Chen BQ, Han XH, Xue YB, Yang YM, Zheng YM, Liu RH. Effect of cis-9, trans-11-conjugated linoleic acid on cell cycle of gastric adenocarcinoma cell line (SGC-7901). *World J Gastroenterol* 2002; **8**: 224-229

19 **Cai L**, Yu SZ. A molecular epidemiologic study on gastric cancer in Changle, Fujian Province. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 652-655

20 **Gao GL**, Yang Y, Yang SF, Ren CW. Relationship between proliferation of vascular andothelial cells and gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 282-284

21 **Xue XC**, Fang GE, Hua JD. Gastric cancer and apoptosis. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 359-361

22 **Niu WX**, Qin XY, Liu H, Wang CP. Clinicopathological analysis of patients with gastric cancer in 1200 cases. *World J Gastroenterol* 2001; **7**: 281-284

23 **Li XY**, Wei PK. Diagnosis of stomach cancer by serum tumor markers. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 568-570

24 **Fang DC**, Yang SM, Zhou XD, Wang DX, Luo YH. Telomere erosion is independent of microsatellite instability but related to loss of heterozygosity in gastric cancer. *World J Gastroenterol* 2001; **7**: 522-526

25 **Morgner A**, Miehlke S, Stolte M, Neubauer A, Alpen B, Thiede C, Klann H, Hierlmeier FX, Ell C, Ehninger G, Bayerdorffer E.

Development of early gastric cancer 4 and 5 years after complete remission of *Helicobacter pylori* –associated gastric low-grade marginal zone B-cell lymphoma of MALT type. *World J Gastroenterol* 2001; **7**: 248-253

26 **Deng DJ**. Progress of gastric cancer etiology: N-nitrosamides in the 1999s. *World J Gastroenterol* 2000; **6**: 613-618

27 **Liu ZM**, Shou NH, Jiang XH. Expression of lung resistance protein in patients with gastric carcinoma and its clinical significance. *World J Gastroenterol* 2000; **6**: 433-434

28 **Guo CQ**, Wang YP, Liu GY, Ma SW, Ding GY, Li JC. Study on *Helicobacter pylori* infection and p53, c-erbB-2 gene expression in carcinogenesis of gastric mucosa. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 313-315

29 **Cai L**, Yu SZ, Ye WM, Yi YN. Fish sauce and gastric cancer: an ecological study in Fujian Province, China. *World J Gastroenterol* 2000; **6**: 671-675

30 **Xue FB**, Xu YY, Wan Y, Pan BR, Ren J, Fan DM. Association of *H pylori* infection with gastric carcinoma: a Meta analysis. *World J Gastroenterol* 2001; **7**: 801-804

31 **Wang RT**, Wang T, Chen K, Wang JY, Zhang JP, Lin SR, Zhu YM, Zhang WM, Cao YX, Zhu CW, Yu H, Cong YJ, Zheng S, Wu BQ. *Helicobacter pylori* infection and gastric cancer: evidence from a retrospective cohort study and nested case-control study in China. *World J Gastroenterol* 2002; **8**: 1103-1107

32 **Hua JS**. Effect of *Hp*: cell proliferation and apoptosis on stomach cancer. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 647-648

33 **Liu DH**, Zhang XY, Fan DM, Huang YX, Zhang JS, Huang WQ, Zhang YQ, Huang QS, Ma WY, Chai YB, Jin M. Expression of vascular endothelial growth factor and its role in oncogenesis of human gastric carcinoma. *World J Gastroenterol* 2001; **7**: 500-505

34 **Cao WX**, Ou JM, Fei XF, Zhu ZG, Yin HR, Yan M, Lin YZ. Methionine-dependence and combination chemotherapy on human gastric cancer cells *in vitro*. *World J Gastroenterol* 2002; **8**: 230-232

35 **Michetti P**, Kreiss C, Kotloff KL, Porta N, Blanco JL, Bachmann D, Herranz M, Saldinger PF, Corthesy-Theulaz I, Losonsky G, Nichols R, Simon J, Stolte M, Acherman S, Monath TP, Blum AL. Orla immunization with urease and Escherichia coli heat-labile enterotoxin is safe and immunogenic in *Helicobacter pylori*-infected adults. *Gastroenterology* 1999; **116**: 804-812

36 **Suganuma M**, Kurusu M, Okabe S, Sueoka N, Yoshida M, Wakatsuki Y, Fujiki H. *Helicobacter pylori* membrane protein 1: a new carcinogenic factor of *Helicobacter pylori*. *Cancer Res* 2001; **61**: 6356-6359

37 **Nakamura S**, Matsumoto T, Suekane H, Takeshita M, Hizawa K, Kawasaki M, Yao T, Tsuneyoshi M, Iida M, Fujishima M. Predictive value of endoscopic ultrasonography for regression of gastric low grade and high grade MALT lymphomas after eradication of *Helicobacter pylori*. *Gut* 2001; **48**: 454-460

38 **Uemura N**, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper RJ. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001; **345**: 8298-8232

39 **Morgner A**, Miehlke S, Fischbach W, Schmitt W, Muller-Hermelink H, Greiner A, Thiede C, Schetelig J, Neubauer A, Stolte M, Ehninger G, Bayerdorffer E. Complete remission of primary high-grade B-cell gastric lymphoma after cure of *Helicobacter pylori* infection. *J Clin Oncol* 2001; **19**: 2041-2048

40 **Kate V**, Ananthakrishnan N, Badrinath S. Effect of *Helicobacter pylori* eradication on the ulcer recurrence rate after simple closure of perforated duodenal ulcer: retrospective and prospective randomized controlled studies. *Br J Surg* 2001; **88**: 1054-1058

41 **Zhuang XQ**, Lin SR. Progress in research on the relationship between *Hp* and stomach cancer. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 206-207

42 **Gao HJ**, Yu LZ, Bai JF, Peng YS, Sun G, Zhao HL, Miu K, Lü XZ, Zhang XY, Zhao ZQ. Multiple genetic alterations and behavior of cellular biology in gastric cancer and other gastric mucosal lesions: *H pylori* infection, histological types and staging. *World J Gastroenterol* 2000; **6**: 848-854

43 **Yao YL**, Zhang WD. Relation between *Helicobacter pylori* and gastric cancer. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 1045-1049

44 **Goto T**, Nishizono A, Fujioka T, Ikewaki J, Mifune K, Nasu M. Local secretory immunoglobulin A and postimmunization gastritis correlated with protection against *Helicobacter pylori* infection after oral vaccination of mice. *Infect Immun* 1999; **67**: 2531-2539

45 **Watanabe T**, Tada M, Nagai H, Sasaki S, Nakao M. *Helicobacter pylori* infection induces gastric cancer in Mongolian Gerbils. *Gastroenterology* 1998; **115**: 642-648

46 **Honda S**, Fujioka T, Tokieda M, Satoh R, Nishizono A, Nasu M. Development of *Helicobacter pylori*-induced gastric carcinoma in Mongolian Gerbils. *Cancer Res* 1998; **58**: 4255-4259

47 **Hatzifoti C**, Wren BW, Morrow WJ. *Helicobacter pylori* vaccine strategies-triggering a gut reaction. *Immuno Today* 2000; **21**: 615-619

48 **Kotloff KL**, Sztein MB, Wasserman SS, Losonsky GA, DiLorenzo SC, Walker RI. Safety and immunogenicity of oral inactivated whole-cell *Helicobacter pylori* vaccine with adjuvant among volunteers with or without subclinical infection. *Infect Immun* 2001; **69**: 3581-3590

49 **Dubois A**, Lee CK, Fiala N, Kleanthous H, Mehlman PT, Monath T. Immunization against natural *Helicobacter pylori* infection in nonhuman primates. *Infect Immune* 1998; **66**: 4340-4346

50 **Ikewaki J**, Nishizono A, Goto T, Fujioka T, Mifune K. Therapeutic oral vaccination induces mucosal immune response sufficient to eliminate long-term *Helicobacter pylori* infection. *Microbiol Immunol* 2000; **44**: 29-39

51 **Eaton KA**, Suerbaum S, Josenhans C, Krakowka S. Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. *Infect Immun* 1996; **64**: 2445-2448

52 **Watanabe S**, Takagi A, Tada U, Kabir AM, Koga Y, Kamiya S, Osaki T, Miwa T. Cytotoxicity and motility of *Helicobacter pylori*. *J Clin Gastroenterol* 1997; **25**(Suppl 1): S169-171

53 **Tomb JF**, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Venter JC, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Karp PD, Smith HO, Fraser CM. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; **388**: 539-547

54 **Alm RA**, Ling SL, Moir DT, King BL, Brown ED, Doig PC, Smith DR, Noonan B, Guild BC, Dejonge BL, Carmel G, Tummino PJ, Caruso A, Uria-Nickelsen M, Mills DM, Lves C, Gibson R, Merberg D, Mills SD, Jiang Q, Taylor DE, Vovis GF, Trust TJ. Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* 1999; **397**: 176-180

55 **Leying H,** Suerbaum S, Geis G, Haas R. Cloning and genetic characterization of a *Helicobacter pylori* flagellin gene. *Mol Microbiol* 1992; **6**: 2863-2874

56 **Suerbaum S,** Josenhans C, Labigne A. Cloning and genetic characterization of the *Helicobacter pylori* and Helicobacter mustelae *flaB* flagellin genes and construction of *H pylori flaA*- and *flaB*-negative mutants by electroporation-mediated allelic exchange. *J Bacteriol* 1993; **175**: 3278-3288

57 **Sambrook J**, Fritsch E F, Maniatis T. Molecular Cloning, A Laboratory Manual [M]. 2nd edition. New York: *Cold Spring Harbor Laboratory Press* 1989: 1.21-1.52, 2.60-2.80, 7.3-7.35, 9.14-9.22

58 **Chen Y**, Wang JD, Shi L. *In vitro* study of the biological activities and immunogenicity of recombinant adhesion of *Helicobacter pylori* rHpaA. *Zhonghua Yixue Zazhi* 2001; **81**: 276-279

59 **Josenhans C,** Labigne A, Suerbaum S. Comparative ultrastructural and functional studies of *Helicobacter pylori* and *Helicobacter* mustelae flagellin mutants: both flagellin subunits, FlaA and FlaB, are necessary for full motility in *Helicobacter species*. *J Bacteriol* 1995; **177**: 3010-3020

60 **Opazo P**, Muller I, Rollan A, Valenzuela P, Yudelevich A, Garcia-de la Guarda R, Urra S, Venegas A. Serological response to *Helicobacter pylori* recombinant antigens in Chilean infected patients with duodenal ulcer, non-ulcer dyspepsia and gastric cancer. *APMIS* 1999; **107**: 1069-1078

**Edited by** Xia HHX and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2251-2257
World Journal of Gastroenterology
 ISSN 1007-9327



# Deletion of *Helicobacter pylori* vacuolating cytotoxin gene by introduction of directed mutagenesis

Jian-Ping Yuan, Tao Li, Xiao-Dong Shi, Bao-Yu Hu, Gui-Zhen Yang, Shan-Qing Tong, Xiao-Kui Guo

**Jian-Ping Yuan, Tao Li, Xiao-Dong Shi, Bao-Yu Hu, Gui-Zhen Yang, Shan-Qing Tong, Xiao-Kui Guo,** Department of Microbiology and Parasitology, Shanghai Second Medical University, Shanghai 200025, China
**Supported by** Ministry of Education Research Foundation for Returned Overseas Chinese Scholars Abroad (2001) 498
**Correspondence to:** Xiao-Kui Guo, Department of Microbiology and Parasitology, Shanghai Second Medical University, 280 Chongqingnan Road, Shanghai 200025, China. xkguo@shsmu.edu.cn
**Telephone:** +86-21-64671226 **Fax:** +86-21-64671226
**Received:** 2003-03-02 **Accepted:** 2003-05-16

## Abstract

**AIM:** To construct a *vacA*-knockout *Helicobacter pylori* mutant strain, whose only difference from the wild strain is its disrupted *vacA* gene.

**METHODS AND RESULTS:** A clone containing kanamycin resistance gene used for homologous recombination was constructed in a directional cloning procedure into pBluescript II SK, and then transformed into *vacA*$^+$ *H pylori* by electroporation. Colonies growing on the selective media containing kanamycin were harvested for chromosomal DNA extraction, and the allelic exchange was determined by polymerase chain reactions and sequencing. Loss of vacuolating activity of the *vacA*-knockout strain was confirmed by examining the gastric cells co-cultured with cell-free supernatants from *H pylori* wild strain or the mutant.

**CONCLUSION:** We constructed a *vacA*-knockout strain of *H pylori* through direct mutagenesis, which creates an important precondition for the future research on virulence comparison with gene expression analysis.

Yuan JP, Li T, Shi XD, Hu BY, Yang GZ, Tong SQ, Guo XK. Deletion of *Helicobacter pylori* vacuolating cytotoxin gene by introduction of directed mutagenesis. *World J Gastroenterol* 2003; 9(10): 2251-2257

http://www.wjgnet.com/1007-9327/9/2251.asp

## INTRODUCTION

*Helicobacter pylori* (*H pylori*) is a Gram-negative bacterium that colonizes the gastric mucosa of humans[1], and plays an important role in pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinomas, and gastric mucosa-associated lymphoid tissue lymphomas[2-6]. Leunk *et al* first reported in 1988 that cell-free supernatants from *H pylori* broth cultures induced striking vacuolar degeneration when added to cultured eukaryotic cells[7]. Subsequently in 1992, this effect was disclosed to be caused by a secreted toxin VacA[8].

The gene encoding the vacuolating cytotoxin has been cloned from an *H pylori* isolate, and termed *vacA*[9]. Analysis of the nucleotide sequence of the *vacA* open reading frame (ORF) suggested that *vacA* encoded a 139-kDa protoxin that has three functional domains: a 33-amino-acid N-terminal signal sequence, a mature cytotoxin domain (approximately 87kDa), and a cleaved C-terminal domain (approximately 50kDa)[10,11]. VacA could induce vacuole formation from the cell cytosol, as determined by transfection of epithelial cells with a plasmid encoding the complete 95-kDa domain of VacA[12]. These vacuoles are acidic, and their membrane contains the vacuolar ATPase proton pump and the small GTP-binding protein rab7. Therefore, they have been suggested to arise from late compartments of the endocytic pathway[13].

Over the past decade, there has been considerable effort directing toward understanding the molecular mechanisms underlying VacA action. But till now, little is known about the mechanisms of vacuole formation and other effects of VacA. In this study, using the technique of direct mutagenesis to disrupt *vacA* gene, we constructed a *vacA*-knockout *H pylori* mutant strain for the further research on virulence comparison between the *H pylori* wild strain and the mutant.

## MATERIALS AND METHODS

### *Bacterial strain and growth conditions*

*H pylori* NCTC 11638 as a gift from Dr. Tong Shi (Shanghai Institute of Digestive Diseases) was cultured routinely on brain heart infusion (BHI) agar plates with 5 % sheep blood in an environment containing 6 % $CO_2$ at 37 ℃. For the preparation of cell-free supernatants from *H pylori* broth cultures, *H pylori* was cultured in BHI broth+10 % fetal bovine serum (FBS) in an environment containing 6 % $CO_2$ at 37 ℃ with agitation (200 rpm) for 48 h. The cultures were centrifuged (15 000 g, 30 min, 4 ℃) and filtrated with a 0.2 μm syringe filter.

### *Disruption of vacA gene*

The strategy for disruption of *vacA* gene by direct mutagenesis is shown in Figure 1, and genetic techniques involved were described as follows.

**DNA isolation** To isolate chromosomal DNA, *H pylori* cells were lysed in lysis buffer (10 mM Tris· HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5 % (w/v) SDS, 20 μg/ml DNase free pancreatic RNase), and then, protease K (Sangon, Shanghai, China) was added in to a final concentration of 100 μg/ml. The lysate was incubated in a water bath at 50 ℃ for 3 h. Then, the solution was cooled to room temperature, and mixed with an equal volume of phenol equilibrated with 0.1 M Tris· HCl (pH 8.0). The two phases were separated by centrifugation at 5 000 g for 15 min at room temperature, and the aqueous phase was extracted with phenol twice again. Afterwards, 0.2 volume of 10 M ammonium acetate and 2 volume of ethanol were added to the aqueous phase. The precipitate was collected by centrifugation at 5 000 g for 2 min, washed twice with 70 % ethanol, and dissolved in an appropriate volume of TE buffer (pH8.0)[14].

**Polymerase chain reactions (PCR)** PCR was carried out in 100 μl volume containing 100 ng of genomic DNA, 1 U of Ex Taq (Takara), 50 pmol of each primer, and 10 nmol of each deoxynucleoside triphosphate in a standard buffer. Oligonucleotide primers (5'-CGTGGAAATCTTATTACT CTTAGC-3' and 5'-TGATGCTGACTAATGCTCCT-3')

were used to amplify a 1.7 kb product from *H pylori* NCTC 11 638. Primers for amplifying kanamycin resistance gene (*kanR*) and two fragments flanking *kanR*, *LA* and *RA*, were designed as shown in Table 1.

**Gel purification and enzyme digestion** PCR products were electrophoresed and excised from a 1 % agarose gel, purified using a Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and digested with corresponding restriction enzymes (Promega, Madison, USA) depending on different restriction sites.

**Cloning of different DNA fragments** Purified PCR products for sequencing were cloned into *pGEM-T* vector (Promega). Fragments *kanR*, *LA*, and *RA* with different restriction sites on both sides were digested with corresponding endonucleases (Promega), and then cloned into *pBluescript SK* II digested with the same enzymes.

**Sequencing** Every clone was sequenced with the ABI DNA sequencer (Bioasia Biotechnology Company, Shanghai, China).

***H pylori* DNA transformation by electroporation** *H pylori* NCTC 11 638 cells were transformed with plasmid *pLKR* by electroporation, and kanamycin-resistant (*Km*$^r$) transformants were selected by a method similar to that described by Clayton *et al*[15]. Briefly, *H pylori* cultured on plates were scraped and suspended in 30 ml cold double-distilled water. Cells were harvested by centrifugation at 4 360 g at 4 ℃ for 5 min, and the pellet was suspended in 20 ml of cold 10 % glycerol. The cells were centrifuged once, and resuspended in 2 ml ice-cold 10 % glycerol. Plasmid DNA (1 μg in 5 μl TE buffer) was mixed with 0.2 ml cell suspension. The mixture was added to a prechilled (-20 ℃) 0.2 cm electroporation cuvet (Bio-Rad, Hercules, USA), and subjected to single-pulse electroporation of initial voltage 2.5 kV, 25 μF and 600Ω in parallel. The sample was transferred onto a cold plate and incubated for 12 h at 37 ℃. Then the cells were inoculated onto selective media with 30 μg/mL kanamycin, followed by incubation for 4 d to allow the growth of transformants.

### *Cell culture and detection of vacuole formation*

Cells of gastric cancer cell line SGC7901 as a gift from Jie Yang (Department of Cell Biology, Shanghai Second Medical University) were grown in DMEM (GIBCO-BRL, Gaithersburg, USA) supplemented with 10 % FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5 % $CO_2$ atmosphere. SGC7901 cells were co-cultured with cell-free supernatants from *H pylori* NCTC11638 wild strain or mutant strain for 12 h, and then observed by contrast microscopy.

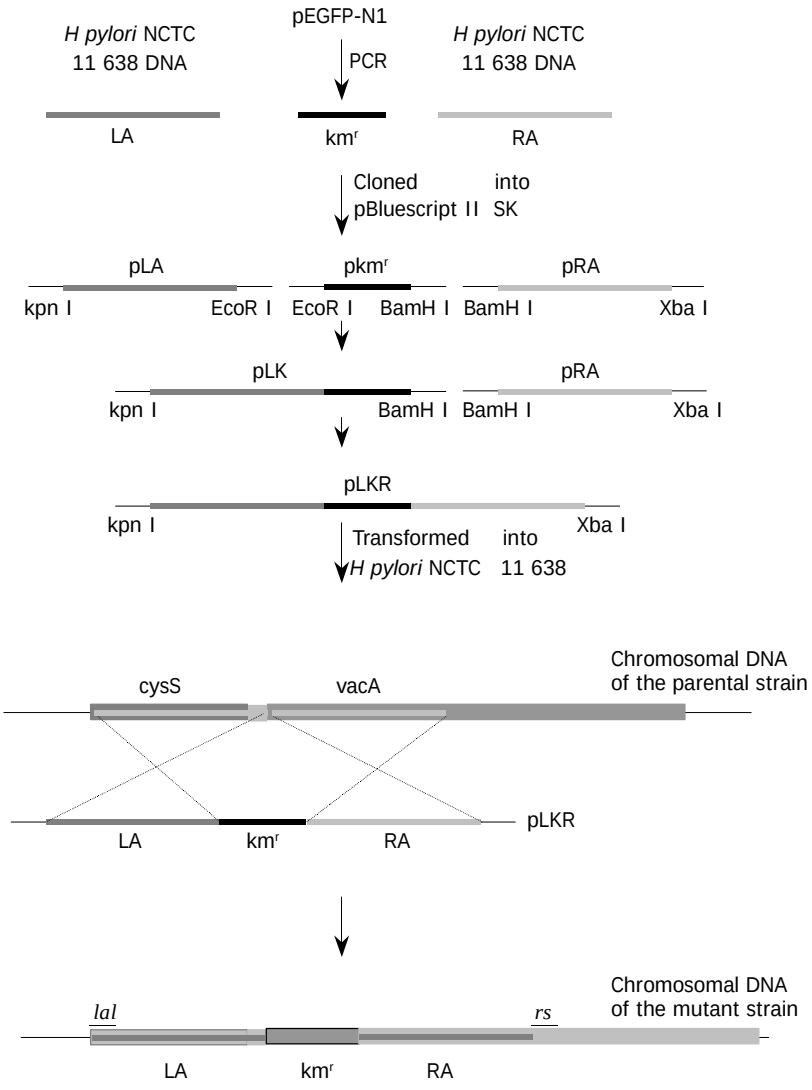



**Figure 1** The strategy for disruption of *vacA* gene by directed mutagenesis. LA and RA were PCR-amplified from *H pylori* NCTC 11638 genomic DNA, and the kanamycin resistance gene, from pEGFP-N1. PCR products with different restriction sites on both sides were digested with corresponding endonucleases, and then cloned into pBluescript II SK digested with the same enzymes. Because there is an *EcoR* II site in RA, *pLA* and *pkm*$^r$ were firstly joined together, resulting in *pLK*, which then was joined with *pRA*, resulting in *pLKR*. The plasmid *pLKR* was transformed into *H. pyloi* NCTC 11638, where the *Km*$^r$ marked mutation was introduced into the genome by homologous recombination, resulting in the *vacA*$^-$Km$^r$ mutant strain.

**Table 1** Primers for amplifying *km*$^r$, LA and RA

| Primer | Sequence (5' to 3') | Site | Coordinates |
|---|---|---|---|
| *la1* | GGGGTACCCTTTTGAGCCTTTAGTT | *Kpn* I | cysS bp36-54 |
| *la2* | CGGAATTCTCCTTTCTTTTTGTAAAAC | *Eco*R I | HPU07145 bp382-400 |
| *km*$^r$*1* | CGGAATTCATGATTGAACAAGATGGATTG | *Eco*R I | pEGFP-N1 bp 2629-2649 |
| *km*$^r$*2* | CGGGATCCTCAGAAGAACTCGTCAAG | *Bam*H I | pEGFP-N1 bp3406-3423 |
| *ra1* | CGGGATCCATCGCCCTCTGGTTTCTC | *Bam*H I | HPU07145 bp 437-454 |
| *ra2* | GCTCTAGACACCCACTTGATTATTCACTCT | *Xba* I | HPU07145 bp 1786-1807 |

cysS *la1*

1 ATGTTTATTT ATGATACCAA ATTAAAACAA AAAGTCCCTT TTGAGCCTTT

51 AGTTGAAAAA AAGGCGAATA TTTATGTGTG CGGGCCTACG GTGTATGATG
101 ACGCTCATTT AGGGCATGCC AGGAGCGCGA TTGCTTTTGA TTTGTTAAGG
151 CGCACGCTTG AATTGAGCGG CTATGAAGTG GTGTTAGTAA GGAATTTCAC
201 GGATATTGAC GATAAAATCA TCAACAAAGC CTTAAAAGAA AACAAAAGCA
251 TTCAAGAATT AAGCAGCATT TACATTGAAT CTTACACGAG GGATTTAAAC
301 GCTTTGAACG TGAAAAAACC CAGCCTAGAG CCTAAAGCGA GCGAGTATTT
351 AGACGCTATG GTGGGCATGA TTGAAACGCT TTTAGAAAAA AATATCGCTT
401 ATCAGGTCTC TAATGGGGAT ATTTATTTAG ACACGAGCAA GGATAAAGAT
451 TACGGCTCTT TGAGCGTGCA TAATAGCAGT ATGGAATTTG GCCGTATTGG
501 TTTGGTGCAA GAAAAACGGC TTGAGCAGGA TTTTGTGCTA TGGAAAAGCT
551 ATAAGGGGGA TAATGATGTG GGTTTTGATA GCCCTTTAGG CAAAGGGCGC
601 CCTGGCTGGC ATATAGAATG CTCTAGCATG GTTTTTGAAA CTTTAGCACT
651 CGCTAACACC CCTTATCAAA TTGACATCCA TGCAGGCGGA GCGGATCTGT
701 TATTCCCCCA CCATGAAAAT GAAGCGTGCC AAACCCGTTG CACCTTTGGC
751 GTGGAGCTTG CTAAATACTG GATGCATAAT GGCTTTGTGA ATATCAACAA
801 CGAAAAAATG TCTAAAAGTT TAGGGAATAG CTTTTTTATT AAAGACGCCC
851 TGAAAAACTA TGATGGCGAG ATTTTGCGCA ATTATTTACT AGGGGTGCAT
901 TATCGCTCTG TTTTGAATTT CAATGAAGAA GACTTGTTAG TGAGTAAAAA
951 ACGCTTGGAT AAAATCTATC GTTTGAAACA GCGCGTTTTA GGGACTTTGG
1001 GAGGAATAAA TCCAAACTTT AAAAAAGAAA TTTTAGAGTG CATGCAAGAT
1051 GATTTAAACG TTTCTAAAGC GTTGAGCGTT TTAGAAAGCA TGCTTTCTTC
1101 TACGAATGAA AAACTGGATC AAAACCCCAA AAACAAGGCT TTGAAGGGCG
1151 AAATTTTAGC GAATTTGAAA TTCATAGAAG AACTGCTTGG TATTGGGTTT
1201 AAAGACCCTA GCGCGTATTT CCAGTTAGGC GTGAGCGAGA GCGAAAAACA
1251 AGAAATTGAA AACAAGATAG AAGAAAGAAA ACGCGCCAAA GAACAAAAAA
1301 TTTTTTTAAA AGCCGATAGC ATCAGAGAAG AACTTTTGAA ACAAAAAATC
1351 GCTTTGATGG ACACCCCACA AGGCACGATT TGGGAGAAGT TTTTTTAAAC
1401 GCCTCCAATT TTACCTTTTT ACACATTCTA GTAACAACCT TCAGCATTTT
1451 TGCTTTTTAA TCTTGTTAAG TTTTATGTTC ATTTACTTTA ATTTGATAAA

1501 AATTGAACAT TGGTTGTAGA TACTATATAT TTATAGCCTT AATCGTAAAT
-35 signal -10 signal

1551 GCAACAGAAA TTTTCTAGTC TAAAGTCGCA CCCTTTGTGC AAAAATCGTT

*la2* km$^r$

1601 TTACAAAAAG AAAGGAGAAT TCATGATTGA ACAAGATGGA TTGCACGCAG
rbs

1651 GTTCTCCGGC CGCTTGGGTG GAGAGGCTAT TCGGCTATGA CTGGGCACAA
1701 CAGACAATCG GCTGCTCTGA TGCCGCCGTG TTCCGGCTGT CAGCGCAGGG

1751 GCGCCCGGTT CTTTTTGTCA AGACCGACCT GTCCGGTGCC CTGAATGAAC
1801 TGCAAGACGA GGCAGCGCGG CTATCGTGGC TGGCCACGAC GGGCGTTCCT
1851 TGCGCAGCTG TGCTCGACGT TGTCACTGAG GCGGGAAGGG ACTGGCTGCT
1901 ATTGGGCGAA GTGCCGGGGC AGGATCTCCT GTCATCTCAC CTTGCTCCTG
1951 CCGAGAAAGT ATCCATCATG GCTGATGCAA TGCGGCGGCT GCATACGCTT
2001 GATCCGGCTA CCTGCCCATT CGACCACCAA GCGAAACATC GCATCGAGCG
2051 AGCACGTACT CGGATGGAAG CCGGTCTTGT CGATCAGGAT GATCTGGACG
2101 AAGAGCATCA GGGGCTCGCG CCAGCCGAAC TGTTCGCCAG GCTCAAGGCG
2151 AGCATGCCCG ACGGCGAGGA TCTCGTCGTG ACCCATGGCG ATGCCTGCTT
2201 GCCGAATATC ATGGTGGAAA ATGGCCGCTT TTCTGGATTC ATCGACTGTG
2251 GCCGGCTGGG TGTGGCGGAC CGCTATCAGG ACATAGCGTT GGCTACCCGT
2301 GATATTGCTG AAGAGCTTGG CGGCGAATGG GCTGACCGCT TCCTCGTGCT
2351 TTACGGTATC GCCGCTCCCG ATTCGCAGCG CATCGCCTTC TATCGCCTTC

*ra1*

2401 TTGACGAGTT CTTCTGAGGA TCCATCGCCC TCTGGTTTCT CTCGCTTTAG

2451 TAGGAGCATT AGTCAGCATC ACACCGCAAC AAAGTCATGC CGCCTTTTTC
2501 ACAACCGTGA TCATTCCAGC CATTGTTGGG GGTATCGCTA CAGGCACCGC
2551 TGTAGGAACG GTCTCAGGGC TTCTTAGCTG GGGGCTCAAA CAAGCCGAAG
2601 AAGCCAATAA AACCCCAGAT AAACCCGATA AAGTTTGGCG CATTCAAGCA
2651 GGAAAAGGCT TTAATGAATT CCCTAACAAG GAATACGACT TATACAGATC
2701 CCTTTTATCC AGTAAGATTG ATGGAGGTTG GGATTGGGGG AATGCCGCTA
2751 GGCATTATTG GGTCAAAGGC GGGCAACAGA ATAAGCTTGA AGTGGATATG
2801 AAAGACGCTG TAGGGACTTA TACCTTATCA GGGCTTAGAA ACTTTACTGG
2851 TGGGGATTTA GATGTCAATA TGCAAAAAGC CACTTTACGC TTGGGCCAAT
2901 TCAATGGCAA TTCTTTTACA AGCTATAAGG ATAGTGCTGA TCGCACCACG
2951 AGAGTGGATT TCAACGCTAA AAATATCTCA ATTGATAATT TTGTAGAAAT
3001 CAACAATCGT GTGGGTTCTG GAGCCGGGAG GAAAGCCAGC TCTACGGTTT
3051 TGACTTTGCA AGCTTCAGAA GGGATCACTA GCGATAAAAA CGCTGAAATT
3101 TCTCTTTATG ATGGTGCCAC GCTCAATTTG GCTTCAAGCA GCGTTAAATT
3151 AATGGGTAAT GTGTGGATGG GCCGTTTGCA ATACGTGGGA GCGTATTTGG
3201 CCCCTTCATA CAGCACGATA AACACTTCAA AAGTAACAGG GGAAGTGAAT
3251 TTTAACCACC TCACTGTTGG CGATAAAAAC GCCGCTCAAG CGGGCATTAT
3301 CGCTAATAAA AAGACTAATA TTGGCACACT GGATTTGTGG CAAAGCGCCG
3351 GGTTAAACAT TATCGCTCCT CCAGAAGGTG GCTATAAGGA TAAACCCAAT
3401 AATACCCCTT CTCAAAGTGG TGCTAAAAAC GACAAAAATG AAAGCGCTAA
3451 AAACGACAAA CAAGAGAGCA GTCAAAATAA TAGTAACACT CAGGTCATTA
3501 ACCCACCCAA TAGTGCGCAA AAAACAGAAG TTCAACCCAC GCAAGTCATT
3551 GATGGGCCTT TTGCGGGCGG CAAAGACACG GTTGTCAATA TCAACCGCAT
3601 CAACACTAAC GCTGATGGCA CGATTAGAGT GGGAGGGTTT AAAGCTTCTC
3651 TTACCACCAA TGCGGCTCAT TTGCATATCG GCAAAGGCGG TGTCAATCTG
3701 TCCAATCAAG CGAGCGGGCG CTCTCTTATA GTGGAAAATC TAACTGGGAA

*ra2*

3751 TATCACCGTT GATGGGCCTT TAAGAGTGAA TAATCAAGTG GGTGGCTATG

*rs*

3801 CTTTGGCAGG ATCAAGCGC

**Figure 2** Nucleotide sequence of cysS gene and the downstream sequence amplified from the *vacA*$^{-}$*Km*$^{r}$ mutant *H pylori*. The 1 398 bp *cysS* ORF and the 795 bp *km*$^{r}$ ORF are shown. Primers *la1*, *la2*, *ra1*, *ra2*, and *rs* for amplifying LA, RA, and ASm are indicated. -35 signal, -10 signal, and rbs of *vacA* gene serving km$^{r}$ gene in the mutant strain are also shown.

## RESULTS

### *Upstream sequence close to* vacA *gene*

Genome of NCTC 11638 was not completely sequenced, and the upstream portion close to *vacA* gene going to be used in the mutagenesis technique was not published in GeneBank. Therefore, the upstream sequences close to *vacA* gene of 26695 and J99, whose genomes were completely sequenced and published, were aligned and searched for conservative sequences. Then a 1.7-kb product was PCR-amplified from *H pylori* NCTC11638 DNA and sequenced. The sequencing result showed the complete *cysS* (cysteinyl-tRNA synthetase) gene of NCTC 11638 (Figure 2).

### *Cloning of pLKR for transforming H pylori*

As shown in Figure 1, LA which contains the *H pylori vacA* promoter and RA were amplified from genomic DNA of NCTC11638, while *kanR* gene which has no promoter was amplified from the plasmid *pEGFP-N1* (Clontech, Palo Alto, USA). PCR-products LA, *kanR* and RA, with restriction sites incorporated at the termini, were joined together in a directional cloning procedure into *pBluescript* II *SK*, resulting in *pLKR*.

### *Construction of vacA-knockout H pylori mutant strain*

*pLKR* which is unable to replicate in *H pylori*, was introduced into *H pylori* NCTC11638 by electroporation. After 4 d of growth, five $Km^r$ single colonies were isolated. To determine whether *vacA* had been disrupted in the transformed strains through allelic exchange, DNA isolated from *H pylori* NCTC11638 wild-type strain and the $Km^r$ mutant strain were PCR-amplified with the primers *la1* and *rs* (5' -GCGCTTGATC CTGCCAAAGCATAGC-3' ) annealing to *H pylori* NCTC 11638 *vacA* at bp 1808 to 1832 flanking *ra2* (Figures 1 and 2). A 3.8-kb product consistent with the expected size was PCR-amplified from $Km^r$ mutant strain, as compared with a 3.0-kb product amplified from wild strain (Figure 3), suggesting a substitution of $Km^r$ gene for a short fragment of *vacA* gene by homologous recombination between plasmid and chromosomal sequences. The sequencing result of the 3.8-kb product confirmed the occurrence of allelic exchange (Figure 2).

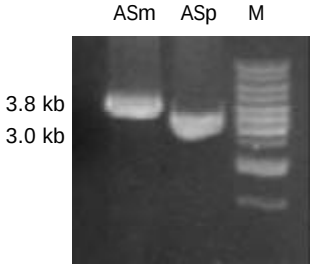



**Figure 3** PCR amplification for the determination of homologous recombination in $Km^r$ mutant strain. Genomic DNA of NCTC 11638 wild strain and $Km^r$ mutant strain were respectively PCR-amplified for the fragments ASm and ASp using the primers *ra1* and *rs*. A single 3.8 kb product ASm was amplified from $Km^r$ mutant strain as compared with the 3.0 kb product ASp amplified from the wild strain.

### *Characterization of vacA-knockout H pylori mutant*

To determine the loss of vacuolating activity of the mutant strain, gastric cells SGC7901 were co-cultured with cell-free supernatants from *H pylori* NCTC11638 wild strain or mutant strain for 12 h, and then observed by contrast microscopy. Intracellular vacuoles developed in cells co-cultured with supernatant from the wild strain, while no vacuoles developed in cells co-cultured with supernatant from $vacA^-Km^r$ mutant strain (Figure 4).

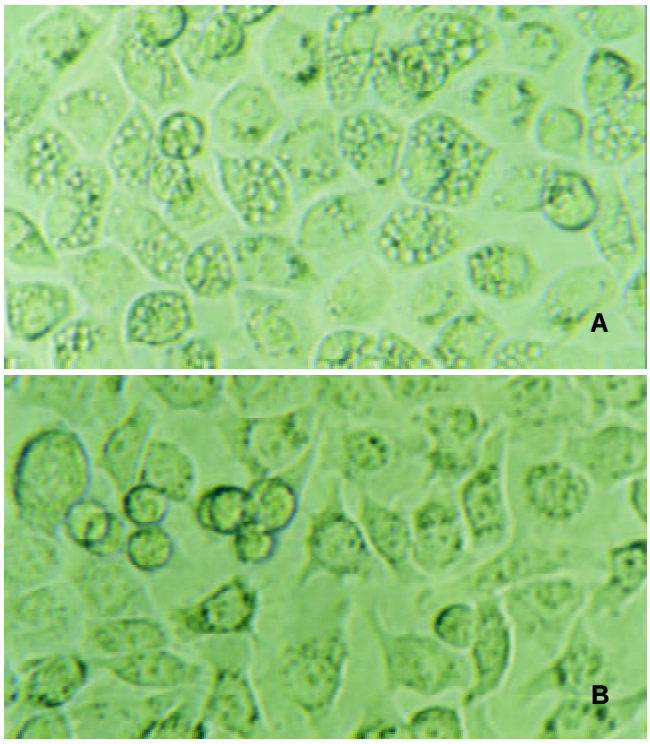



**Figure 4** Gastric cancer cells SGC-7901 were co-cultured with the supernatant either from *Helicobacter pylori* NCTC 11638 or its *vacA*-knockout mutant strain. 12 h after the incubation, A: cells co-cultured with NCTC 11638 developed vacuoles in the cytosol; B: cells co-cultured with $vacA^-$ mutant strain developed no vacuoles at all.

## DISCUSSION

VacA, produced by pathogenic strains of *H pylori*, was a major virulence factor in pathogenesis of gastroduodenal ulcers[8,16]. VacA induced the formation of membrane-delimited vacuoles in intoxicated cells[7], and showed many other effects on cellular functions and viability, such as causing mitochondrial depolarization[17], inducing apoptosis in gastric cells[18], affecting or interacting with various components of cytoskeleton to cause actin rearrangements[19], and even disorganizing microtubular network[20]. To study VacA machanism of action, we have tried to get purified VacA used as single virulence determinant, to study its effect on the expression profile of eukaryocyte. However, like Manetti *et al*[21], we did not successfully get the expressed VacA as a functional recombinant protein in *E. coli*, probably due to its incorrect fold. We have also considered letting VacA directly expressed in the cytosol to induce vacuole formation. In our experiments, vacuoles were induced in only 10 % of cells transfected with plasmids expressing VacA, because the efficiency of the transfection method was relatively low. In addition, when VacA acts outside the cells, the pathway by which it interacts with the cells is quite different from that when the protein is produced in the cytosol. Under natural conditions, association of VacA with the eukaryotic cell surface was the first step in the intoxication of cells[22]. The initial interaction of VacA with target cells was through high-affinity cell surface receptors, and this interaction was necessary for its biologic activity[23,24]. A 250 kDa receptor protein tyrosine phosphatase (RPTP) β served as a receptor for VacA on AZ521 cells, and another protein, p140, was also commonly detected in VacA-susceptible cells[25,26]. Increased binding of acid- or alkali-activated VacA to RPTPβ may alter its activity and possibly accelerates or inhibits dephosphorylation of tyrosine on cytosolic proteins. Moreover, VacA acting outside the cells

is a kind of exogenous antigen, having different pathways of processing and presentation from that of VacA expressed in the cytosol as an endogenous antigen. All of these processes may affect gene expression of the host cells.

Direct mutagenesis was probably the most useful technique for assessing the contribution to virulence of specific bacterial gene products[27]. In our study, *vacA* gene encoding vacuolating cytotoxin that has been identified by conventional biochemical means was disrupted by gene replacement. This technique requires a means for introducing DNA into the pathogen, as well as suitable selective markers and an inherent capacity for homologous recombination. In previous studies, the $Km^r$ determinant often came from *Campylobacter coli*[28]. Here we introduced a simple method to get the $Km^r$ gene from commercialized plasmids such as *pEGFP-N1*. Coding sequence of the gene without a promotor was PCR-amplified from *pEGFP-N1* and ligated downstream with the promotor of *H pylori vacA* gene. Upon insertion into chromosomal DNA of *H pylori* through homologous recombination, this gene could be efficiently transcribed because the *vacA* promoter was recognized by *H pylori* transcriptional machinery, introducing kanamycin resistance characteristics into *H pylori*. Due to the stop coden of $Km^r$, *vacA* would not be translated at all although most of the sequences still existed. The results of PCR and sequencing confirmed the occurrence of allelic exchange. Therefore, using the direct mutagenesis technique, we obtained the isogenic mutant strain of *H pylori*, which differed from the wild strain only in that the *vacA* gene was knocked out. Through co-culture of cell-free supernatants from the wild or mutant *H pylori* strain with gastric cells, loss of vacuolating activity of the *vacA*-knockout strain was confirmed. These results clearly show that VacA is an indispensable toxin secreted by *H pylori* for the induction of vacuole formation.

Such kind of technique has been used to yield $vacA^-$ mutant *H pylori*[9,28,29]. But no further experiment has been done to compare the virulence between the mutant and the parental strain. On the other hand, microarray analysis has been used in several studies to screen gene expression profiles in gastric epithelial cells induced by *H pylori*[30-32]. Our group has also analyzed different expression profiles of gastric cancer cells co-cultured with supernatants of $VacA^+$ or $VacA^-$ *H pylori* isolates. However, VacA has not been used as a single virulence determinant to stimulate host cells, thus one can not determine which virulent factors result in the alteration of the expression. In this study, we successfully constructed the $vacA^-$ mutant strain, using the direct mutagenesis technique, which creates an important precondition for the further research on virulence comparison with gene expression analysis.

## REFERENCES


1 **Dunn BE**, Cohen H, Blaser MJ. *Helicobacter pylori*. *Clin Microbiol Rev* 1997; **10**: 720-741

2 **Marshall BJ**, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; **1**: 1311-1315

3 **Graham DY**, Lew GM, Klein PD, Evans DG, Evans DJ Jr, Saeed ZA, Malaty HM. Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann Intern Med* 1992; **116**: 705-708

4 **Parsonnet J**, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131

5 **Forman D**, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, Sitas F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991; **302**: 1302-1305

6 **Xue FB**, Xu YY, Wan Y, Pan BR, Ren J, Fan DM. Association of *H pylori* infection with gastric carcinoma: a Meta analysis. *World J Gastroenterol* 2001; **7**: 801-804

7 **Leunk RD**, Johnson PT, David BC, Kraft WG, Morgan DR. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J Med Microbiol* 1988; **26**: 93-99

8 **Cover TL**, Blaser MJ. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. *J Biol Chem* 1992; **267**: 10570-10575

9 **Cover TL**, Tummuru MK, Cao P, Thompson SA, Blaser MJ. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J Biol Chem* 1994; **269**: 10566-10573

10 **Schmitt W**, Haas R. Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Mol Microbiol* 1994; **12**: 307-319

11 **Hou P**, Tu ZX, Xu GM, Gong YF, Ji XH, Li ZS. *Helicobacter pylori vacA* genotypes and cagA status and their relationship to associated diseases. *World J Gastroenterol* 2000; **6**: 605-607

12 **De Bernard M**, Arico B, Papini E, Rizzuto R, Grandi G, Rappuoli R, Montecucco C. *Helicobacter pylori* toxin VacA induces vacuole formation by acting in the cell cytosol. *Mol Microbiol* 1997; **26**: 665-674

13 **Papini E**, de Bernard M, Milia E, Bugnoli M, Zerial M, Rappuoli R, Montecucco C. Cellular vacuole induced by *Helicobacter pylori* originate from late endosomal compartments. *Proc Natl Acad Sci U S A* 1994; **91**: 9720-9724

14 **Sambrook J,** Russell DW. Molecular Cloning-A Laboratory Manual. 3thed, Cold Spring Harbor, New York: *Cold Spring Harbor Laboratory Press* 2001; Vol 1: 6.4-6.11

15 **Clayton CL**, Mobley HLT. Methods in Molecular Medicine, *Helicobacter pylori* Protocols. 1sted. Totowa: *Humana Press Inc* 1997: 145-152

16 **Cover TL**. The vacuolating cytotoxin of *Helicobacter pylori*. *Mol Microbiol* 1997; **20**: 241-246

17 **Kimura M**, Goto S, Wada A, Yahiro K, Niidome T, Hatakeyama T, Aoyagi H, Hirayama T, Kondo T. Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microb Pathog* 1999; **26**: 45-52

18 Kuck D, Kolmerer B, Iking-Konert C, Krammer PH, Stremmel W, Rudi J. Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. *Infect Immun* 2001; **69**: 5080-5087

19 **Ashorn M**, Cantet F, Mayo K, Megraud F. Cytoskeletal rearrangements induced by *Helicobacter pylori* strains in epithelial cell culture: possible role of the cytotoxin. *Dig Dis Sci* 2000; **45**: 1774-1780

20 **Pai R**, Cover TL, Tarnawski AS. *Helicobacter pylori* vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. *Biochem Biophys Res Commun* 1999; **262**: 245-250

21 **Manetti R**, Massari P, Burroni D, de Bernard M, Marchini A, Olivieri R, Papini E, Montecucco C, Rappuoli R, Telford JL. *Helicobacter pylori* cytotoxin: importance of native conformation for induction of neutralizing antibodies. *Infect Immun* 1995; **63**: 4476-4480

22 **Papini E**, Zoratti M, Cover TL. In search of the *Helicobacter pylori* VacA mechanism of action. *Toxicon* 2001; **39**: 1757-1767

23 **Garner JA**, Cover TL. Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infect Immun* 1996; **64**: 4197-4203

24 **Massari P**, Manetti R, Burroni D, Nuti S, Norais N, Rappuoli R, Telford JL. Binding of the *Helicobacter pylori* vacuolating cytotoxin to target cells. *Infect Immun* 1998; **66**: 3981-3984

25 **Yahiro K**, Niidome T, Kimura M, Hatakeyama T, Aoyagi H, Kurazono H, Imagawa K, Wada A, Moss J, Hirayama T. Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase β. *J Biol Chem* 1999; **274**: 36693-36699

26 **Yahiro K**, Niidome T, Hatakeyama T, Aoyagi H, Kurazono H, Padilla PI, Wada A, Hirayama T. *Helicobacter pylori* vacuolating cytotoxin binds to the 140-kDa protein in human gastric cancer cell lines, AZ-521 and AGS. *Biochem Biophys Res Commun* 1997; **238**: 629-632

27 **Henderson B**, Wilson M, McNab R, Lax AJ. Cellular Microbiology: Bacteria-host interactions in health and disease. Hoboken: *John Wiley Sons Ltd* 1999: 163-188
28 **Copass M**, Grandi G, Rappuoli R. Introduction of unmarked mutations in the *Helicobacter pylori vacA* gene with a sucrose sensitivity marker. *Infect Immun* 1997; **65**: 1949-1952
29 **Burroni D**, Lupetti P, Pagliaccia C, Reyrat JM, Dallai R, Rappuoli R, Telford JL. Deletion of the major proteolytic site of the *Helicobacter pylori* cytotoxin does not influence toxin activity but favors assembly of the toxin into hexameric structures. *Infect Immun* 1998; **66**: 5547-5550
30 **Sepulveda AR**, Tao H, Carloni E, Sepulveda J, Graham DY, Peterson LE. Screening of gene expression profiles in gastric epithelial cells induced by *Helicobacter pylori* using microarray analysis. *Aliment Pharmacol Ther* 2002; **16**(Suppl 2): 145-157
31 **Cox JM**, Clayton CL, Tomita T, Wallace DM, Robinson PA, Crabtree JE. cDNA array analysis of *cag* pathogenicity island-associated *Helicobacter pylori* epithelial cell response genes. *Infect Immun* 2001; **69**: 6970-6980
32 **Maeda S**, Otsuka M, Hirata Y, Mitsuno Y, Yoshida H, Shiratori Y, Masuho Y, Muramatsu M, Seki N, Omata M. cDNA microarray analysis of *Helicobacter pylori*-mediated alteration of gene expression in gastric cancer cells. *Biochem Biophys Res Commun* 2001; **284**: 443-449

**Edited by** Xia HHX

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2258-2260
World Journal of Gastroenterology
 ISSN 1007-9327



# Distribution of *cagG* gene in *Helicobacter pylori* isolates from Chinese patients with different gastroduodenal diseases and its clinical and pathological significance

Can Xu, Zhao-Shen Li, Zhen-Xing Tu, Guo-Ming Xu, Yan-Fang Gong, Xiao-Hua Man

**Can Xu, Zhao-Shen Li, Zhen-Xing Tu, Guo-Ming Xu, Yan-Fang Gong, Xiao-Hua Man,** Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
**Supported by** the National Natural Science Foundation of China, No. 30170427
**Correspondence to:** Dr. Can Xu, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China. xucan9@hotmail.com
**Telephone:** +86-21-25070556 **Fax:** +86-21-25074635
**Received:** 2003-05-12 **Accepted:** 2003-06-12

## Abstract

**AIM:** To determine the distribution of *cagG* gene of *Helicobacter pylori* (*H pylori*) isolates cultured from patients with various digestive diseases and its relationship with gastroduodenal diseases.

**METHODS:** *cagG* was amplified by polymerase chain reaction in 145 *H pylori* isolates cultured from patients with chronic gastritis ($n$=72), duodenal ulcer ($n$=48), gastric ulcer ($n$=17), or gastric and duodenal ulcer ($n$=8), and the relationship between *cagG* status and the grade of gastric mucosal inflammation was determined.

**RESULTS:** *cagG* was present in 91.7 % of the 145 *H pylori* isolates, with the rates were 90.3 %, 93.8 %, 88.2 % and 100.0 %, respectively, in those from patients with chronic gastritis, duodenal ulcer, gastric ulcer, and gastric and duodenal ulcer. There was no significant difference among the four groups ($P$>0.05). The average grade of gastric mucosal inflammation in the antrum and corpus was 1.819±0.325 and 1.768±0.312, respectively in *cagG* positive patients, whereas the average inflammation grade was 1.649±0.297, 1.598±0.278 respectively in *cagG* negative cases ($P$>0.05).

**CONCLUSION:** *cagG* gene of *H pylori* was quite conservative, and most *H pylori* strains in Chinese patients were *cagG* positive. *cagG* status was not related to clinical outcome or the degree of gastric mucosal inflammation. Therefore, *cagG* can not be used as a single marker for discrimination of *H pylori* strains with respect to a specific digestive disease.

Xu C, Li ZS, Tu ZX, Xu GM, Gong YF, Man XH. Distribution of *cagG* gene in *Helicobacter pylori* isolates from Chinese patients with different gastroduodenal diseases and its clinical and pathological significance. *World J Gastroenterol* 2003; 9(10): 2258-2260

http://www.wjgnet.com/1007-9327/9/2258.asp

## INTRODUCTION

*Helicobacter pylori* is a well-recognized pathogen that chronically infects more than 50 % of the world population. *H pylori* is associated with the development of acute or chronic gastritis, peptic ulcer diseases, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Most infected subjects will remain asymptomatic throughout life with only about 20 % developing peptic ulcer diseases or gastric carcinoma[1-5]. What determines the outcome of an infection remains unclear. The reasons for these different outcomes of *H pylori* infection may be related to both bacterial factors and host responses. The major *H pylori* disease-associated genetic factor is the whole *cag* pathogenicity island (PAI), which contains 25 open reading frames and at least 30 genes. The *cag* PAI is associated with increased interleukin (IL)-8 production by gastric epithelial cells[6,7].

The cytotoxin-associated gene A (*cagA*) is located in the most downstream part of the *cag* PAI. The presence of this gene or its encoded protein, CagA, has been regarded as a marker for the *cag* PAI. Many clinical studies have demonstrated that *cagA* gene or CagA protein is associated with a more severe clinical outcome. CagA was reported to increase the risk of development of duodenal ulcer, atrophic gastritis and gastric adenocarcinoma. In contrast to the *cagA*-negative patients, gastric mucosal inflammation of *cagA*-positive patients was more severe[8-10]. *cagG* is located within the *cag* PAI upstream of *cagA*. The distribution of this gene in *H pylori* strains isolated from Chinese digestive patients and its relation with the gastroduodenal diseases remain unclear. In the present study, a set of specific primers were designed to detect the *cagG* gene in 145 clinical *H pylori* strains, and the relationship between *cagG* and different digestive diseases was determined.

## MATERIALS AND METHODS

### *H pylori isolates*

*H pylori* isolates obtained from 145 patients (80 males, 65 females, aged 18-69 years, mean age 42.5 years old) who underwent upper endoscopy in our department were included in this study. These patients were diagnosed endoscopically as chronic gastritis ($n$=72), duodenal ulcer ($n$=48), gastric ulcer ($n$=17), or gastric and duodenal ulcer ($n$=8). Informed consents were obtained from all patients. The standard strains CCUG17874 (NCTC11638) and Tx30a were kindly provided by the Italian IRIS Research Center.

### *H pylori culture*

Two antral biopsy specimens taken during endoscopy were immediately cultured on the *H pylori* selective agar plates with 10 % defibrillated sheep blood and antibiotics (Merck Company, Germany) at 37 ℃ under microaerophilic conditions with 5 % $O_2$ 10 % $CO_2$ and 85 % $N_2$ for 3-6 days. The colonies were identified as *H pylori* if Gram stain morphology and biochemical tests were positive for urease, oxidase and catalase. All stock cultures were preserved at -80 ℃ in Brucella broth with 20 % glycerol, and subcultured for genomic DNA extraction. The passage number of *H pylori* used in this study averaged six.

### *Histopathologic examination*

Two biopsy specimens each taken from the gastric corpus and antrum endoscopically were used for histopathologic examination to grade the severity of gastritis after they were embedded in paraffin and stained with hematoxylin and eosin. The severity of gastritis (i.e. mononuclear cell and polymorphonuclear leukocyte infiltration) was evaluated, and graded on a scale of 0-3 (i.e. 0=no, 1=mild, 2=moderate, and 3=marked) according to the updated Sydney system[11].

### *Genomic DNA extraction*

Subcultured *H pylori* cells were collected from the agar plates, then genomic DNA was extracted and purified from each *H pylori* isolate using cetyltrimethyl ammonium bromide (CTAB), phenol-chloroform-isoamyl alcohol, and ethanol precipitation.

### *Detection of cagG with polymerase chain reaction (PCR)*

The primers to amplify *cagG* gene and give a 497 base pair (bp) product were designed based on the published gene sequence[12]. *cagG*F: 5' -GCCATGTTAACACCCCCTAG-3' , and *cagG*R:5' -TTAATGCGCTAGAATAGTGC-3' . PCR was performed in an Eppendorf thermal cycler using a PCR kit (Takara, Dalian, China) according to the manufacturer' s instructions. Briefly, the reaction was performed in a total volume of 50 μl containing 20 ng genomic DNA as a template and 200 μM each deoxynucleotide, 1.5U Taq polymerase, 0.4 μM each primer and PCR buffer. The PCR amplification program comprised at 95 ℃ for 5 min, then 35 cycles at 95 ℃ for 1 min, at 52 ℃ for 1 min and at 72 ℃ for 1 min, followed by at 72 ℃ for 7 min, then cooled at 4 ℃. The PCR products were analyzed on 1.5 % agarose gels with ethidium bromide. CCUG17874 was taken as a positive control, Tx30a as a negative control, and deionized water as a blank control.

### *Statistical analysis*

The data were expressed as the mean ±SD. The *t* test and $\chi^2$ test were used for statistical analysis. A *P* value <0.05 was considered to be statistically significant.

## RESULTS

### *Amplification of cagG gene*

After PCR amplification of the *cagG* gene, the products were electrophoresed on 1.5 % agarose gels, and stained with ethidium bromide. Under ultraviolet light, *cagG* appeared as a specific band with 497 bp (Figure 1).

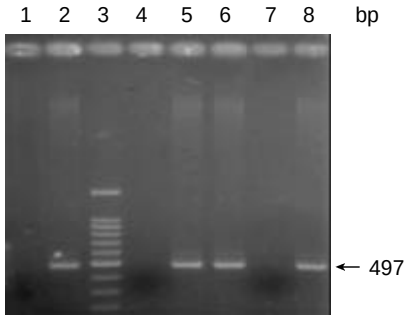



**Figure 1** Electrophoresis of PCR products. Lane 3: 100 bp DNA Marker; Lanes 1, 2 and 4: controls: Tx30a, CCUG17874, and deionized water; Lanes 5, 6, 7 and 8: clinical *H pylori* isolates.

### *CagG status in H pylori isolates from patients with various diseases*

*H pylori cagG* gene was detected in 91.7 % of the 145 isolates, the rate was 90.3 %, 93.8 %, 88.2 % and 100.0 % in patients with chronic gastritis, duodenal ulcer, gastric ulcer and gastric/duodenal ulcer, respectively (Table 1). There was no significant difference among the groups (*P*>0.05).

**Table 1** *cagG* in *H pylori* isolates from patients with different gastroduodenal diseases

| Group | Number | *cagG* positive | *cagG* negative | *cagG* positive rate (%)[a] |
|---|---|---|---|---|
| Chronic gastritis | 72 | 65 | 7 | 90.3 |
| Duodenal ulcer | 48 | 45 | 3 | 93.8 |
| Gastric ulcer | 17 | 15 | 2 | 88.2 |
| Gastric duodenal ulcer | 8 | 8 | 0 | 100.0 |
| Total | 145 | 133 | 12 | 91.7 |

[a]*P*>0.05 between any two groups.

### *CagG status and gastric mucosal inflammation*

The average grade of gastric mucosal inflammation in the antrum and corpus was 1.819±0.325 and 1.768±0.312 in *cagG* positive patients, respectively, whereas the average grade was 1.649±0.297, 1.598±0.278 in *cagG* negative group, respectively (*P*>0.05, both in the antrum and body).

## DISCUSSION

*H pylori* infects human gastric mucosa which evokes a mucosal inflammatory response by neutrophil recruitment from the microcirculation. Persistent inflammation may lead to the development of digestive diseases such as chronic gastritis, peptic ulcer disease and gastric cancer. Although pathogenicity of *H pylori* infection is not well understood, there were several putative virulence factors that might contribute to mucosal damage by *H pylori* infection[13,14].

The *cag* PAI is an approximately 40-kb cluster of genes in *H pylori* chromosome, and a quite conservative entity. Many of *H pylori* strains had an intact *cag* PAI divided into two regions: *cag*I in downstream and *cag*II in upstream, and some with an insert sequence IS605 or IS606[15,16]. There were at least 14 and 16 open reading frames in *cag*I and *cag*II, several of which were virulence factors[17]. Several studies suggested that *cagA* gene could be used as a marker for *cag* PAI[16]. *cagG* is another gene in *cag*I region which is located upstream of *cagA* gene. The function of this gene is not well known. Recently, Hsu *et al*[17] reported that an intact *cag* PAI was identified in 95 % and 100 % of the strains that possessed *cagA* and *cagG* respectively, whereas the *cagA* and *cagG* genes were found in 100 % and 95 % of the strains containing a partial or complete set of *cag* PAI, indicating that *cagA* gene is not associated with a complete *cag* PAI in 5 % of the strains, and cannot be considered as an absolute marker for the presence of a complete set of *cag* PAI, but *cagG* gene may be a better indicator for the presence of an intact *cag* PAI.

Extensive studies of *cagA* gene indicated that CagA protein encoded by *cagA* gene was associated with severe clinical outcomes, such as peptic ulcer disease and gastric cancer. Therefore, it was considered as a main virulence factor of *H pylori*[16]. Some reports suggested that the presence of *cagE* gene within *cag*I might be related to more severe clinical outcomes. For example, Day *et al*[18] revealed that *H pylori* isolates containing *cagE* were associated with duodenal ulcer in Canadian children. Fallone *et al*[19] reported that *cagE*-positive isolates were more prevalent in Canadian adult patients with peptic ulcer or gastric cancer than in those with gastritis only. In the present study, we designed a set of primers to amplify *cagG* gene of 145 clinical *H pylori* isolates, and

determined the correlation of *cagG* status with endoscopic presentation, and histological findings. The results showed that *cagG* was present in 91.7 % *H pylori* isolates examined. 100 % of *H pylori* isolates from patients with gastric and duodenal ulcer were *cagG* positive, which was higher, but not statistically significant than that in other groups ($P>0.05$). Lack of difference in *cagG* positive rate might be due to patient selection or the relatively small number of patients with gastric and duodenal ulcer. Nevertheless, our study suggested that positive rate of *cagG* in *H pylori* was high and *cagG* was quite conservative in Chinese population, and that there was no difference in the frequencies of *cagG*-positive isolates among patients with gastritis, duodenal ulcer, gastric ulcer or gastric duodenal ulcer. Our results are supported by the study by Jenks *et al*[20] who demonstrated that no specific genes within the *cag* PAI could reliably predict the clinical outcome of *H pylori* infection in French patients, and also by Hsu *et al*[17] who concluded that any of the *cag* PAI genes such as *cagE* could not predict the clinical presentation in Korean patients.

Hsu *et al* reported that of the 120 clinical isolates from Korean patients with various gastrointestinal diseases, 86.7 % (104/120) were *cagG* positive[17]. Mizushima *et al*[21] used PCR and Southern blot to investigate the prevalence of *cagG* gene in 236 clinical *H pylori* isolates from Japanese patients, and found that *cagG* was present in 97 % of the isolates. These results were similar with ours. The same Japanese research group[21] further used flow cytometry to assay the ability of *H pylori* with or without *cagG* to adhere to KATOIII and ELISA to detect the IL-8 secreted from gastric epithelial cells induced by *H pylori*. They observed that in comparison with the *cagG*-positive strains, all *cagG*-deleted strains decreased adherence to KATOIII cells, and abolished IL-8 induction despite the presence of *cagE*, which was reported to be essential for IL-8 induction. *H pylori* genome is known to be diversified and may differ between geographic regions. However, there has no reports so far about *cagG* gene distribution in the Western countries.

Infection with *cagA* positive *H pylori* induces stronger gastric chemokine mRNA expression such as IL-8 in the antral mucosa, which may be relevant to the increased mucosal damage associated with *cagA* positive *H pylori* infection. The levels of the chemokines were correlated with cellular infiltration in the antrum and inflammation of the gastric mucosa[22,23]. We compared the severity of gastric mucosal inflammation in the antrum and corpus in *cagG*-positive and *cagG*-negative patients, and observed that, the average grade of inflammation was only slightly higher in *cagG*-positive group than that in *cagG* negative group both in the antrum and in the corpus ($P>0.05$). Therefore, the *cagG* status has no relation to the severity of gastritis.

In conclusion, *cagG* gene was quite conservative in clinical *H pylori* isolates from Chinese patients with different gastroduodenal diseases, since most *H pylori* isolates were *cagG* positive. There was no difference in the frequency of *cagG* -positive isolates among patients with different diseases. The *cagG* status was not related to gastric mucosal inflammation grade. Therefore, *cagG* cannot reliably predict the clinical and histological outcomes.

## REFERENCES


1 **Kapadia CR**. Gastric atrophy, metaplasia, and dysplasia: a clinical perspective. *J Clin Gastroenterol* 2003; **36**: S29-36

2 **Matsuhisa TM**, Yamada NY, Kato SK, Matsukura NM. *Helicobacter pylori* infection, mucosal atrophy and intestinal metaplasia in Asian populations: a comparative study in age-, gender- and endoscopic diagnosis-matched subjects. *Helicobacter* 2003; **8**: 29-35

3 **Kelley JR**, Duggan JM. Gastric cancer epidemiology and risk factors. *J Clin Epidemiol* 2003; **56**: 1-9

4 **McColl KE**, El-Omar E. How does *H pylori* infection cause gastric cancer? *Keio J Med* 2002; **51**(Suppl): 53-56

5 **Sepulveda AR**, Graham DY. Role of *Helicobacter pylori* in gastric carcinogenesis. *Gastroenterol Clin North Am* 2002; **31**: 517-535

6 **Figura N**, Valassina M. *Helicobacter pylori* determinants of pathogenicity. *J Chemother* 1999; **11**: 591-600

7 **Megraud F**. Impact of *Helicobacter pylori* virulence on the outcome of gastroduodenal diseases: lessons from the microbiologist. *Dig Dis* 2001; **19**: 99-103

8 **Audibert C**, Burucoa C, Janvier B, Fauchere JL. Implication of the structure of the *Helicobacter pylori cag* pathogenicity island in induction of interleukin-8 secretion. *Infect Immun* 2001; **69**: 1625-1629

9 **Li CQ**, Pignatelli B, Ohshima H. Increased oxidative and nitrative stress in human stomach associated with *cagA+ Helicobacter pylori* infection and inflammation. *Dig Dis Sci* 2001; 46: 836-844

10 **Perez-Perez GI**, Peek RM, Legath AJ, Heine PR, Graff LB. The role of CagA status in gastric and extragastric complications of *Helicobacter pylori*. *J Physiol Pharmacol* 1999; **50**: 833-845

11 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181

12 **Yamaoka Y**, Kodama T, Kita M, Imanishi J, Kashima K, Graham DY. Relation between clinical presentation, *Helicobacter pylori* density, interleukin 1β and 8 production, and *cagA* status. *Gut* 1999; **45**: 804-811

13 **Suzuki H**, Masaoka T, Miyazawa M, Suzuki M, Miura S, Ishii H. Gastric mucosal response to *Helicobacter pylori*. *Keio J Med* 2002; **51**(Suppl): 40-44

14 **McGee DJ**, Mobley HL. Mechanisms of *Helicobacter pylori* infection: bacterial factors. *Curr Top Microbiol Immunol* 1999; **241**: 155-180

15 **Zhang M**, Zhang J, He L, Guo H, Yin Y, Zhou Z. Dissemination of insertion sequences IS605, IS606 among clinical isolates of *Helicobacter pylori* in China. *Zhonghua Liuxing Bingxue Zazhi* 2002; **23**: 366-369

16 **Owen RJ**, Peters TM, Varea R, Teare EL, Saverymuttu S. Molecular epidemiology of *Helicobacter pylori* in England: prevalence of *cag* pathogenicity island markers and IS605 presence in relation to patient age and severity of gastric disease. *FEMS Immunol Med Microbiol* 2001; **30**: 65-71

17 **Hsu PI**, Hwang IR, Cittelly D, Lai KH, El-Zimaity HM, Gutierrez O, Kim JG, Osato MS, Graham DY, Yamaoka Y. Clinical presentation in relation to diversity within the *Helicobacter pylori cag* pathogenicity island. *Am J Gastroenterol* 2002; **97**: 2231-2238

18 **Day AS**, Jones NL, Lynett JT, Jennings HA, Fallone CA, Beech R, Sherman PM. *cagE* is a virulence factor associated with *Helicobacter pylori*-induced duodenal ulceration in children. *J Infect Dis* 2000; **181**: 1370-1375

19 **Fallone CA**, Barkun AN, Gottke MU, Best LM, Loo VG, Veldhuyzen van Zanten S, Nguyen T, Lowe A, Fainsilber T, Kouri K, Beech R. Association of *Helicobacter pylori* genotype with gastroesophageal reflux disease and other upper gastrointestinal diseases. *Am J Gastroenterol* 2000; **95**: 659-669

20 **Jenks PJ,** Mégraud F, Labigne A. Clinical outcome after infection with *Helicobacter pylori* does not appear to be reliably predicted by the presence of any of the genes of the *cag* pathogenicity island. *Gut* 1998; **43**: 752–758

21 **Mizushima T**, Sugiyama T, Kobayashi T, Komatsu Y, Ishizuka J, Kato M, Asaka M. Decreased adherence of *cagG*-deleted *Helicobacter pylori* to gastric epithelial cells in Japanese clinical isolates. *Helicobacter* 2002; **7**: 22-29

22 **Shimoyama T**, Everett SM, Dixon MF, Axon AT, Crabtree JE. Chemokine mRNA expression in gastric mucosa is associated with *Helicobacter pylori cagA* positivity and severity of gastritis. *J Clin Pathol* 1998; **51**: 765-770

23 **Yamaoka Y**, Kita M, Kodama T, Sawai N, Tanahashi T, Kashima K, Imanishi J. Chemokines in the gastric mucosa in *Helicobacter pylori* infection. *Gut* 1998; **42**: 609-617


**Edited by** Xia HHX

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2261-2265
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Effect of oral epidermal growth factor on mucosal healing in rats with duodenal ulcer

Jane CJ Chao, Kuo-Yu Liu, Sheng-Hsuan Chen, Chia-Lang Fang, Chih-Wei Tsao

**Jane CJ Chao, Kuo-Yu Liu,** School of Nutrition and Health Sciences, Taipei Medical University, 110 Taiwan
**Sheng-Hsuan Chen,** Department of Gastroenterology, Taipei Medical University Hospital, 110 Taiwan
**Chia-Lang Fang,** Department of Pathology, Taipei Medical University, 110 Taiwan
**Chih-Wei Tsao,** Department of Surgery, National Defense Medical Center, Tri-Service General Hospital, 110 Taiwan
**Supported by** the National Council Science of Taiwan (grant NSC88-2314-B-038-008)
**Correspondence to:** Jane CJ Chao, School of Nutrition and Health Sciences, Taipei Medical University, 250 Wu Hsing Street, Taipei, 110 Taiwan. chenjui@tmu.edu.tw
**Telephone:** +886-2-2736-1661 #6551-6556 Ext. 117
**Fax:** +886-2-2737-3112
**Received:** 2003-06-28 **Accepted:** 2003-08-02

## Abstract

**AIM:** To investigate the effect of epidermal growth factor (EGF) on mucosal healing in rats with duodenal ulcer.

**METHODS:** Male Sprague-Dawley rats were randomly divided into sham operation without EGF, sham operation with EGF, duodenal ulcer without EGF, or duodenal ulcer with EGF groups. Additionally, normal rats without operation served as the control group. Duodenal ulcer was induced in rats by 300 mL/L acetic acid. Rats with EGF were orally administered at a dose of 60 μg/kg/day in drinking water on the next day of operation (day 1). Healing of duodenal ulcer was detected by haematoxylin and eosin staining. Cell growth of damaged mucosa was determined by the contents of nucleic acids and proteins. The level of EGF in duodenal mucosa was measured by ELISA.

**RESULTS:** The pathological results showed that duodenal ulcer rats with EGF improved mucosal healing compared with those without EGF after day 5. Duodenal ulcer rats with EGF significantly increased duodenal DNA content compared with those without EGF on day 15 (6.44±0.54 mg/g *vs* 1.45±0.52 mg/g mucosa, $P<0.05$). Duodenal RNA and protein contents did not differ between duodenal ulcer rats with and without EGF during the experimental period. Sham operation and duodenal ulcer rats with EGF significantly increased duodenal mucosal EGF content compared with those without EGF on day 5 (76.0±13.7 ng/g *vs* 35.7±12.9 ng/g mucosa in sham operation rats, and 68.3±10.9 ng/g *vs* 28.3±9.2 ng/g mucosa in duodenal ulcer rats, $P<0.05$).

**CONCLUSION:** Oral EGF can promote mucosal healing of the rats with duodenal ulcer by stimulating mucosal proliferation accompanied by an increase in mucosal EGF content.

Chao JCJ, Liu KY, Chen SH, Fang CL, Tsao CW. Effect of oral epidermal growth factor on mucosal healing in rats with duodenal ulcer. *World J Gastroenterol* 2003; 9(10): 2261-2265
http://www.wjgnet.com/1007-9327/9/2261.asp

## INTRODUCTION

Epidermal growth factor (EGF) is present in various body fluids and tissues, and is continuously secreted into the gastrointestinal lumen in humans by submandibular glands, mucous neck cells of the stomach, Brunner's glands of the duodenum, Paneth cells of the small intestine, and ulcer-associated cell lineage (a recently identified glandular structure induced at the sites of injury)[1-3]. EGF and EGF family of related peptides are involved as key constituents in the maintenance and repair of gastrointestinal mucosa[4]. There has been evidence that increases in the EGF receptor and EGF producing cells around acetic acid-induced gastric ulcer in rats, and a novel cell lineage in human mucosal ulceration secreting EGF adjacent to peptic ulcer[3,5]. The results suggest that EGF plays an important role in ulcer healing.

Previous studies showed that EGF administration regulated the healing of ulcers in rats[6,7] and humans[8]. Oral administration of EGF, given at 30 μg/kg/day in the drinking water for 25 or 50 days, promoted the healing of cysteamine HCl-induced duodenal ulcer in rats to the same extent as cimetidine, a $H_2$-receptor antagonist[6]. However, Kuwahara *et al*.[7] demonstrated human EGF, given orally twice daily at 30 and 100 μg/kg for 2 weeks or at 100 μg/kg for 4 weeks, had no effect on natural healing of acetic acid-induced gastric ulcer in rats. It has been controversial if orally administered EGF, a feasible and easy way in clinical therapy, is effective to promote the healing of duodenal ulcer. Therefore, the purpose of the study was to investigate the effect of orally administered EGF on the healing of intestinal mucosa and the content of EGF in acetic acid-induced duodenal ulcer rats.

## MATERIALS AND METHODS

### *Animals and duodenal ulcer operation*

Male Sprague-Dawley rats (-200 g) were purchased from the National Laboratory Animal Center (National Science Council, Taipei, Taiwan). The rats were housed in individual cages and had free access to food (powdered laboratory autoclavable rodent diet 5 010, PMI Nutrition International Inc., Brentwood, MO), except for the fasting period. The light cycle was 12 h and the room temperature was kept at 22-24 ℃. The rats were randomly divided into four operated groups: sham operation without EGF, sham operation with EGF, duodenal ulcer without EGF, and duodenal ulcer with EGF groups ($n$=10 on each sacrificed day, 6 rats for biological analysis, and 4 rats for pathological examination and photography). Additionally, normal rats ($n$=10) without operation served as the control group. Duodenal ulcer was induced in rats by acetic acid according to the modified method of Konturek *et al*[9]. Prior to operation, the rats were fasted overnight, anesthetized by intraperitoneal injection with 50 mg/kg body weight thiopental sodium (Abbott Australia Pty.Ltd., Kurnell, Australia), and the abdomen was then opened. A plastic tube (4.5 mm inner diameter) filled with 70 μL of 300 mL/L acetic acid was applied tightly to the surface of the duodenum for 10 sec. Due to different tolerance to acetic acid in various layers of the duodenum, it only caused immediate necrosis in the mucosal and submucosal

layers exactly within the area (4.5 mm diameter) of acetic acid application without penetration or perforation to the surrounding organs. Normal saline instead of 300 mL/L acetic acid was applied to sham operation rats. After operation, the rats were allowed to recover from anesthesia. The operated rats received only water on the day of operation (day 0), and were fed a normal chow diet *ad libitum* next day (day 1). Body weight, food intake, and water intake of the rats were routinely recorded. All protocols were conducted under the guidelines of Animal Care and Use Committee, Taipei Medical University.

### *Treatment and pathological observation*

The next day after operation at approximately 15:00, the rats were orally administered recombinant human EGF (60 μg/kg body weight) (Biosource International, Camarillo, CA) in 35 mL (minimal intake during the adaptation period) sterile deionized drinking water, and remaining water was exactly recorded to determine actual intake of EGF. The rats without oral EGF were given the same amount of sterile deionized drinking water. The operated rats were killed on day 1 to identify the formation of duodenal ulcer, and on days 5 and 15 at 15:00 for pathological and biological analyses. The duodenum (5×5 mm) was excised, preserved in 10 % formaldehyde, and stained with haematoxylin and eosin. The diameter of ulcer size was measured in sectioned samples by microscopy. Coded mucosal specimens were evaluated under a light microscope at ×100 or ×200 magnification by a pathologist in a blinded fashion.

### *DNA, RNA, and protein in duodenal mucosa*

The ulcer area of duodenal mucosa in duodenal ulcer rats and a similar area of intact duodenal mucosa in sham operation rats were excised. Duodenal mucosa was entirely scraped off, weighed, and immediately frozen at -80 ℃ for further analysis. DNA, RNA, and protein in duodenal mucosa were purified using a commercial TRIZOL reagent (Life Technologies, Inc., Rockville, MD) to evaluate mucosal growth[10]. Duodenal mucosa (0.2-0.5 g wet weight) was homogenized in 1 mL of TRIZOL reagent followed by the addition of 200 μL chloroform. After centrifugation, RNA remained exclusively in the aqueous phase, and DNA and protein were then recovered by sequential precipitation. RNA was precipitated with isopropanol. DNA was precipitated with ethanol from the interphase, and protein was precipitated with isopropanol from the organic phase after separation from DNA. DNA, RNA, and protein pellets were resuspended in 8 mmol/L NaOH, diethylpyrocarbonate-water, or 1 mol/L NaOH, respectively. DNA and RNA were quantitated spectrophotometrically at 260 nm. Protein content was determined spectrophotometrically at 690 nm by a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA).

### *EGF in duodenal mucosa*

Duodenal mucosal EGF content was measured by a commercial EGF immunoassay kit (Quantikine™, DEG00, Research and Diagnostics Systems, Inc., Minneapolis, MN)[11]. Duodenal mucosa (0.2-0.3 g wet weight) was homogenized with RD1 reagent. The mucosal homogenate (200 μL) was incubated with EGF antibody coated in a 96-well plate for 2 h at room temperature, washed 3 times with 400 μL wash buffer, and then incubated with 200 μL polyclonal EGF antibody conjugated to horseradish peroxidase for 2 h. After several washes, samples were incubated with 200 μL substrate (tetramethylbenzidine: $H_2O_2$=1:1) for 20 min. The reaction was terminated by 50 μL of 1 mol/L sulfuric acid. Mucosal EGF content was determined at 450 nm and corrected at 540 nm using an ELISA reader (Multiskan RC, Labsystems, Helsinki, Finland).

### *Statistical analysis*

All values were expressed as mean ± SE. Data were analyzed by three-way ANOVA to determine the main effects of duodenal ulcer, oral EGF, and time using SAS 6.12 (SAS Institute, Cary, NC). *Post hoc* multiple comparisons between two groups were performed either by Fisher's least significant difference or Dunnett's test. Differences were considered significant at $P<0.05$.

## RESULTS

### *Weight gain, EGF and food intake*

Body weight of sham operation and duodenal ulcer rats with or without EGF on different sacrificed days is shown in Table 1. At the beginning of the experiment, body weight of the rats was similar in all operated groups and the control group (189.0±4.5 g). Body weight of sham operation and duodenal ulcer rats with or without EGF significantly increased ($P<0.05$) on day 15 compared with that of the control group on day 0 and the corresponding group on day 5. Oral EGF did not affect body weight.

**Table 1** Body weight of sham operation and duodenal ulcer rats with or without oral EGF before operation and sacrifice

| Group | Sham operation | | Duodenal ulcer | |
|---|---|---|---|---|
| | - EGF | + EGF | - EGF | + EGF |
| | Body weight before operation (g)[2] | | | |
| Day 5 | 192.0±3.9 | 199.8±3.8 | 200.6±5.0 | 195.2±5.0 |
| Day 15 | 192.4±4.8 | 198.9±4.2 | 194.9±4.7 | 195.9±6.0 |
| | Body weight before sacrifice (g)[2] | | | |
| Day 5 | 203.6±4.0* | 209.9±3.6* | 208.1±4.7* | 200.5±4.9 |
| Day 15 | 271.4±5.5*† | 279.8±7.7*† | 267.1±7.7*† | 278.8±5.3*† |

[1]Data are mean ± SE ($n$=10). [2]Values were not significantly different ($P>0.05$) among different groups in each row by Fisher's least significant difference test. *Body weight was significantly different ($P<0.05$) from that in the control group (189.0±4.5 g, $n$=10) on day 0 by Dunnett's test. †$P<0.05$ compared with day 5 within the same group by Fisher's least significant difference test.

**Table 2** EGF and food intake of sham operation and duodenal ulcer rats with or without oral EGF[1]

| Group | Sham operation | | Duodenal ulcer | |
|---|---|---|---|---|
| | - EGF | + EGF | - EGF | + EGF |
| | EGF intake (μg / kg / day)[2] | | | |
| Day 5 | -[3] | 59.9±3.8 | - | 57.8±0.4 |
| Day 15 | - | 57.3±0.6 | - | 58.1±0.3 |
| | Food intake before operation (g / day)[2] | | | |
| Day 5 | 24.5±1.1 | 25.2±0.8 | 26.5±0.5 | 25.4±0.9 |
| Day 15 | 25.1±0.5 | 25.3±0.4 | 26.6±0.8 | 24.8±0.6 |
| | Food intake before sacrifice (g / day) | | | |
| Day 5 | 27.9±4.3[a] | 27.4±6.4[a] | 24.2±3.6[a] | 25.6±5.4[a] |
| Day 15 | 27.2±1.8[b] | 26.7±1.0[b] | 25.1±1.4[a] | 25.2±1.8[ab] |

[1]Data are mean ± SE ($n$=10). Values in a row not sharing a superscript letter were significantly different ($P<0.05$) by Fisher's least significant difference test. [2]Values were not significantly different ($P>0.05$) among different groups in each row and column by Fisher's least significant difference test. [3]Data were not measured.

Exact oral EGF intake of the rats was close to 60 μg/kg/day, and not significantly different between sham operation and duodenal ulcer groups, and between different days in the same group (Table 2). The initial food intake of the rats before

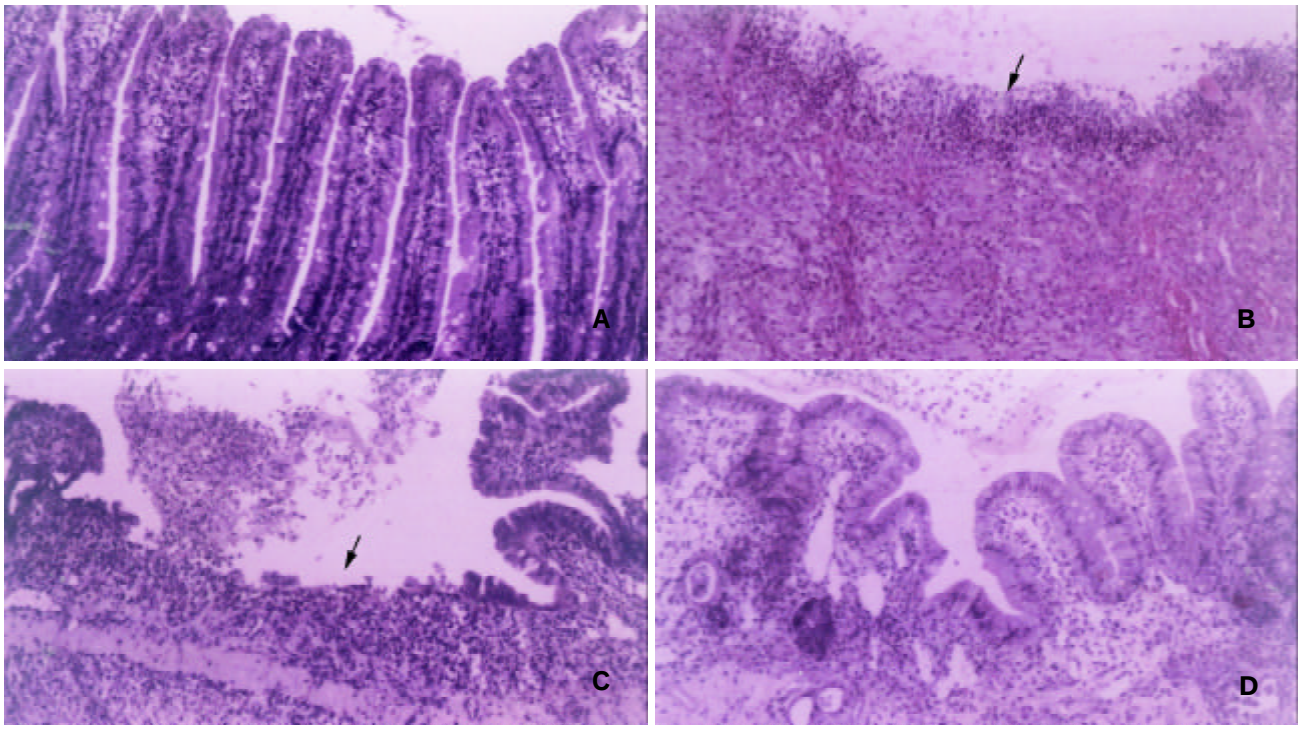



**Figure 1** Representative micrographs of duodenal mucosa stained by haematoxylin and eosin were selected from 4 sectioned samples per group. A: control rats on day 0 (×200), B: duodenal ulcer rats without EGF on day 1 (×100), C: duodenal ulcer rats without EGF on day 5 (×100), D: duodenal ulcer rats with EGF on day 5 (×200). Arrow represents the discontinuous lining of the duodenal mucosa.

operation was similar to that in the control group (26.7±3.1 g/day), and not significantly different among the groups. On day 15, decreased food intake was only observed in duodenal ulcer rats without EGF compared with that in sham operation rats. Neither EGF nor time in sham operation or duodenal ulcer rats affected food intake.

### *Morphological appearance*

To identify the formation and healing of duodenal ulcer in rats after operation, the morphological appearance magnified by ×100 or ×200 is shown in Figure 1. Compared with the control group (Figure 1A), duodenal ulcer rats on day 1 had the discontinuous lining of the mucosal and submucosal layers, and serious inflammation (Figure 1B). The mean diameter of ulcer damage was 2 mm. Sham operation rats had similar morphology as the control rats (data not shown). The morphology was not different between sham operation and the control rats on days 5 and 15. On day 5, the mean diameter of ulcer damage reduced to 1 mm in duodenal ulcer rats without EGF. Although some microvilli proliferated, but the discontinuous lining of the mucosal layer and inflammation were still observed in the ulcer area of duodenal ulcer rats without EGF (Figure 1C). Duodenal ulcer rats with EGF had obvious mucosal healing and proliferation (Figure 1D), and the mean diameter of ulcer damage was undetectable on day 5. On day 15, the morphology was not different between duodenal rats with and without EGF (data not shown).

### *DNA, RNA, and protein in duodenal mucosa*

The results of mucosal DNA, RNA, and protein contents in the duodenum are shown in Table 3 to evaluate the growth of duodenal mucosa in rats after operation. Mucosal DNA content in the duodenum significantly increased ($P$<0.05) in sham operation rats without EGF (2.96±1.27 mg/g mucosa) and duodenal ulcer rats with EGF (6.44±0.54 mg/g mucosa) on day 15 compared with that in the control rats (0.86±0.06 mg/g mucosa) on day 0 and the corresponding group on day 5 (Table 3). Duodenal ulcer rats with EGF had higher duodenal DNA content ($P$<0.05) on day 15 compared with other groups. Three-way ANOVA showed both oral EGF and time significantly increased duodenal DNA content ($P$<0.05).

Mucosal RNA content in the duodenum did not change with EGF treatment and time in sham operation and duodenal ulcer rats (Table 3). However, duodenal ulcer significantly decreased mucosal RNA content ($P$<0.05). Duodenal ulcer rats had significantly lower RNA content ($P$<0.05) in duodenal mucosa on day 5 compared with the control rats (15.9±9.3 mg/g mucosa) on day 0. Sham operation rats with EGF had higher duodenal RNA content ($P$<0.05) than other operated groups on day 5. On day 15, duodenal RNA content did not differ among four operated groups.

Three-way ANOVA showed that oral EGF and time affected duodenal protein content ($P$<0.05). Duodenal protein content in sham operation rats without EGF significantly decreased ($P$<0.05) with time, but significantly increased ($P$<0.05) compared with that in the control group (25.1±1.4 mg/g mucosa) on day 0 and other operated groups on day 5 (Table 3). On day 15, duodenal protein content did not differ among four operated groups.

### *EGF in duodenal mucosa*

Duodenal EGF content significantly increased ($P$<0.05) with time in sham operation rats without EGF, but significantly decreased ($P$<0.05) in duodenal ulcer rats with EGF (Table 4). Sham operation or duodenal ulcer rats with EGF significantly increased duodenal EGF content ($P$<0.05) compared with those without EGF on day 5. However, duodenal EGF content did not differ between sham operation and duodenal ulcer rats with or without EGF on day 5. On day 15, duodenal EGF content significantly decreased ($P$<0.05) in duodenal ulcer rats without EGF (12.9±3.9 ng/g mucosa) compared with that in the control rats (61.1±5.4 ng/g mucosa) on day 0 and sham operation rats without EGF (84.8±22.8 ng/g mucosa). However, duodenal EGF content did not differ between

sham operation or duodenal ulcer rats with and without EGF. Three-way ANOVA showed only duodenal ulcer significantly decreased mucosal EGF content ($P<0.05$).

**Table 3** Duodenal mucosal DNA, RNA, and protein contents in sham operation and duodenal ulcer rats with or without oral EGF[1]

| Group | Sham operation | | Duodenal ulcer | |
|---|---|---|---|---|
| | -EGF | +EGF | -EGF | +EGF |
| | Duodenal mucosal DNA (mg/g mucosa) | | | |
| Day 5 | 1.06±0.20[a] | 1.51±0.57[a] | 1.31±0.45[a] | 0.70±0.25[a] |
| Day 15 | 2.96±1.27[a*†] | 1.77±0.64[a] | 1.45±0.52[a] | 6.44±0.54[b*†] |
| | Duodenal mucosal RNA (mg/g mucosa) | | | |
| Day 5 | 4.89±0.42[a] | 7.10±0.20[b] | 4.71±0.35[a*] | 3.32±0.82[a*] |
| Day 15 | 5.24±1.60[a] | 6.00±0.30[a] | 5.38±0.44[a] | 4.79±0.91[a] |
| | Duodenal mucosal protein (mg/g mucosa) | | | |
| Day 5 | 49.8±2.9[a*] | 20.5±5.4[b] | 22.7±2.8[b] | 25.5±3.3[b] |
| Day 15 | 25.2±2.8[a†] | 19.3±2.7[a] | 24.3±1.6[a] | 26.2±1.3[a] |

[1]Data are mean ± SE ($n$=6). Values in a row not sharing a superscript letter were significantly different ($P<0.05$) by Fisher's least significant difference test. *The contents of DNA, RNA, and protein in duodenal mucosa were significantly different ($P<0.05$) from those in the control group (0.86±0.06 mg/g, 6.57±0.40 mg/g, 25.1±1.4 mg/g mucosa, $n$=6) on day 0 by Dunnett's test. †$P<0.05$ compared with day 5 within the same group by Fisher's least significant difference test.

**Table 4** Duodenal mucosal EGF content in sham operation and duodenal ulcer rats with or without oral EGF[1]

| Group | Sham operation | | Duodenal ulcer | |
|---|---|---|---|---|
| | - EGF | + EGF | - EGF | + EGF |
| | Duodenal mucosal EGF (ng/g mucosa) | | | |
| Day 5 | 35.7±12.9[ab] | 76.0±13.7[c] | 28.3±9.2[a] | 68.3±10.9[bc] |
| Day 15 | 84.8±22.8[a†] | 47.5±19.3[ab] | 12.9±3.9[b*] | 28.3±10.5[b†] |

[1]Data are mean ± SE ($n$=6). Values in a row not sharing a superscript letter were significantly different ($P<0.05$) by Fisher's least significant difference test. *The content of EGF in duodenal mucosa was significantly different ($P<0.05$) from that in the control group (61.1±5.4 ng/g mucosa, $n$=6) on day 0 by Dunnett's test. †$P<0.05$ compared with day 5 within the same group by Fisher's least significant difference test.

## DISCUSSION

Duodenal ulcer *per se* and oral EGF at a dose of 60 μg/kg/day did not obviously affect body weight of the rats in this study. However, Majumdar[12] found administration of EGF (20 μg/kg/day, ip. injection) to undernourished weanling rats for 7 days significantly reversed the decreased weight of whole body, small intestine, and oxyntic gland in the stomach caused by undernutrition. Similar to the results of body weight, oral EGF did not affect food intake. Because food intake was similar, body weight of the rats did not differ among four operated groups.

From the pathological observation, the mean diameter of ulcer area was undetectable in duodenal ulcer rats with EGF on day 5, and the damaged mucosa apparently recovered. However, the damaged mucosa was still found in duodenal ulcer rats without EGF on day 5. The data revealed that oral EGF reversed the damaged mucosa of duodenal ulcer on day 5, but duodenal ulcer could be self-recovered after 15 days. The results for cell growth of duodenal ulcer evaluated by mucosal DNA, RNA, and protein levels in the duodenum showed that mucosal DNA content did not significantly increase in duodenal ulcer rats with EGF until day 15 compared with those without EGF. Although we only measured DNA, RNA, and protein contents in the ulcer area of duodenal mucosa in duodenal ulcer rats and a similar area of intact duodenal mucosa in sham operation rats, these levels could be overestimated due to mixed cell types while collecting the samples. According to the results of pathological observation, the healing of damaged mucosa obviously occurred after day 5 in duodenal ulcer rats with EGF. Mucosal DNA content could reflect cell proliferation or cell division, but not measure DNA synthesis in the real time. While the pathological observation in the healing of damaged mucosa included the overall results of both cell proliferation and DNA synthesis. Oral EGF did not affect mucosal RNA and protein contents in duodenal ulcer rats. The data suggested that oral EGF had hyperplastic rather than hypertrophic effect on duodenal mucosa in duodenal ulcer rats. Although oral EGF did not influence cell proliferation in sham operation rats, EGF supplementation might temporarily cause cell hypertrophy due to increased ratio of RNA to protein caused by an increase in RNA and a decrease in protein on day 5. However, a previous study[7] demonstrated that orally administered EGF twice daily at 30 and 100 μg/kg body weight for 2 weeks had no effect on ulcer area and healing rate in Donryu rats with gastric ulcer induced by a submucosal injection of 20 mL/L acetic acid into the antrum. Different strain of animals, method of ulcer induction, severity of ulcer, and duration of EGF treatment could cause different results.

Our data showed that sham operation and duodenal ulcer rats with EGF significantly elevated mucosal EGF to 2.1- and 2.4-fold, respectively, compared with those without EGF on day 5. However, mucosal EGF did not differ between sham operation and duodenal ulcer rats with or without EGF on day 5. The results indicated that exogenous EGF increased mucosal EGF in the duodenum of both sham operation and duodenal ulcer rats to the similar extent on day 5. Therefore, exogenous EGF could be directly uptaken by the mucosa, and increased mucosal EGF in the duodenum of sham operation and duodenal ulcer rats could be derived from both exogenous and endogenous EGF. Increased mucosal EGF in sham operation and duodenal ulcer rats with EGF reduced to the similar level as in those without EGF on day 15, probably because of the adaptation to exogenous EGF through down-regulation of endogenous EGF. The EGF receptor was localized in the apical membrane of the enterocytes of rat duodenum[13,14], therefore the hypertrophic and hyperplastic effect of EGF on the mucosa of sham operation and duodenal ulcer rats, respectively, in this study could be induced via the interaction with the EGF receptor. Whereas if and how exogenous EGF regulates endogenous production of EGF or the interaction with the EGF receptor in the gastrointestinal tract of sham operation or duodenal ulcer rats, a further study to measure EGF mRNA and EGF receptors in the duodenum is required.

Our study showed that oral EGF significantly increased EGF content in the duodenal mucosa on day 5, accompanied by grossly improved healing in duodenal ulcer rats after day 5, and followed by elevated mucosal DNA content on day 15. The data suggested that oral EGF improved mucosal healing in duodenal ulcer rats by increasing EGF content in the duodenal mucosa to accelerate cell proliferation. Although EGF could be cleaved to smaller, less active forms in acidic gastric juice, the proportion of intact EGF increased to about 60 % if the pH was maintained above 4[15], which allowed it to survive passage through the intestinal tract. After administration of $^{125}$I-labeled EGF, reversed-phase HPLC identified >90 % and 46-51 % of C18-extracted radioactivity from gastric and

midjejunal luminal contents as intact $^{125}$I-EGF, whereas <3 % of C18-extracted radioactivity in extracts of duodenal, jejunal, and ileal luminal contents was intact $^{125}$I-EGF in adult mice[16]. The result indicated that EGF, given by oral administration, in the gut lumen, more or less, was still intact. Additionally, Tsujikawa *et al*[17] suggested that the luminal EGF might play a role only under tissue damage, where enhanced permeability allowed passage of luminal EGF to its receptor on the membranes. The mechanism for the healing effect of EGF on the damaged intestine is now uncertain. Most studies have focused on the mitogenic and antisecretory actions of EGF[6,9,18-21]. Konturek *et al*.[18] suggested that the mechanism for protective and ulcer healing effects of EGF involved the activation of ornithine decarboxylase, the key enzyme in the biosynthesis of polyamines, which play a crucial role in the growth-promoting action of EGF. Additionally, EGF administration (50 μg/kg) accelerated the healing of acetic acid-induced duodenal ulcer in rats via an increase in collagen proliferation and secretion without affecting gastric acid secretion[19]. A previous study[9] reported that subcutaneous injection of EGF increased duodenal DNA and RNA contents in rats with duodenal ulcer after 7-day treatment, but oral administration of EGF did not. In addition, they indicated that oral dose (10 μg/kg) of EGF had no influence on gastric secretion in rats with chronic gastroduodenal ulcer but subcutaneous injection of EGF (10 μg/kg) decreased gastric acid output by 59 % compared with the control rats without EGF administration. They suggested that the ulcer-healing effects of EGF were mediated by factors other than the inhibition of acid secretion, because oral EGF did not have any influence on gastric secretion. However, decreased gastric acid secretion in rats with chronic duodenal ulcer was observed after intravenous administration of EGF at a dose of 36 μg/kg but not at doses of 0.36 or 3.6 μg/kg[6]. Furthermore, EGF has been reported to inhibit gastric acid and stimulate duodenal bicarbonate secretion[20]. The physiological effect of EGF on acid secretion was mediated by induction of gastric $H^+$, $K^+$-ATPase gene expression[21,22].

In conclusion, oral administration of EGF (60 μg/kg/day) can increase EGF content in the duodenal mucosa and promote the healing of the rats with duodenal ulcer by its mitogenic action.

## REFERENCES


1 **Marti U**, Burwen SJ, Jones AL. Biological effects of epidermal growth factor, with emphasis on the gastrointestinal tract and liver: an update. *Hepatology* 1989; **9**: 126-138

2 **Poulsen SS**, Nexo E, Olsen PS, Hess J, Kirkegaard P. Immunohistochemical localization of epidermal growth factor in rat and man. *Histochemistry* 1986; **85**: 389-394

3 **Wright NA**, Pike C, Elia G. Induction of a novel epidermal growth factor- secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature* 1990; **343**: 82-85

4 **Jones MK**, Tomikawa M, Mohajer B, Tarnawski AS. Gastrointestinal mucosal regeneration: role of growth factors. *Front Biosci* 1999; **4**: D303-309

5 **Konturek PC**, Bielanski W, Bobrzynski A, Hahn EG, Konturek SJ. Gastric mucosal expression and luminal release of growth factors in gastric carcinoma and duodenal ulcer patients before and after eradication of *Helicobacter pylori*. *J Physiol Pharmacol* 1997; **48**: 375-382

6 **Skov Olsen P**, Poulsen SS, Therkelsen K, Nexo E. Oral administration of synthetic human urogastrone promotes healing of chronic duodenal ulcers in rats. *Gastroenterology* 1986; **90**: 911-917

7 **Kuwahara Y**, Sunagawa Y, Imoto Y, Okabe S. Effects of orally administered human epidermal growth factor on natural and delayed healing of acetic acid-induced gastric ulcers in rats. *Jpn J Pharmacol* 1990; **52**: 164-166

8 **Palomino A**, Hernandez-Bernal F, Haedo W, Franco S, Mas JA, Fernandez JA, Soto G, Alonso A, Gonzalez T, Lopez-Saura P. A multicenter, randomized, double-blind clinical trial examining the effect of oral human recombinant epidermal growth factor on the healing of duodenal ulcers. *Scand J Gastroenterol* 2000; **35**: 1016-1022

9 **Konturek SJ**, Dembinski A, Warzecha Z, Brzozowski, Gregory H. Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats. *Gastroenterology* 1988; **94**: 1300-1307

10 **Chomczynski P**. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 1993; **15**: 532-534, 536-537

11 **Sizemore N**, Dudeck RC, Barksdale CM, Nordblom GD, Mueller WT, McConnell P, Wright DS, Guglietta A, Kuo BS. Development and validation of two solid-phase enzyme immunoassays (ELISA) for quantitation of human epidermal growth factors (hEGFs). *Pharm Res* 1996; **13**: 1088-1094

12 **Majumdar AP**. Postnatal undernutrition: effect of epidermal growth factor on growth and function of the gastrointestinal tract in rats. *J Pediatr Gastroenterol Nutr* 1984; **3**: 618-625

13 **Montaner B**, Perez-Tomas R. Epidermal growth factor receptor (EGF-R) localization in the apical membrane of the enterocytes of rat duodenum. *Cell Biol Int* 1999; **23**: 475-479

14 **Montaner B**, Asbert M, Perez-Tomas R. Immunolocalization of transforming growth factor-alpha and epidermal growth factor receptor in the rat gastroduodenal area. *Dig Dis Sci* 1999; **44**: 1408-1416

15 **Playford RJ**, Marchbank T, Calnan DP, Calam J, Royston P, Batten JJ, Hansen HF. Epidermal growth factor is digested to smaller, less active forms in acidic gastric juice. *Gastroenterology* 1995; **108**: 92-101

16 **Rao RK**. Luminal processing of epidermal growth factor in mouse gastrointestinal tract *in vivo*. *Peptides* 1995; **16**: 505-513

17 **Tsujikawa T**, Itoh A, Yasuoka T, Fukunaga T, Satoh J, Uda K, Ihara T, Sasaki M, Fujiyama Y. Mucosal permeability regulates receptor binding of luminal epidermal growth factor in the adult rat intestine. *Int J Mol Med* 2003; **11**: 349-352

18 **Konturek JW**, Brzozowski T, Konturek SJ. Epidermal growth factor in protection, repair, and healing of gastroduodenal mucosa. *J Clin Gastroenterol* 1991; **13**(Suppl 1): S88-S97

19 **Perez Aisa A**, Sopena Biarge F, Arceiz Gonzalo E, Sainz Samitier R, Ortego Diez De Retana J, Lanas Arbeloa A. Effect of exogenous administration of platelet-derived growth factor and epidermal growth factor on duodenal ulcer healing in rats treated with indomethacin. *Gastroenterol Hepatol* 2002; **25**: 299-305

20 **Szabo S**, Vincze A. Growth factors in ulcer healing: lessons from recent studies. *J Physiol Paris* 2000; **94**: 77-81

21 **Kaise M**, Muraoka A, Yamada J, Yamada T. Epidermal growth factor induces $H^+$, $K^+$-ATPase alpha-subunit gene expression through an element homologous to the 3' half-site of the c-fos serum response element. *J Biol Chem* 1995; **270**: 18637-18642

22 **Kusayanagi S**, Takeuchi Y, Todisco A, Mitamura K. Extracellular signal-regulated protein kinases mediate H(+),K(+)-ATPase alpha-subunit gene expression. *Biochem Biophys Res Commun* 2002; **290**: 1289-1294


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2266-2269
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Role of oxygen free radicals in patients with acute pancreatitis

Byung Kyu Park, Jae Bock Chung, Jin Heon Lee, Jeong Hun Suh, Seung Woo Park, Si Young Song, Hyeyoung Kim, Kyung Hwan Kim, Jin Kyung Kang

**Byung Kyu Park, Jae Bock Chung, Jin Heon Lee, Jeong Hun Suh, Seung Woo Park, Si Young Song, Jin Kyung Kang**, Department of Internal Medicine, Institute of Gastroenterology, College of Medicine, Yonsei University, Seoul, Korea
**Hyeyoung Kim, Kyung Hwan Kim**, Department of Pharmacology, College of Medicine, Yonsei University, Seoul, Korea
**Correspondence to:** Jae Bock Chung, M.D. Department of Internal Medicine, College of Medicine, Yonsei University, 134 Shinchon-Dong, Seodaemun-ku, Seoul, 120-752 Korea. jbchung@yumc.yonsei.ac.kr
**Telephone:** +82-2-361-5427 **Fax:** +82-2-393-6884
**Received:** 2003-06-04 **Accepted:** 2003-08-02

## Abstract

**AIM:** The generation of oxygen free radicals has been implicated in the pathogenesis of experimental pancreatitis. The aim of this study was to determine the role of oxygen free radicals in patients with acute pancreatitis.

**METHODS:** The plasma levels of C-reactive protein (CRP), lipid peroxide (LPO), myeloperoxidase (MPO) and superoxide dismutase (SOD) were measured in 13 patients with acute pancreatitis and 14 healthy volunteers.

**RESULTS:** Among the patients with acute pancreatitis, there were higher plasma levels of LPO and MPO and lower SOD activity in patients with severe pancreatitis than in those with mild pancreatitis. However, there was no significant difference in the serum marker of oxidative stress no matter what the etiology was. The LPO level was especially correlated with the concentration of serum CRP and CT severity index.

**CONCLUSION:** The oxygen free radicals may be closely associated with inflammatory process and the severity of acute pancreatitis. Especially, the concentration of plasma LPO is a meaningful index for determining the severity of the disease.

Park BK, Chung JB, Lee JH, Suh JH, Park SW, Song SY, Kim H, Kim KH, Kang JK. Role of oxygen free radicals in patients with acute pancreatitis. *World J Gastroenterol* 2003; 9(10): 2266-2269

http://www.wjgnet.com/1007-9327/9/2266.asp

## INTRODUCTION

Oxygen free radicals are molecules produced continuously in cells by several mechanisms. The generation of oxygen free radicals is physiologic. In most circumstances, oxygen free radicals are neutralized immediately by enzymatic scavengers. However, when formation of oxygen free radicals overwhelms radical neutralization in cells, oxidative stress occurs. As they are very reactive, they react well with all biological substances such as proteins, polysaccharides and nucleic acids, resulting in tissue injury[1-3]. It has been suggested that oxygen free radicals are responsible for a wide variety of diseases or conditions[1,4-8].

Oxygen free radicals have been known to play an important role in the pathogenesis of pancreatitis of some experimental models[6-12]. Oxygen free radicals are involved in initiation of pancreatitis[11]. Also, it was reported that oxygen free radicals acted as important mediators of tissue damage in experimental acute pancreatitis[10]. Therapeutic effects of antioxidants and radical scavengers have been shown in experimental models of acute pancreatitis[11,13,14].

However, there is doubt that experimental models of acute pancreatitis will match clinically acute pancreatitis in humans. Although there were some data that reflected enhanced oxygen stress in patients with acute pancreatitis[15-17], the role of oxygen free radicals in patients with acute pancreatitis has not been well clarified.

Therefore, this study was conducted to evaluate the role of oxygen free radicals in human acute pancreatitis and to analyze the correlation between oxygen free radicals and the severity of acute pancreatitis.

## MATERIALS AND METHODS

### *Patients*

Thirteen patients admitted to Medical Center of Yonsei University with a diagnosis of acute pancreatitis were included in this study. The diagnosis was established on the basis of acute abdominal pain, at least 3-fold elevated levels of serum amylase and computed tomography (CT). All patients had no previous history of acute pancreatitis. Fourteen healthy subjects without previous medical history were enrolled as controls. The mean age was 53.6 years in patients and 32.5 years in control group. The male to female ratio was 11:2 in patients and 10:4 in control groups.

### *Measurement of oxygen free radicals*

Peripheral blood samples were taken on recruitment. After the blood was centrifuged, the plasma was stored at -70 ℃ until analysis. The blood samples of control group underwent the same process. C-reactive protein was measured by the routine method. Serum lipid peroxide (LPO) and myeloperoxide (MPO) as markers of oxygen free radicals were measured. Also the activity of superoxide dismutase (SOD) that diminished in oxidative stress due to its role as enzymatic scavenger was measured.

### *Lipid peroxidation*

LPO was analyzed by measuring the amount of thiobarbituric acid reactive substances (TBARS). It was preceded that the standard solution was prepared by diluting 1 000 nmol/ml tetraethoxy propane, which was made by mixing with tetraethoxy propane and tertiary distilled water. Tertiary distilled water was used as blank solution. Plasma, blank, and standard solution 200 μL, respectively, were mixed with 20 % acetic acid. TBARS resulted from the reaction with thiobarbituric acid (TBA) 400 μL, was detected as reading fluorescence at 535 nm emission. The result was compared with standard curve.

### *Myeloperoxidation*

MPO was analyzed as a marker of neutrophil activation by

Bioxytech®MPO enzyme immunoassay kit (OXIS International Inc., Portland, USA). This assay detects the fluorescence of solution at 405 nm emission using sandwich enzyme-linked immunosorbent assay (ELISA) against biotin-labeled goat polyclonal anti-MPO.

### *Superoxide dismutase*

SOD activity was measured by using Bioxytech® SOD-525™ assay kit (OXIS International Inc., Portland, USA). This assay detects the fluorescence of solution at 525 nm, based on the principle that autoxidation of tetracyclic cathechol accelerates in condition of SOD.

### *Other parameters*

To determine the severity and prognosis of acute pancreatitis, the level of C-reactive protein (CRP) in serum was used for a single indicator, Ranson' s score for multiple indicator, and CT severity index[18] for morphological indicator. Atlanta classification[19] was used as a main determining index for the severity of acute pancreatitis.

### *Statistical analysis*

We used chi-square test and independent sample *t*-test for comparison in each group. A *P* value less than 0.05 was considered significant.

## RESULTS

### *Levels of oxygen free radicals*

The mean plasma level of LPO was 21.9±26.5 nmol/ml in acute pancreatitis and 5.7±1.7 nmol/ml in control group (*P*=0.030). Also, the mean plasma level of MPO was 12.3±11.7 nmol/ml in acute pancreatitis and 2.7±1.2 nmol/ml in control group (*P*=0.005). Oxygen free radicals significantly increased more in acute pancreatitis patients than in control group. The mean level of SOD activity was 48.2±41.4 U/dl in acute pancreatitis patients and 100.4±20.2 U/dl in control group (*P*=0.002). The decrease of SOD activity was more prominent in acute pancreatitis than in control group (Table 1).

**Table 1** Laboratory parameters in patients with acute pancreatitis and control group

| Parameters | Control (*n*=14) | Acute pancreatitis (*n*=13) | *P*-value |
|---|---|---|---|
| Amylase (IU / dL) | 141.9±179.3 | 393.3±243.1 | 0.005 |
| LPO (nmol / mL) | 5.7±1.7 | 21.9±26.5 | 0.030 |
| MPO (nmol / mL) | 2.7±1.2 | 12.3±11.7 | 0.005 |
| SOD (U / dL) | 100.4±20.2 | 48.2±41.4 | 0.002 |

Values are means ± SD, LPO = lipid peroxide, MPO = myeloperoxidase, SOD=superoxide dismutase.

### *Oxygen free radicals according to etiology of acute pancreatitis*

The etiologies of acute pancreatitis were gallstone (*n*=5), alcohol (*n*=4), hyperlipidemia (*n*=1), and unexplained (*n*=3). Comparing gallstone pancreatitis patients with alcohol induced pancreatitis, the plasma levels of CRP, LPO, MPO, and SOD showed no significant differences between the two groups (Table 2).

### *Association with severity of acute pancreatitis*

According to the Atlanta classification, acute pancreatitis was classified as mild in 8 patients and as severe in 5 patients based on their clinical manifestations. There were no differences of age and sex between the two groups (Table 3). The mean level of serum CRP in patients with severe pancreatitis was higher than the one in patients with mild pancreatitis (12.3±9.0 mg/dl *vs* 6.7±4.5 mg/dl, *P*=0.311). The mean level of SOD activity more markedly decreased in patients with severe pancreatitis than in patients with mild pancreatitis (27.3±15.4 U/dl *vs* 65.7±49.2 U/dl, *P*=0.130).

**Table 2** Laboratory parameters according to etiology of acute pancreatitis

| Paramerters | Gallstone group (*n*=5) | Alcohol group (*n*=4) | *P*-value |
|---|---|---|---|
| Age (years) | 55.4±19.7 | 44.5±14.2 | 0.368 |
| Amylase (IU / dL) | 364.6±271.9 | 366.5±252.5 | 0.992 |
| Lipase (IU / dL) | 10 004.0±15873.2 | 6 035.8±8,265.2 | 0.646 |
| CRP (mg / dL) | 6.0±2.5 | 12.3±9.8 | 0.296 |
| LPO (nmol / mL) | 15.6±10.5 | 38.7±45.1 | 0.385 |
| MPO (nmol / mL) | 11.3±15.3 | 15.5±14.3 | 0.689 |
| SOD (U / dL) | 59.2±49.3 | 21.0±17.1 | 0.171 |

Values are means ±SD, LPO = lipid peroxide, MPO = myeloperoxidase, SOD = superoxide dismutase.

**Table 3** Clinical characteristics of patients with acute pancreatitis classified according to Atlanta classification

| | Mild group | Severe group |
|---|---|---|
| No. of cases | 8 | 5 |
| Age (years) | 53.5±17.1 | 53.8±15.7 |
| Sex (M:F) | 7:1 | 5:1 |
| Etiology | | |
| Gallstone | 2 | 3 |
| Alcohol | 2 | 2 |
| Others* | 4 | 0 |
| No. of mortality | 0 | 0 |

*One case was due to hyperlipidemia and 3 cases were idiopathic.

**Table 4** Laboratory parameters in patients with mild and severe pancreatitis according to Atlanta classification

| Parameters | Mild group (*n*=8) | Severe group (*n*=5) | *P*-value |
|---|---|---|---|
| Amylase (IU / dL) | 374.7±252.4 | 423.0±253.1 | 0.745 |
| Lipase (IU / dL) | 10 082.8±12 565.6 | 30 008.0±3 314.9 | 0.167 |
| CRP (mg / dL) | 6.7±4.5 | 12.3±9.0 | 0.311 |
| LPO (nmol / mL) | 11.5±6.8 | 38.7±38.1 | 0.068 |
| MPO (nmol / mL) | 7.5±4.1 | 20.1±15.4 | 0.053 |
| SOD (U / dL) | 65.7±49.2 | 27.3±15.4 | 0.130 |
| Ranson's score | 1.4±0.7 | 2.8±1.9 | 0.177 |
| CT severity index | 2.0±1.1 | 5.8±3.1 | 0.008 |

Values are means ±SD, LPO = lipid peroxide, MPO = myeloperoxidase, SOD = superoxide dismutase.

The mean plasma levels of LPO and MPO were higher in patients with severe pancreatits than those in patients with mild pancreatitis (LPO: 38.7±38.1 nmol/ml *vs* 11.5±6.8 nmol/ml, *P*=0.068, MPO: 20.1±16.1 nmol/dl *vs* 7.5±4.1 nmol/dl, *P*=0.053). The significant difference was for CT severity index between the two groups (5.8±3.1 in severe pancreatitis, 2.0±1.1 in mild pancreatitis, *P*=0.008) (Table 4). To analyze the relationship between oxygen free radicals and CT severity index, serum CRP that is widely used as an index for severity of pancreatitis, plasma LPO showed a close correlation with CT severity index and serum CRP ($r^2$=0.373, and 0.675, *P*=0.027, and 0.001, respectively) (Figure 1). Plasma MPO showed a close correlation

with CT severity index but not with serum CRP ($r^2$=0.319 and 0.202, $P$=0.044 and 0.143, respectively) (Figure 2). But SOD activity showed no significant correlation with either CT severity index or serum CRP ($r^2$=0.026 and 0.307, $P$=0.638 and 0.096, respectively ) (Figure 3). CT severity index was correlated with serum CRP and Ranson' s score ($r^2$=0.334 and 0.382, $P$=0.049 and 0.024, respectively).

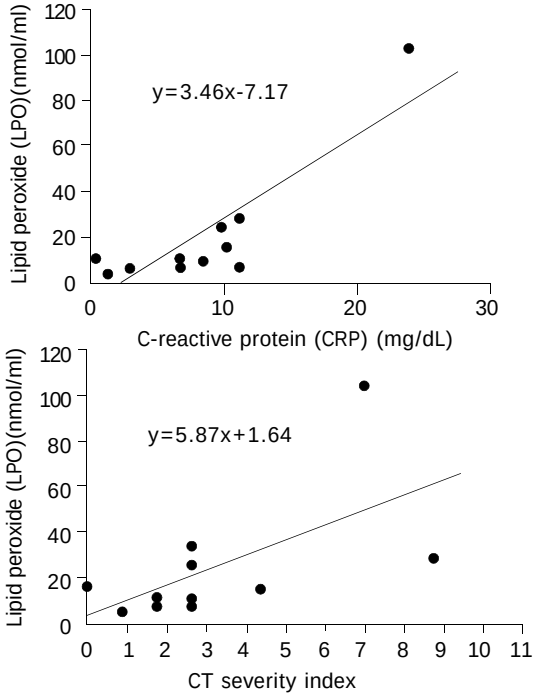



**Figure 1** Relationships between serum levels of LPO and prognostic indices (CRP and CT severity index). There was a statistically significant correlation between LPO levels with CRP and CT severity index (γ2=0.675 and 0.373, $P$=0.001 and 0.027, respectively).

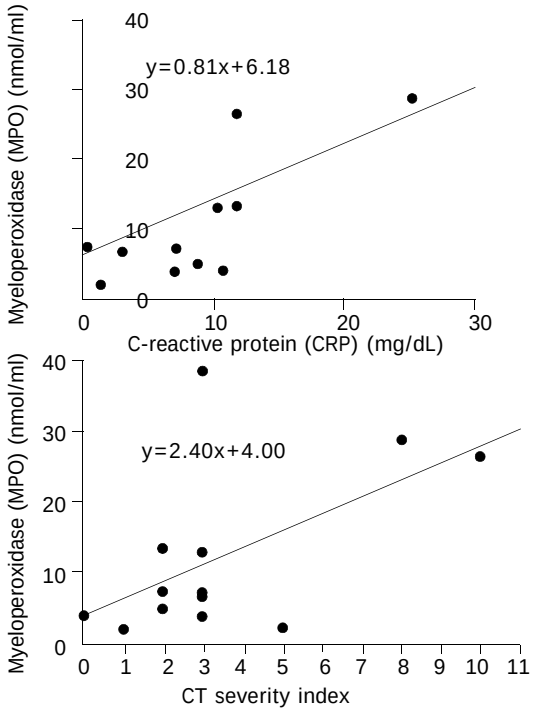



**Figure 2** Relationships between serum levels of MPO and prognostic indices (CRP and CT severity index). There was no statistically significant correlation between MPO level and CRP (γ2=0.202, $P$=0.143). However, MPO level correlated positively and significantly with CT severity index (γ2=0.319, $P$=0.044).

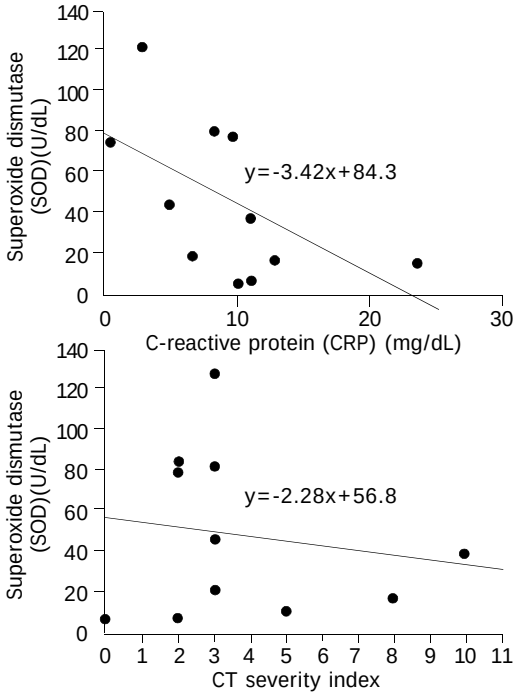



**Figure 3** Relationships between serum enzymatic activity of SOD and prognostic indices (CRP and CT severity index). There was no statistically significant correlation between serum SOD concentrations and CRP and CT severity index (γ2=0.307 and 0.026, $P$=0.096 and 0.638, respectively).

## DISCUSSION

Acute pancreatitis led to various degrees of interstitial edema, acinar cell damage, hemorrhage and necrosis[8]. Although the inflammation initiates in pancreas, the disease may lead to systemic multi-organ failure. Several factors (complement activation, cytokines, oxygen free radicals, ischemia, and autodigestion of pancreatic enzyme) have been known to be involved in the pathogenesis of acute pancreatitis. But the role of these factors remains still unclear. Among them, oxygen free radicals could damage extracellular tissue by degrading hyaluronic acid and collagen in the intercellular matrix and directly attack biological membrane through the peroxidation of structurally and functionally important lipids[20]. Furthermore, they could denature enzymes and other important proteins, and damage nucleic acid. In addition, they could indirectly trigger the accumulation of polymorphonulclear (PMN) leukocyte in the tissue. Activated PMN leukocytes could secrete various enzymes such as myeloperoxidase, protease, and elastase[21]. As a result, the inflammatory reaction accelerated. Also, oxygen free radicals could indirectly stimulate arachidonic acid metabolism with increased production of prostaglandins, thromboxane, and leukotrienes, and eventually could lead to microcircular derangement and cellular damage[2,15].

There are some reports concerning mechanisms of enhanced production of oxygen free radicals. Xanthine oxidase, PMN leukocyte, and cytochromes P-450 have been introduced as the source of oxygen free radicals[3,12]. However, the true sources of oxygen free radicals that are responsible for the pathogenesis of acute pancreatitis have not yet been identified. Since the first study by Sanfey *et al*[9], many studies have demonstrated the role of oxygen free radicals in the pathogenesis of acute pancreatitis in experimental models and patients. Oxygen free radicals have been known to mediate an important step in the initiation of acute pancreatitis[11]. But most results have been derived from experimental animal models in which acute pancreatitis was induced by stress, cerulein

injection, choline-deficient diet, or taurocholate injection into pancreatic duct. Only a few data have been derived from patients. Furthermore, there are many limitations on studying patients with acute pancreatitis in clinical settings.

Because of the nature of their high reactivity, oxygen free radicals are difficult to measure directly. Direct measurement of oxygen free radicals with electron spin resonance (ESR) technique was limited to *in vitro* studies due to short half time of free radicals and toxicity of compounds[22]. For these reasons, the alternative of measuring stable metabolites was met with broad acceptance. In most studies, measurements of the effects of radical reaction with biological substances (i.e., lipid peroxides and changes of glutathione metabolism) have replaced the assessment of oxygen free radicals.

In acute pancreatitis patients, increased production of lipid peroxidation, decreased level of vitamin C, and direct correlation between oxidative stress and severity of acute pancreatitis have been reported[15-17]. Equally, our results showed that plasma levels of LPO and MPO increased more in acute pancreatitis patients than in normal control group, and that increase of LPO and MPO was more prominent in severe pancreatitis than in mild pancreatitis, and that SOD activity, as a scavenger of oxygen free radical, was lower in acute pancreatitis patients than in normal control group. From these results, we can suggest that oxygen-derived free radicals play a pivotal role in the pathogenesis of acute pancreatitis in patients. In our study, it was probable that lipid peroxidation was correlated with severity of acute pancreatitis. There was a close correlation of lipid peroxidation with serum CRP and CT severity index. Therefore, there is some possibility that LPO could be a predictor of severity of acute pancreatitis. Similarly it was shown that lipid peroxidation was significantly increased in patients with septic shock[23]. In contrast, Abu-Zidan *et al*[16] have reported that lipid peroxidation was highly correlated with the severity of pancreatitis but not a good predictor of it. This discordance may be due to different method of measuring lipid peroxidation and study design.

In our study, the level of SOD activity, enzymatic scavenger, was significantly decreased in the plasma of patients with acute pancreatitis, and although not significant, it was related to the severity of acute pancreatitis. The effects of antioxidant, such as vitamins C and E, in experimental models have been reported[11,13,14]. However, the results have varied according to different models. Because in these most studies, antioxidant therapy was performed before acute pancreatitis was induced, the results were unlikely to be applied to clinical conditions. As well, the therapeutic effect of antioxidants and radical scavenger in patients has not been demonstrated yet.

There was no difference of oxygen free radicals between alcohol-induced pancreatitis and gallstone pancreatitis in our study. In many various experimental models and patients with acute pancreatitis, oxygen free radicals have been related to the disease[2,15,24,25]. Thus in our opinion, the role of oxygen free radicals may not be different no matter what the etiology of the disease is.

In conclusion, oxygen-derived free radicals may be closely associated with inflammatory process and the severity of acute pancreatitis, and the plasma level of LPO is a meaningful index for determining the severity of the disease.

## REFERENCES


1 **Kerr ME**, Bender CM, Monti EJ. An introduction to oxygen free radicals. *Heart Lung* 1996; **25**: 200-209
2 **Schoenberg MH**, Birk D, Beger HG. Oxidative stress in acute and chronic pancreatitis. *Am J Clin Nutr* 1995; **62**: 1306S-1314S
3 **Schulz HU**, Niederau C, Klonowski-Stumpe H, Halangk W, Luthen R, Lippert H. Oxidative stress in acute pancreatitis. *Hepato-Gastroenterol* 1999; **46**: 2736-2750
4 **Janero DR**. Ischemic heart disease and antioxidants: mechanistic aspects of oxidative injury and its prevention. *Crit Rev Food Sci Nutr* 1995; **35**: 65-81
5 **Thomas MJ**. The role of free radicals and antioxidants: how do we know that they are working? *Crit Rev Food Sci Nutr* 1995; **35**: 21-39
6 **Correa P**. The role of antioxidants in gastric carcinogenesis. *Crit Rev Food Sci Nutr* 1995; **35**: 59-64
7 **Machlin LJ**. Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. *Crit Rev Food Sci Nutr* 1995; **35**: 41-50
8 **Bulkley GB**. The role of oxygen free radicals in human disease processes. *Surgery* 1983; **94**: 407-411
9 **Sanfey H**, Bulkley GB, Cameron JL. The role of oxygen-derived free radicals in the pathogenesis of acute pancreatitis. *Am Surg* 1984; **200**: 405-413
10 **Rau B**, Poch B, Gansauge F, Bauer A, Nüssler AK, Nevalainen T, Schoenberg MH, Beger HG. Pathophysiologic role of oxygen free radicals in acute pancreatitis initiating event or mediator of tissue damage? *Ann Surg* 2000; **231**: 352-360
11 **Schoenberg MH**, Büchler M, Gaspar M, Stinner A, Younes M, Melzener I, Bültmann B, Beger HG. Oxygen free radicals in acute pancreatitis of the rat. *Gut* 1990; **31**: 1138-1143
12 **Sanfey H**, Bulkley GB, Cameron JL. The pathogenesis of acute pancreatitis the source and role of oxygen-derived free radicals in three different experimental models. *Ann Surg* 1985; **201**: 633-639
13 **Sarr MG**, Bulkley GB, Cameron JL. Temporal efficacy of allopurinol during the induction of pancreatitis in the ex *vivo* perfused canine pancreas. *Surgery* 1987; **101**: 342-346
14 **Shikata A**, Suganuma M, Marugami Y, Sakurai Y, Ochiai M, Kamei K, Funabiki T, Shinohara R. The role of oxyhen-derived free radicals and their scavengers in experimental acute pancreatitis. *Digestion* 1996; **57**: 264
15 **Tsai K**, Wang SS, Chen TS, Kong CW, Chang FY, Lee SD, Lu FJ. Oxidative stress: an important phenomenon with pathogenetic significance in the progression of acute pancreatitis. *Gut* 1998; **42**: 850-855
16 **Abu-Zidan FM**, Bonham MJD, Windsor JA. Severity of acute pancreatitis: a multivariate analysis of oxidative stress markers and modified Glasgow criteria. *Br J Surg* 2000; **87**: 1019-1023
17 **Bonham MJD**, Abu-Zidan FM, Simovic MO, Sluis KB, Wilkinson A, Winterbourn CC, Windsor JA. Early ascorbic acid depletion is related to the severity of acute pancreatitis. *Br J Surg* 1999; **86:** 1296-1301
18 **Balthazar EJ**, Robinson DL, Megibow AJ, Ranson JHC. Acute pancreatitis: value of CT in establishing prognosis'. *Radiology* 1990; **174**: 331-336
19 **Bradley EL**. A clinically based classification system for acute pancreatitis. *Arch Surg* 1993; **128**: 586-590
20 **Slater TF**. Free-radical mechanisms in tissue injury. *Biochem J* 1984; **222**: 1-15
21 **Babior BM**. Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* 1978; **298**: 659-668
22 **Schoenberg MH**, Büchler M, Beger HG. Oxygen radicals in experimental acute pancreatitis. *Hepato-Gastroenterol* 1994; **41**: 313-319
23 **Goode HF**, Cowley HC, Walker BE, Howdle PD, Webster NR. Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. *Crit Care Med* 1995; **23**: 646-651
24 **Uden S**, Schofield D, Miller PF, Day JP, Bottiglieri T, Braganza JM. Antioxidant therapy for recurrent pancreatitis: biochemical profiles in a placebo-controlled trial. *Aliment Pharmacol Ther* 1992; **6**: 229-240
25 **Gut A**, Shiel N, Kay PM, Segal I, Braganza JM. Heightened free radical activity in blacks with chronic pancreatitis at Johannesburg, *South Africa*. *Clin Chim Acta* 1994; **230**: 189-199


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2270-2273
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Parenteral versus early intrajejunal nutrition: Effect on pancreatitic natural course, entero-hormones release and its efficacy on dogs with acute pancreatitis

Huan-Long Qin, Zhen-Dong Su, Lei-Guang Hu, Zai-Xian Ding, Qing-Tian Lin

**Huan-Long Qin, Zhen-Dong Su, Lei-Guang Hu, Zai-Xian Ding, Qing-Tian Lin,** Department of Surgery, Sixth People' s Hospital, Shanghai Jiaotong University Shanghai 200233, China
**Supported by** Shanghai Science Fund for the Morning Star Young Scholars, No.99QB14010
**Correspondence to:** Dr. Huan-Long Qin, Department of Surgery, Sixth People' s Hospital, Shanghai Jiaotong University Shanghai 200233, China. sshosp @public.sta.net.cn
**Telephone:** +86-21-64368920 **Fax:** +86-21-64368920
**Received:** 2003-05-11 **Accepted:** 2003-06-02

## Abstract

**AIM:** To evaluate the effect of early intrajejunal nutrition (EIN) on the natural course, entero-hormone secretion and its efficacy on dogs with acute pancreatitis.

**METHODS:** An acute pancreatitis model was induced by injecting 1 ml/kg of combined solution (2.5 % sodium taurocholate and 8 000-10 000 BAEE units trypsin/ml) into the pancreas via pancreatic duct. Fifteen dogs were divided into parenteral nutrition (PN) group and EIN group. Two groups were isonitrogenous and isocaloric. EIN was used at postoperative 24 h. Serum glucose, calcium, amylase and lysosomal enzymes were determined before and 1, 4, 7 d after acute pancreatitis was induced. All the dogs were injected 50 uCi $^{125}$I-BSA 4 h before sacrificed on the 7$^{th}$ day. The $^{125}$I -BSA index of the pancreas/muscle, pancreas/blood, and pancreas pathology score (PPS) were determined. The peripheral plasma cholecystokinin (CCK), secretin (SEC) and gastrin were measured by ELISA and RIA, and was quantitative analysis of pancreatic juice and amylase, pancreatolipase and $HCO_3^-$, $Cl^-$, $Na^+$ and $K^+$ performed by an autochemical analyzer at 30, 60, 120 and 180 min after beginning PN or EIN on the first day.

**RESULTS:** There was no difference between two groups in the contents of serum calcium, amylase and lysosomal enzymes, $^{125}$I-BSA index of pancreas/muscle and pancreas/blood and PPS. The contents of CCK and gastrin in EIN were higher than those in PN group at 60 and 120 min ($P$<0.05). The content of SEC post-infusion of nutrition solution was higher than that of pre-infusion of nutrition solution in both groups, and only at 60 min SEC in EIN group was higher than that in PN group. The content of gastrin in EIN was higher than that in PN group at 120 and 180 min ($P$<0.05). The changes of pancreatic juice, amylase, pancreatolipase and $HCO_3^-$, $Cl^-$, $Na^+$ and $K^+$ between two groups did not reach significantly statistical difference ($P$>0.05).

**CONCLUSION:** EIN does not stimulate entero-hormone and pancreatic juice secretion, and enzyme-protein synthesis and release. EIN has no effect on the natural course of acute pancreatitis.

Qin HL, Su ZD, Hu LG, Ding ZX, Lin QT. Parenteral versus early intrajejunal nutrition: Effect on pancreatitic natural course, entero-hormones release and its efficacy on dogs with acute pancreatitis. *World J Gastroenterol* 2003; 9(10): 2270-2273
http://www.wjgnet.com/1007-9327/9/2270.asp

## INTRODUCTION

Total parenteral nutrition (TPN) has been the standard practice for providing exogenous nutrients to patients with acute pancreatitis in order to improve their nutritional status and to avoid pancreatic stimulation. However, TPN is associated with certain disadvantages. In particular, there is an increased risk of central catheter infection, severe hyperglycaemia, and other metabolic and electrolyte disturbances and a possible exacerbation of metabolic disturbances. TPN may also result in gut barrier function alterations due to increasing intestinal permeability[1-10].

Benefits from the use of total enteral nutrition (TEN) have been noted in a number of other diseases, such as burns, trauma, and sepsis. In comparison with TPN, use of TEN reduces nosocomical infection, multiple organ failure (MOF), and length of hospitalization[3,4]. The use of early enteral feeding for nutritional support in patients with acute pancreatitis has not been evaluated systematically. The commonly encountered problems of gastric atony and outlet obstruction have limited the successful delivery of enteral formulas to patients with severe acute pancreatitis. In addition, many surgeons hold scrupulously that the EIN may exacerbate the clinical pathological features, and lead to recurrence of symptoms and delayed complications to be cured[11-18]. However, these problems could be overcome if enteral nutrition is delivered to the distal ileum far away from the Treitz' s ligament, to avoid stimulation of the cephalic and gastric phase, and those effects are not so pronounced as nutrients are delivered directly into jejunum[3,9-13]. Therefore, it is necessary to investigate the efficiency of early intrajejunal nutrition on pancreatitic clinicopathological changes, entero- hormone release and its efficacy on dogs with acute pancreatitis.

## MATERIALS AND METHODS

### *Animal model*

A total of 22 dogs weighing 18-22 kg, were allowed *ad libitum* intake of water. After fasted for 12-14 hours, all the dogs were induced anesthesia by intramuscular injection of ketamine 10 ml/kg, and intravenous injection of sodium pentobarbital 30 mg/kg. Under sterile conditions, a middle laparotomy and a duodenotomy were performed. The duodenum papilla was found and a silastic catheter was inserted into pancreatic tube and fixed for collecting pancreatic juice. Acute pancreatitis model was induced by injecting 1 mg/kg of combined solution of 2.5 % sodium taurocholate and 8 000-10 000 BAEF units trypin/ml into pancreas via pancreatic duct with a pressure of 30 cm$H_2O$, and the common biliary duct was clamped. After the model

was established, the duodenum and abdomen were closed. A catheter via jejunostomy was set at 30 cm away from the Treitz' s ligament. The neck region of the dogs was shaved and prepared in a sterile manner for catheterization. A silastic catheter (1.0 mm inner diameter and 1.5 mm outer diameter) was inserted through the external jugular vein to reach the superior vena cava. The catheter was fixed to connect the infusion solution. Fifteen dogs with acute pancreatitis survived over 7 days, and the death rate was 32 % (7/22). The trial was approved by the Institutional Animal Committee.

### *Experimental groups and preparation of nutritional solution*

Fifteen dogs having survived over 7 days with acute pancreatitis were randomly divided into PN group ($n$=7) and EIN group ($n$=8). The two groups were isocaloric and isonitrogenous. The PN solutions were consisted of 7 % Vamin (SSPC, 9.4 g/1 000 ml) and 20 % Intralipid (SSPC) and 50 % glucose (GS). Non-protein calorie was 50 kC (209.2 kJ/kg) and nitrogen was 0.3 g/kg/d. The total volume of solution infused was 70 ml/kg/d. Energy index supported with glucose and fat emulsion was 1∶1. Multivitamins and electrolytes were also contained in TPN solutions. The normal saline was infused by 250 ml/kg during operation time and 8 h postoperation, and then infused with 125±25 ml/kg. The nutrient solution was infused at a constant infusion rate by a pump (100-120 ml/h).

The EIN solution was Nutrison (Nutricia). At the 24$^{th}$ h after acute pancreatitis was induced, the jejunum through jejunostomy catheter was infused 250 ml Nutrison and 500 ml NS. At the 48$^{th}$ h after acute pancreatitis, 500 ml Nutrison and 250 ml NS were infused for 5 days. The infusion rate was controlled by a microcomputer-pump (Nutricia). During EIN supporting, the content with insufficient calories and nitrogen were supplemented by partial parenteral nutrition[3,16,18].

### *Laboratory tests, $^{125}$I-BSA index and pancreatic pathology*

Serum glucose, calcium, amylase and lysosomal enzymes (according to Kit' s indication) were determined before and 1, 4, 7 d after the occurrence of acute pancreatitis. All the dogs were injected 50 uCi $^{125}$I-BSA 4 h before sacrificed on the seventh day. The $^{125}$I-BSA volume in the pancreas (/g), muscle of the right leg (/g) and blood (/ml) were tested by a r-accounter radioimmunology analyzer. The $^{125}$I -BSA index of the pancreas/muscle and pancreas/blood was measured. Fixed tissues of the pancreatic head, body, tail and the total pancreas were sectioned and the histological change was observed. Pancreatitic pathological scores (PPS) were taken by the extent of pancreas tissue edema, inflammation, hemorrhage and necrosis according to scores 1, 2, 3 and 4, and PPS of the different parts of the pancreas was determined.

### *Entero-hormone determination*

Twenty-four hours after the occurrence of acute pancreatitis, serum was used to determine the CCK and SEC (Peninsula Laboration, Inc.USA), and gastrin (Beijing Furui Bioengineer Co.) at the same time before and 30, 60, 120 and 180 min after PN or EIN. The former two samples were measured by competitive ELISA. The serum gastrin was measured by competitive binding RIA. The serum amylase, pancreaolipase and pancreatic juice, electrolytes ($HCO_3^-$, $Cl^-$, $Na^+$ and $K^+$) were determined by a 1 600 full-automatic biochemical analyser.

### *Statistical analysis*

These data were expressed as means ± SEM, and comparison between two groups was made using $\chi^2$ analysis of variance. A $P$ value less than 0.05 was considered statistically significant.

## RESULTS

### *Changes of serum glucose, calcium, amylase and lysosomal enzymes*

After the acute pancreatitis model was prepared, serum glucose level in PN group was higher as compared with that in EIN group during the whole experimental period. Serum calcium was markedly decreased after acute pancreatitis was induced, and there was no difference in the changes of serum calcium in the latter days between two groups. Serum amylase and lysosomal enzymes (LE) increased and gradually decreased later during the experimental period, there was no difference between two groups ($P$>0.05) (Table 1).

**Table 1** Effect of different nutrition support methods on serum glucose, calcium amylase and lysosomal enzymes

| Groups | Time | Glu (umol/L) | Ca (mmol/L) | Amylase (SU) | LE(U) |
|---|---|---|---|---|---|
| PN group | Pre-AP | 4.0±0.5 | 2.50±0.10 | 328±96 | 22±10 |
| | 30 min | 8.3±0.8[a] | 2.42±0.11 | 636±100[a] | 53±11 |
| | 1 d | 9.7±0.7[a] | 2.35±0.09 | 1 689±298[a] | 68±17 |
| | 4 d | 10.3±1.0[a] | 2.36±0.13 | 1 150±416[a] | 45±14 |
| | 7 d | 9.80±1.1[a] | 2.20±0.11 | 1 060±260[a] | 47±13 |
| EIN group | Pre-AP | 4.7±0.8 | 2.34±0.16 | 400±110 | 26±9 |
| | 30 min | 8.4±1.0[a] | 2.39±0.11 | 734±164 | 64±17 |
| | 1 d | 8.9±0.8[a] | 2.31±0.09 | 1 289±439[b] | 71±13 |
| | 4 d | 6.7±1.0 | 2.34±0.15 | 1 215±416 | 50±19 |
| | 7 d | 6.6±0.7 | 2.30±0.09 | 1 169±362 | 52±20 |

[a]$P$<0.05, *vs* pre-SAP, [b]$P$<0.05, *vs* PN group, Pre-AP: before acute pancreatitis.

### *$^{125}$I-BSA index and pancreatitic pathological scores*

$^{125}$I-BSA index of pancreas/muscle and pancreas/blood in EIN group (4.22±0.18 cpm/g, 0.22±0.03 cpm/ml) and PN group (3.69±0.26 cpm/g, 0.17±0.02 cpm/ml) did not reach statistical difference ($P$>0.05). Pancreatitic pathological scores (PPS) of different parts including head, body, tail and total pancreas in EIN group (1.40±0.24, 2.3±0.20, 2.1±0.20, 1.9±0.23) and PN group (1.30±0.38, 2.4±0.22, 2.2±0.22, 1.9±0.06) also did not reach statistical difference ($P$>0.05).

### *Entero-hormone, pancreatic juice and their components analysis*

**CCK** The content of CCK in EIN group was higher than that in PN group at 30 and 60 min ($P$<0.05). There was no difference before and after nutrition liquid infusion in PN group (Table 2).

**Table 2** Changes of serum CCK at different times (ng/ml)

| Group | 0 min | 30 min | 60 min | 120 min | 180 min |
|---|---|---|---|---|---|
| PN group | 0.24±0.02 | 0.17±0.01 | 0.23±0.01 | 0.23±0.02 | 0.33±0.03 |
| EIN group | 0.22±0.02 | 0.29±0.06[a] | 0.33±0.05[a] | 0.29±0.03[a] | 0.31±0.05 |

[a]$P$<0.05 *vs* PN group.

**Table 3** Changes of serum SEC at different times (ng/ml)

| Group | 0 min | 30 min | 60 min | 120 min | 180 min |
|---|---|---|---|---|---|
| PN group | 0.33±0.03 | 0.65±0.14 | 0.74±0.17 | 0.61±0.20 | 0.56±0.23 |
| EIN group | 0.35±0.06 | 0.61±0.17 | 0.88±0.25[a] | 0.64±0.13 | 0.61±0.25 |

[a]$P$<0.05 *vs* PN group.

**SEC** SEC after infusion of nutrition liquid was higher than that before infusion of nutrition liquid ($P<0.05$), but it did not reach statistical difference between two groups at 30, 120 and 180 min ($P>0.05$). SEC only at 60 min in EIN group was higher than that in PN group (Table 3).

**Gastrin** The changes of gastrin in the PN group did no reach statistical difference before and after nutrition liquid infusion. Gastrin in EIN group gradually increased, and was higher than that in PN group at 120 and 180 min ($P<0.05$) (Table 4).

**Table 4** Changes of serum gastrin at different times (pg / ml)

| Group | 0 min | 30 min | 60 min | 120 min | 180 min |
|---|---|---|---|---|---|
| PN group | 12.5±3.7 | 13.9±4.1 | 20.8±5.1 | 16.4±5.2 | 17.6±6.3 |
| EIN group | 12.2±3.2 | 14.4±4.6 | 20.7±5.5 | 24.2±6.3[a] | 25.3±6.5[a] |

[a]$P<0.05$ *vs* PN group.

### *Pancreatic secretion and its component analysis*

The changes of pancreatic juice, amylase, pancreatolipase, $HCO_3^-$, $Cl^-$, $Na^+$ and $K^+$ did not reach statistical difference between two groups (Table 5).

**Table 5** Changes of pancreatic juice, amylase, pancreatolipase, $HCO_3^-$, $Cl^-$, $Na^+$ and $K^+$

| Component | PN group | EIN group |
|---|---|---|
| Pancreatic juice (ml) | 6±1 | 9±3 |
| Amylase (U / L) | 6 717±540 | 7 121±670 |
| pancreatolipase (U / L) | 629±78 | 661±101 |
| $HCO_3^-$ (mmol / L) | 17±3 | 22±4 |
| $Cl^-$(mmol / L) | 117±11 | 126±9 |
| $Na^+$(mmol / L) | 133±21 | 147±17 |
| $K^+$(mmol / L) | 4.1±1.0 | 5.7±1.4 |

## DISCUSSION

Autodigestion of the pancreas is the main mechanism of acute pancreatitis. The conception of pancreatic rest stems from the belief that stimulation of pancreatic exocrine function in patients with acute pancreatitis releases large quantities of proteolytic enzymes that result in autodigestion of the inflammatory pancreas and peripancreatic tissues, causing a deterioration in the patient' s condition. The presence of food in the stomach and duodenum elicits gastropancreatic and duodenopancreatic reflexs that result in stimulation of pancreatic exocrine secretions. Therefore, traditionally, enteral nutrition could be adopted after parenteral nutrition support for over 2-3 weeks. That means to keep the pancreas in rest and rehabilitation for a long time. However, these effects are not so pronounced when nutrients are delivered directly into the jejunum[11-18].

Heidenhain in 1 875 first demonstrated the effect of vagal and entero- hormone stimulation on pancreatic secretion, in which hormones played a more important role than vagal stimulation. There were three classic phases, namely cephalic, gastric and intestinal phases of digestion that describe the response of the pancreas to a meal. The hormones served a major function in mediating pancreatic exocrine secretion[16,19]. Normally, during the cephalic and gastric phases, oral nutrients can stimulate the release of gastric acid, duodenum juice and pancreatic enzyme, and activation of proteins and peptide in the nutrients commences after the peptidase enters the duodenum, where mucosal enterokinase cleaves trypsinogen to trypsin, leaving trypsin to further activate the other peptidases, and then stimulates entero-hormones secretion such as cholecystokinin (CCK), secretin (SEC) and gastrin to increase pancreatic secretion. It is known that CCK and SEC are synthesized in the mucosal I and S cells of the crypts of Liberkuhn in the proximal small intestine and released in the presence of luminal acid and bile. The gastic G cell product, gastrin, which serves a major function to promote gastric acid release also serves as a weak stimulator of pancreatic enzyme secretion. CCK is one of the most important entro-hormones known to stimulate pancreatic enzyme secretion. Some authors also found that avoidance of cephalic, gastric and duodenual stimuli by jejunal tube feeding did not result in pancreatic stimulation. They concluded that bypassing the stomach, and minimizing acid secretion, played an important role in keeping the pancreas at rest[16,21,22].

Some authors[20-25] described an experience of early enteral nutrition in severe acute pancreatitis using nasoenteral feeding. No patients developed relapse, hypertri-glyceridaemia or abnormalities of liver function, indicating that jejunal feeding can be used safely in acute pancreatitis without reactivation of the inflammatory process[26-32]. Our experimental results showed that the changes of serum glucose, calcium, and amylase did not reach statistical difference between two groups. The serum lysosomal enzymes is believed to be the gold standard for reflecting the extent of pancreatic tissue necrosis and inflammation and more attention has been paid to them in international medicine. Once the pancreatic tissue necrosis stoped, the volume of systemic lysosomal enzymes discharging from pancreatitis tissue would be attenuated. Our results indicated that serum lysosomal enzymes was markedly increased after acute pancreatitis was induced, but did not reach statistical difference between two groups. In addition, the $^{125}$I-BSA index of pancreas/muscle and pancreas/blood reflected the permeability of the pancreas microcirculation. If this $^{125}$I-BSA index decreased, microvessal permeability would be improved. Once the $^{125}$I- BSA index in pancreatic tissue increased, the microvessal permeability elevated and deteriorated pancreatitis. Our study showed that administration of EIN did not increase the content of serum lysosomal enzymes, and deteriorate the course of acute pancreatitis. As to the PPS in different parts of the pancreas, there was no difference between EIN group and PN group. Kalfarentzos *et al*[17], reported that EIN was well tolerated following acute pancreatitis, and was of comparable efficacy to PN. In fact, EIN did not deteriorate pancreatitic pathology, and might be safely adopted in dogs with acute pancreatitis[26-32].

Normally, it is known that secretion of CCK, SEC and gastrin is mainly located in the duodenum and jejunum. The number of CCK-produced cells is 11-30 per square millimeter both in duodenum and in proximal jejunum, and their amount is 52.5±8 pmol/g, and 26±5 pmol/g respectively; The number of pancreatic SEC-produced cells is >31 in duodenum, 1-10 in proximal jejunum per square millimeter respectively, and their amount is 73±7 pmol/g and 32±4 pmol/g. The number of gastrin CCK-produced cells is >31 in gastric antrum, 11-30 in duodenum and 1-10 in proximal jejunum per square millimeter respectively, and their amount is 2 342±14 pmol/g, 1 397±192 pmol/g, 190±24 pmol/g, respectively. Therefore, the secretory locations of entero-hormones are mostly in gastric antrum, duodenum and proximal jejunum, and less in the distal jejunum. Theoretically, if the nutrients were infused from proximal jejunum to distal jejunum, it would decrease stimulatory activation of pancreatic secretion to a minimum degree[16].

To further study the possibility of EIN stimulated entero-hormones and pancreatic juice release, we observed the effects of different nutrition support methods on the CCK, SEC, gastrin and pancreatic secretion and their components. Our results suggested that the serum CCK increased at 60 min after nutrient was infused in EIN group as compared with PN group.

It was interesting that SEC was elevated after nutrition infusion in both groups, but it was only higher at 60 min in EIN group than in PN group. The serum gastrin was gradually increased in EIN group at 120 and 180 min as compared with PN group. Based on pancreatic juice and its component analysis, our results suggested that the amount of pancreatic juice was higher in EIN than in PN group. But the changes of the amylase, pancreatolipase and electrolytes were not significant. The study suggested that EIN indeed stimulated entero- hormones secretion at some degrees, but did not increase enzyme-proetin and pancreatic juice secretion. The reason was not clear, maybe due to the fact that the pancreatic acinar cells swelling, hemorrhage and necrosis decreased the physiological efficiency of entero-hormones by altering the membrane receptor number and activity.

In recent years, effect of human EIN or oral feeding on the natural course and entero-hormones secretion was seldom reporte. It was well tolerable, feasible and desirable as TPN in the management of acute pancreatitis, but it failed to reveal any detrimental effect on the clinical pathologic features of AP, and increase pancreatic secretion. Therefore, EIN can contribute to the study of pancreatic natural course, and may play an important role in keeping the pancreas at rest, bypassing the stomach, and minimizing acid secretion.

## REFERENCES


1 **Kalfarentzos FE**, Karavias DD, Karatzas TM, Alevizatos BA, Androulakis JA. Total parenteral nutrition in severe acute pancreatitis. *J Am Coll Nutr* 1991; **10**: 156-162

2 **Buchman AL**, Moukarzel AA, Bhutta S, Belle M, Ament ME, Eckhert CD, Hollander D, Gornbein J, Kopple JD, Vijayaroghavan SR. Parenteral nutrition is associated with intestinal morphologic and functional changes in humans. *JPEN* 1995; **19**: 453-460

3 **Qin HL**, Su ZD, Hu LG, Ding ZX, Lin QT. Effect of early intrajejunal nutrition on pancreatic pathological features and gut barrier function in dogs with acute pancreatitis. *Clin Nutr* 2002; **21**: 469-473

4 **Mitchell RM**, Byrne MF, Baillie J. Pancreatitis. *Lancet* 2003; **26**: 1447-1455

5 **Ammori BJ**. Gut barrier dysfunction in patients with acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 411-412

6 **McClave SA**, Greene LM, Snider HL, Makk LJ, Cheadle WG, Owens NA, Dukes LG, Goldsmith LJ. Comparison of the safety of early enteral vs parenteral nutrition in mild acute pancreatitis. *JPEN* 1997; **21**: 14-20

7 **Vison N**, Hecketsweiler P, Butel J, Bernier JJ. Effect of continuous jejunal perfusion of elemental and complex nutritional solutions on pancreatic enzyme secretion in human subjects. *Gut* 1978; **19**: 194-198

8 **Maillet P**. Enteral nutrition by alimentation jejunostomy in 11 cases of severe acute pancreatitis. In controversies in Acute pancreatitis. *Hollender LF(ed.). Beilin* 1982: P293

9 **Lobo DN**, Memon MA, Allison SP, Rowlands BJ. Evolution of nutritional support in acute pancreatitis. *Br J Surg* 2000; **87**: 695-707

10 **Windsor AC**, Kanwar S, Li AG, Barnes E, Guthrie JA, Spark JI, Welsh F, Guillou PJ, Reynolds JV. Compared with parenteral nutrition,enteral feeding attenuates the acute phase response and improves disease severity in acute pancreatitis. *Gut* 1998; **42**: 431-435

11 **Abou-Assi S**, Craig K, O'Keefe SJ. Hypocaloric jejunal feeding is better than total parenteral nutrition in acute pancreatitis: results of a randomized comparative study. *Am J Gastroenterol* 2002; **97**: 2255-2262

12 **Olah A**, Pardavi G, Belagyi T, Nagy A, Issekutz A, Mohamed GE. Early nasojejunal feeding in acute pancreatitis is associated with a lower complication rate. *Nutrition* 2002; **18**: 259-262

13 **Chen QP**. Enteral nutrition and acute pancreatitis. *WJG* 2001; **7**: 185-192

14 **Hallay J**, Kovacs G, Szatmari K, Szatmari K, Bako A, Szentkereszty Z, Lakos G, Sipka S, Sapy P. Early jejunal nutrition and changes in the immunological parameters of patients with acute pancreatitis. *Hepatogastroenterology* 2001; **48**: 1488-1492

15 **Eatock FC**, Brombacher GD, Steven A, Imrie CW, McKay CJ, Carter R. Nasogastric feeding in severe acute pancreatitis may be practical and safe. *Int J Pancreatol* 2000; **28**: 23-29

16 **Qin HL**, Su ZD, Hu LG, Ding ZX, Lin QT. Effect of early intrajejunal nutrition on secretion of entero-hormone and its efficiency in acute pancreatitic dogs. *Chang Wai Chang Nei Ying Yang* 2002; **9**: 193-195

17 **Kalfarentzos F**, Kehagias J, Mead N, Kokkinis K, Gogos CA. Enteral nutrition is superior to parenteral nutrition in severe acute pancreatitis:results of a randomized prospective trial. *Br J Surg* 1997; **84**: 1665-1669

18 **Qin HL**, Su ZD, Ding ZX, Lin QT. Effects of enteral nutrition on uptake of amino acid and enzyme-protein synthesis of pancreatic acinar cell in acute pancreatic dogs. *Zhonghua Waike Zazhi* 2003; **41**: 146-149

19 **Takacs T**, Hajnal F, Nemeth J, Lonovics J, Pap A. Stimulated gastrointestinal hormone release and gallbladder contraction during continuous jejunal feeding in patients with pancreatic pseudocyst is inhibited by octreotide. *Int J Pancreatol* 2000; **28**: 215-220

20 **MacFie J**. Enteral versus parenteral nutrition. *Br J Surg* 2000; **87**: 1121-1122

21 **Neoptolemos JP**, Raraty M, Finch M, Sutton R. Acute pancreatitis: the subatantial human and financial costs. *Gut* 1998; **42**: 886-891

22 **Al-Omran M**, Groof A, Wilke D. Enteral versus parenteral nutrition for acute pancreatitis. *Cochrane Database Syst Rev* 2003; **3**: CD002837

23 **Austrums E**, Pupelis G, Snippe K. Postoperative enteral stimulation by gut feeding improve outcomes in severe acute pancreatitis. *Nutrition* 2003; **19**: 487-491

24 **Powell JJ**, Murchison JT, Fearson KC, Ross JA, Siriwardena AK. Randomized controlled trial of the effect of early enteral nutrition on markers of the inflammatory response in predicted severe acute pancreatitis. *Br J Surg* 2000; **87**: 1375-1381

25 **McGregor CS**, Marshall JC. Enteral feeding in acute pancreatitis: just do it. *Curr Opin Crit Care* 2001; **7**: 89-91

26 **Sanabria A**. Randomized controlled trial of the effect of early enteral nutrition on markers of the inflammatory response in prevent severe acute pancreatitis. *Br J Surg* 2001; **88**: 728

27 **Eckerwall G**, Andersson R. Early enteral nutrition in severe acute pancreatitis:a providing nutrients, gut barrier protection, immunomodul or all of them? *Scand J Gastroenterol* 2001; **36**: 449-458

28 **Pupelis G**, Austrums E, Jansone A, Sprucs R, Wehbi H. Randomised trial of safety and efficacy of postoperative enteral feeding in patients with severe pancreatitis: preliminary report. *Eur J Surg* 2000; **166**: 383-387

29 **Sahin M**, Ozer S, Vatansev C, Akoz M, Vatansev H, Aksoy F, Dilsiz A, Yilmaz O, Karademir M, Aktan M. The impact of oral feeding on the severity of acute pancreatitis. *Am J Surg* 1999; **178**: 394-398

30 **Hamvas J**, Schwab R, Pap A. Jejunal feeding in necrotising acute pancreatitis- a restrospective study. *Acta Chir Hung* 1999; **38**: 177-185

31 **Tesinsky P**. Nutritional care of pancreatitis and its complication. *Curr Opin Clin Nutr Metab Care* 1999; **2**: 395-398

32 **Duerksen DR**, Bector S, Yaffe C, Parry DM. Does jejunal feeding with a polymeric immune-enhancing for increase pancreatic exocrine output as compared with TPN: case report. *Nutrition* 2000; **16**: 47-49


**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2274-2277
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Protective effects of transplanted and mobilized bone marrow stem cells on mice with severe acute pancreatitis

Hui-Fei Cui, Zeng-Liang Bai

**Hui-Fei Cui,** Biochemical and Biotechnological Institute of Materia Medica, Pharmaceutical College of Shandong University, Jinan 250012, Shandong Province, China
**Zeng-Liang Bai,** College of Life Sciences, Shandong University, Jinan 250100, Shandong Province, China
**Correspondence to:** Hui-Fei Cui, Associate Professor, Biochemical and Biotechnological Institute of Materia Medica, Pharmaceutical College of Shandong University, Jinan 250012, Shandong Province, China. cuihuifei@sdu.edu.cn
**Telephone:** +86-531-8380288 **Fax:** +86-531-8380288
**Received:** 2003-09-01 **Accepted:** 2003-09-15

## Abstract

**AIM:** To evaluate the protective effects of transplanted and mobilized bone marrow stem cells (BMSCs) on mice with severe acute pancreatitis (SAP) and to probe into their possible mechanisms.

**METHODS:** A mouse model of SAP induced by intraperitoneal injections of L-arginine was employed in the present study. Two hundred female Balb/c mice weighing 18-22 g were randomly assigned into 4 groups. Group A was the stem cell mobilized group treated by injection of granulocyte-colony stimulating factor (G-CSF) into mice for 4 days at a dose of 40 μg· $kg^{-1}$· $d^{-1}$ before induction of SAP. Group B was the group of BMSCs transplantation, in which the mice were given the isolated BMSCs via the tail vein 4 days prior to induction of SAP. Group C served as the model control and only SAP was induced. The mice without induction of SAP in group D acted as the normal control. At the time of animal sacrifice at 24, 48 and 72 h after induction of SAP, blood samples were obtained and prepared to detect serum amylase, while the abdominal viscera were examined both grossly and microscopically for the observation of pathological changes.

**RESULTS:** The mortality of mice in the model control, groups A and B was 34 %, 8 % and 10 % respectively within 72 h after induction of SAP. The serum level of amylase in the model control was significantly increased at all time points after induction of SAP as compared with that of the normal control ($P$<0.05-0.01). When the mice were pretreated with BMSCs' transplantation or G-CSF injection, their serum level of amylase was significantly reduced at 48 h and 72 h after induction of SAP in comparison with that of the model control ($P$<0.05-0.01). In accordance with these observations, both gross and microscopic examinations revealed that the pathological changes of SAP in mice pretreated with BMSCs transplantation or G-CSF injection were considerably attenuated as compared with those in the model control at all observed time points.

**CONCLUSION:** Both transplanted allogenic and mobilized autologous BMSCs can protect mouse pancreas from severe damage in the process of SAP.

Cui HF, Bai ZL. Protective effects of transplanted and mobilized bone marrow stem cells on mice with severe acute pancreatitis. *World J Gastroenterol* 2003; 9(10): 2274-2277
http://www.wjgnet.com/1007-9327/9/2274.asp

## INTRODUCTION

Severe acute pancreatitis (SAP) is a life-threatening disease with a mortality rate of 20 to 30 percent[1,2]. Despite recent improvements in our understanding of the disease process and the development of a range of supportive measures, today's treatment approaches for SAP are still less than ideal. It has been demonstrated recently that multipotent somatic stem cells in adult bone marrow can exhibit tremendous functional plasticity[3-9] and reprogram in a new environmental tissue niche to give rise to cell lineages specific for the new organ site. Stem cells from bone marrow, autologous or allogenic, have been used to treat myocardial infarction[10-13], hepatic disease[14-20], nervous system dysfunction[21-25] and severe autoimmune diseases[26, 27]. However, there have been fewer reports concerned about the treatment of SAP with BMSCs as yet. Since the two critical determinants, tissue damage and higher level of pluripotent cells, seem to be the prerequisite for the transdifferentiation of transplanted BMSCs and G-CSF has been proved to have a great potency in mobilizing both hematopoietic stem cell (HSC) and mesenchymal stem cells (MSCs) of bone marrow, we hypothesized that the transplanted allogenic BMSCs, as well as the autologous BMSCs mobilized by G-CSF would exert a protective role in the treatment of SAP. The present study is therefore designed to verify our hypothesis in attempt to develop new protocols for the improvement of SAP therapy.

## MATERIALS AND METHODS

### *Animals and experimental protocol*

Two hundred female Balb/c mice weighing 18-22 g were randomly assigned into 4 groups according to different treatment protocols with 50 mice each. Group A was the stem cell mobilized group treated by injection of sc G-CSF into mice for 4 days at a dose of 40 μg· $kg^{-1}$· $d^{-1}$ before induction of SAP. Group B was the group of BMSCs transplantation, in which the mice were given BMSCs isolated from male mice bone marrow at a dose of $2\times10^7$ per mouse via tail vein 4 days prior to induction of SAP. Group C served as the model control and only SAP was induced. The mice in group D acted as the normal control treated only with an equal amount of saline as sc G-CSF in group A and without induction of SAP.

Mouse model of SAP was prepared in all animals except that in group D according to the scheme described elsewhere[28]. Briefly, the mice were fasted overnight but allowed to free access to water. The SAP inducer, a 20 g/L of L-arginine (Sigma) solution, was freshly prepared with saline just prior to use. In induction of SAP, the animals were injected intraperitoneally L-arginine solution at a dose of 2 g· $kg^{-1}$ twice at an interval of 1 h.

The mice in groups A, B and C were sacrificed at 24, 48 and 72 h after induction of SAP. In the meanwhile, the mice in group D were killed at the corresponding time points. At the time of animal sacrifice, blood samples were obtained and prepared to

detect serum amylase, while the abdominal viscera were examined both grossly and microscopically for the observation of pathological changes.

### *Transplantation of primary BMSCs*

Primary BMSCs to be transplanted to group B animals were isolated from donor male mice. The whole bone marrow cells were harvested by rinsing the thighbone and shankbone' s medullary cavities with cold DMEM (Gibco, Grand Island, NY) and then fractionated by density gradient centrifugation with lymphocytic separating medium. Mononuclear component, the constituent rich in BMSCs, was obtained from the interface after centrifugation at 2 500 r/min for 30 min. After repeatedly washed in cool D-Hanks solution, BMSCs were resuspended and adjusted to a cell density of $10^8$/mL with the same solution. The transplantation of primary BMSCs into group B animals was performed via the tail vein injection at a dose of 0.2 ml cell suspension per mouse 4 days prior to induction of SAP.

The transplanted stem cells were identified at the end of the experiment (72 h post SAP induction) by examining the existence of Y chromosome Sry region in recipient female mice with a PCR scheme. Briefly, the female recipients were killed by cervical dislocation and DNA samples were extracted respectively from the pancreas, bone marrow, liver and spleen. The sequence of the sense primer was 5' -ATTTATGGTG TGGTCCCG-3' and that of the antisense primer was 5' -GCTGTAAAATGCCACTCC-3' . PCR consisted of an initial denaturation step at 96 ℃ for 6 min, followed by 35 cycles at 94 ℃ for 1 min, at 52 ℃ for 1 min and at 72 ℃ for 1 min each, and a final extension at 72 ℃ for 10 min. The resulting products were analyzed by electrophoresis on a 2 % agarose gel and stained with ethidium bromide. The expected size of amplified DNA fragment was 239 base pair.

### *Statistical analysis*

All the data were expressed as $\bar{x}\pm s$. Comparisons between the means of different groups were performed using analysis of variance followed by Student' s *t*-test. $P<0.05$ was selected to be the level of statistical significance.

## RESULTS

### *Animal mortality*

The mortality of mice in the model control was 34 % within 72 h after induction of SAP, which was considerably decreased in the mices pretreated with BMSCs' transplantation or sc G-CSF injection. The mortality of mice in groups A and B was 8 % and 10 % respectively.

**Table 1** Changes of serum amylase activity after SAP induction ($\bar{x}\pm s$, $n$=10)

| Group | Serum amylase activity (μkat/L) | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| A | 25.3±4.8 | 40.8±7.4[b,c] | 27.6±6.2[a,c] |
| B | 25.9±4.9 | 40.7±6.7[b,c] | 27.3±6.3[a,c] |
| C | 46.1±12.8[a] | 78.4±15.8[b] | 50.2±13.3[a] |
| D | 17.2±6.2 | 16.9±2.7 | 19.0±3.4 |

[a]$P<0.05$, [b]$P<0.01$ *vs* group D; [c]$P<0.05$ *vs* group C.

### *Alterations of serum amylase*

The serum level of amylase was significantly increased in the mice of model control at all time points after induction of SAP as compared with that of the normal control ($P<0.05$-0.01). When the mice were pretreated with BMSCs transplantation or G-CSF injection, their serum level of amylase was significantly reduced at 48 h and 72 h after induction of SAP as compared with that of the model control ($P<0.05$-0.01), although the amylase value was still significantly higher than that of the normal control ($P<0.05$, Table 1).

### *Pathological changes*

Grossly, a typical appearance of SAP changes was observed in animals of the model control. These pathological changes were progressively aggravated after SAP induction and manifested in a time- dependent manner. Twenty-four hours after L-arginine injection, the pancreas was sprinkled with hemorrhagic spots and focal necrosis with a dark-color appearance. At the same time, a little bloody ascites was noted in the abdominal cavity. At 48 h, different sizes of more hemorrhagic and necrotic focus appeared on the pancreatic surface with the increment of bloody ascites. Seventy-two hours after induction SAP, the pancreatic necrosis and blood ascites were even more prominent with saponification of fatty tissue around. Massive necrosis of multiple organs such as the intestine, lungs and kidneys was found in the mice died from SAP (Figure 1).

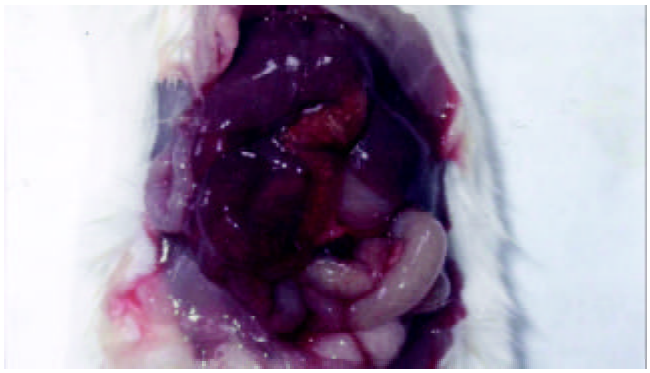


**Figure 1** Gross appearance of abdominal cavity in SAP model control.

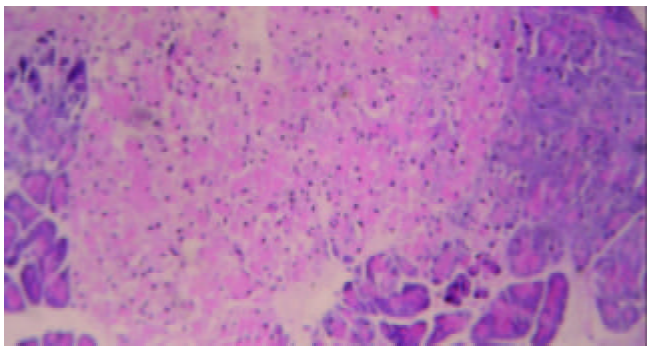


**Figure 2** Pathological changes of pancreas at 24 h post SAP induction in mice of model control (HE, ×200).

Attenuated pathological lesions were noted both in group A and in group B mice, which were manifested mainly as minor pancreatic edema and congestion with less bloody or non-bloody ascites generated, no fat saponification and necrosis were observed.

In the microscopic examination, various degrees of pancreatic impairments were found in all mice injected with L-arginine. In mice of the model control, pancreatic congestion, interstitial edema, disorganized lobular architecture, as well as the focal hemorrhage and necrosis appeared at 24 h after induction of SAP, along with an obvious mesenchymal infiltration by inflammatory cells (Figure 2). The changes above were even more aggravated at 48 h and large areas of

coagulation necrosis occurred in the pancreatic parenchyma acompanied by the destroyed lobular architecture and massive inflammatory cell infiltration at the time of 72 h after induction of SAP (Figure 3).

In contrast, these pathological changes were obviously attenuated in mice pretreated with BMSCs' transplantation or G-CSF injection (Figures 4, 5). Most lobular architecture in these animals remained recognizable with alleviated hemorrhage, necrosis and infiltration of inflammatory cells, whereas some focal necrosis remained in the periphery of pancreas.

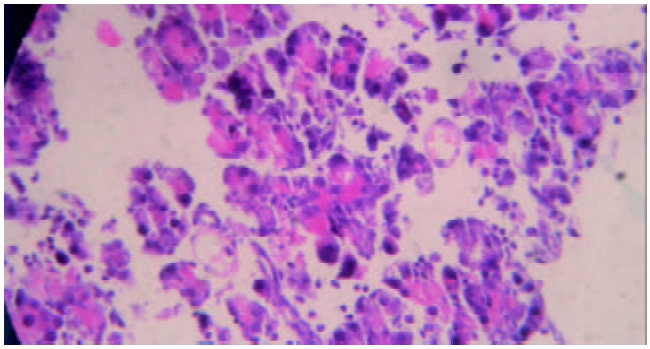


**Figure 3** Pathological changes of pancreas at 72 h post SAP induction in mice of model control (HE, ×200).

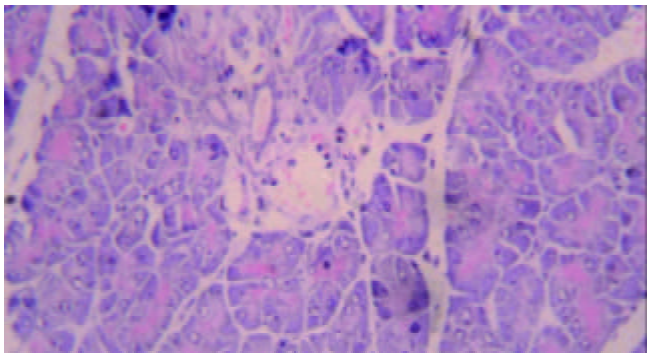


**Figure 4** Microscopic changes of pancreas at 72 h after induction of SAP in mice pretreated with G-CSF (HE, ×200).

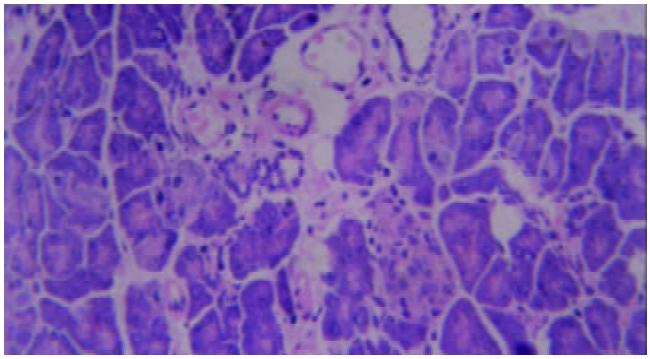


**Figure 5** Microscopic changes of pancreas at 72 h post SAP induction in mice pretreated with BMSCs transplantation (HE, ×200).

### *Identification of transplanted BMSC*

All sampled tissues from the pancreas, liver, spleen and bone marrow of the female recipients were demonstrated harboring a DNA fragment, 239 bp in length, of Y chromosome Sry region of donor mice, indicating that the engrafted BMSCs could migrate and survive in the impaired pancreas of SAP animals (Figure 6).

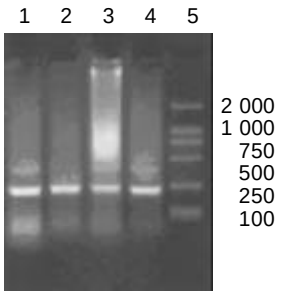



**Figure 6** PCR products of Y chromosome Sry region gene existed in some organs of recipient female mice after male BMSCs transplantation demonstrated by agarose gel electrophoresis. Lane 1: pancreas, Lane 2: liver, Lane 3: liver, Lane 4: spleen, Lane 5: DNA marker.

## DISCUSSION

The management of SAP has changed significantly over the past 20 years. In contrast to surgical intervention, there is now a strong tendency towards a more conservative therapy. The established treatment of SAP included aggressive fluid resuscitation, oxygen supplementation, prophylactic use of antibiotics, enteral feeding and intensive care support of any failing organ or system[29]. Nevertheless, the fact that these therapeutic modalities did not aim directly at the etiological factors made the treatment lack of specificity. Therefore, it remains a great challenge for the improvement of SAP therapy in the daily clinical practice. Recently, it has been shown that somatic stem cells were capable of regenerating injured tissue and improving the functions of involved organs[6,30-32], which might be used also as a new potential therapeutic modality for SAP treatment.

Bone marrow is an ideal source of stem cells. The multidirectional differentiation potential of BMSCs has been proved to be more than what we expected[32]. It has been reported that BMSCs could transdifferentiate into a variety of cell types derived from three embryonic layers such as endoblast-derived hepatic cells and lung alveolar epithelial cells, mesoblast-derived kidney and muscle cells, and ectoblast-derived neurons and epidermic cells. Furthermore, some authors have shown that bone marrow might serve as an extra-pancreatic hideout for the pancreatic stem cells[33,34] that contributed to the adult islet neogenesis. Transplantation with bone marrow cells has been used in several animal experiments for the treatment of types I and II diabetes mellitus, which has been achieved some promising results. All these facts indicate that BMSCs harbor a biological basis which can be used as an excellent candidate for SAP therapy.

In the present study, we observed the protective effects of BMSCs transplantation in a mouse model of SAP. It showed that a considerably reduced mortality and serum amylase activity, as well as obviously attenuated pancreatic pathological changes in the animals treated by transplantation of BMSCs or by injection of G-CSF, which presented a striking contrast to those of the model control. Regarding the mechanisms involved, these protective effects might be mediated by the rehabilitative action of BMSCs, which was partially supported by the existence of transplanted stem cells in the assaulted pancreas with a PCR scheme. The transplanted BMSCs might be ' tweaked' from the peripheral blood circulation to the injured tissues whenever SAP took place, where they exerted the function of pancreatic stem cells to regenerate the destroyed cells. As a result, gradual necrosis of impaired pancreas was effectively prevented. On the other hand, critical ill conditions such as SAP can usually caused serious injuries to multiple organs or systems, leading to a poor capability for the tissue repair. At

this time, transplanted stem cells in peripheral circulation were no doubt helpful for regeneration of injured tissues[38]. However, the direct evidence of BMSCs transdifferentiating to pancreatic cells should be further demonstrated by *in situ* hybridization and immuneohistochemistry.

An important phenomenon that deserved to note in the present study was the protective effects of autologous BMSCs mobilized by G-CSF injection in this model of SAP, whose efficiency was similar to that of alligeneic BMSCs transplantation. It implies that BMSCs, no matter autologous or allogenic, could get to and reside in the impaired tissue via blood circulation to repair the dysfunctional organs. Recently, Jensen and his associates[31] have shown that a normal physiological process of tissue regeneration and repair could be achieved by *in situ* mobilization of autologous stem cells from the bone marrow. Through the stimulation of normal stem cell migration, therapeutic benefits could be achieved with less invasive regimens than the removal and re-injection of stem cells. In this way, some obstacles in the allograft such as rejection and shortness of tissue donor supply would be also overcome easily. However, there were fewer BMSCs with weaker expansion and differentiation capacity in the peripheral circulation. Once mobilized by G-CSF, BMSCs in the peripheral circulation were estimated to be increased about 250 times higher than the baseline, along with an enhanced expansion and differentiation capacity. Thereby, autologous BMSCs transplantation could be performed safely and conveniently. Further research should be focused on the more efficient mobilization of autologous BMSCs to attract them to the injured region for tissue repair. This cell-restoring therapy may serve as a new modality in the future management of SAP and other serious diseases.

## REFERENCES


1 **Ponette J,** Wilmer A. Update on the management of acute severe pancreatitis. *Acta Clin Belg* 2001; **56:** 135-145
2 **Bank S,** Singh P, Pooran N, Stark B. Evaluation of factors that have reduced mortality from acute pancreatitis over the past 20 years. *J Clin Gastroenterol* 2002; **35:** 50-60
3 **Anderson DJ**, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? *Nat Med* 2001; **7**: 393-395
4 **Tao H,** Ma DD. Evidence for transdifferentiation of human bone marrow-derived stem cells: recent progress and controversies. *Pathology* 2003; **35:** 6-13
5 **Goodell MA.** Stem-cell "plasticity": befuddled by the muddle. *Curr Opin Hematol* 2003; **10:** 208-213
6 **Prockop DJ.** Further proof of the plasticity of adult stem cells and their role in tissue repair. *J Cell Biol* 2003; **160:** 807-809
7 **Zubair AC**, Silberstein L, Ritz J. Adult hematopoietic stem cell plasticity. *Transfusion* 2002; **42:** 1096-1101
8 **Avots A,** Harder F, Schmittwolf C, Petrovic S, Muller AM. Plasticity of hematopoietic stem cells and cellular memory. *Immunol Rev* 2002; **187:** 9-21
9 **Forbes SJ,** Vig P, Poulsom R, Wright NA, Alison MR. Adult stem cell plasticity: new pathways of tissue regeneration become visible. *Clin Sci* 2002; **103:** 355-369
10 **Jackson KA,** Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest* 2001; **107**: 1395-1402
11 **Orlic D**, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infracted myocardium. *Nature* 2001; **410**: 701-705
12 **Strauer BE**, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P. Repair of infracted myocardium by auotologous intracoronary mononuclear bone marrow cells transplantation in humans. *Circulation* 2002; **106**: 1913-1918
13 **Kocher AA**, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblast prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001; **7**: 430-436
14 **Lagasse E.** Purified hematopoietic stem cells can differentiate to hepatocytes *in vivo*. *Nature Med* 2000; **6**: 1229-1234
15 **Theise ND,** Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, Krause DS. Derivation of hepatocyte from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* 2000; **31**: 235-240
16 **Theise ND**. Liver from bone marrow in human. *Hepatology* 2000; **32**: 11-16
17 **Okumoto K,** Saito T, Hattori E, Ito JI, Adachi T, Takeda T, Sugahara K, Watanabe H, Saito K, Togashi H, Kawata S. Differentiation of bone marrow cells into cells that express liver-specific genes *in vitro*: implication of the Notch signals in differentiation. *Biochem Biophys Res Commun* 2003; **304:** 691-695
18 **Petersen BE**, Bowen WC, Patrene KD, Mars WM, Sulivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284:** 1168-1170
19 **Austin TW,** Lagasse E. Hepatic regeneration from hematopoietic stem cells. *Mech Dev* 2003; **120:** 131-135
20 **Gao ZH,** McAlister V, Williams G. Repopulation of liver endothelium by bone marrow-derived cells. *Lancet* 2001; **357:** 932-933
21 **Woodbury D**, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000; **61**: 364-370
22 **Prockop DJ,** Azizi SA, Colter D, Digruolamo C, Kopen G, Phinney DG. Potential use of stem cells from bone marrow to repair the extracellular matrix and the central nervous system. *Biochem Soc Trans* 2000; **28**: 341-345
23 **Kopen G**, Prockop D, Phinney D. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A* 1999; **96:** 10711-10716
24 **Brazelton TR,** Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* 2000; **290:** 1775-1779
25 **Hess DC,** Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells After stroke. *Stroke* 2002; **33**: 1362-1368
26 **Ikehara S.** Bone marrow transplantation: a new strategy for intractable diseases. *Drugs Today* 2002; **38**: 103-111
27 **Marmont AM**. Stem cell transplantation for severe autoimmune diseases: progress and problems. *Haematologica* 1998; **83:** 733-743
28 **Czako L**, Takacs T, Varga IS, Hai DQ, Tiszlavicz L, Hegyi P, Mandi Y, Matkovics B, Lonovics J. The pathogenesis of L-arginine-induced acute necrotizing pancreatitis: inflammatory mediators and endogenous cholecystokinin. *J Physiol Paris* 2000; **94**: 43-50
29 **Zulewski H,** Abraham EJ, Gerlach MJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 2001; **50:** 521-533
30 **Poulsom R,** Alison MR, Forbes SJ, Wright NA. Adult stem cell plasticity. *J Pathol* 2002; **197**: 441-456
31 **Jensen GS**, Drapeau C. The use of in situ bone marrow stem cells for the treatment of various degenerative diseases. *Med Hypotheses* 2002; **59:** 422-428
32 **Krause DS,** Theise ND, Collector ML, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001; **105:** 369-377
33 **Ianus A,** Holz GG, Theise ND, Hussain MA. *In vivo* derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest* 2003; **111**: 843-850
34 **Lee VM,** Stoffel M. Bone marrow: an extra-pancreatic hideout for the elusive pancreatic stem cell? *J Clin Invest* 2003; **111**: 799-801


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2278-2283
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Biomechanical properties of ileum after systemic treatment with epithelial growth factor

Jian Yang, Jing-Bo Zhao, Yan-Jun Zeng, Hans Gregersen

**Jian Yang, Yan-Jun Zeng,** Biomedical Engineering Center, Beijing Polytechnic University, Beijing,100022, China
**Jian Yang, Jing-Bo Zhao, Hans Gregersen,** Center for Sensory-Motor Interaction, Aalborg University and Department of Medicine M and Department of Surgery A, Aalborg Hospital, Denmark
**Supported by** Karen Elise Jensens Foundation and the Danish Technical Research Council
**Correspondence to:** Hans Gregersen, M.D.Sci., Center for Sensory-Motor Interaction, Aalborg University, Fredrik Bajers Vej 7D-3, DK-9200 Aalborg, Denmark. hag@smi.auc.dk
**Telephone:** +45-96358843 **Fax:** +45-98133060
**Received:** 2002-11-26 **Accepted:** 2003-02-26

## Abstract

**AIM:** Systemic treatment with epidermal growth factor (EGF) leads to growth of all parts of the small intestine in normal functioning rats. In this study, we investigated the effect of this growth process on morphometric and biomechanical parameters of ileum.

**METHODS:** Rats were treated with EGF (150 μg· $kg^{-1}day^{-1}$) or placebo via osmotic minipumps for 2, 4, 7, and 14 days. A segment of ileum was removed. The morphology at no-load state and zero-stress state was measured and passive biomechanical properties were assessed using a biaxial test machine (combined inflation and axial stretching).

**RESULTS:** The ileum weight increased after EGF administration. After 4 days' EGF treatment, the wall thickness was increased. Significantly smaller inner perimeters were seen in 4 day and 7 day EGF treatment groups. The opening angle and residual strain began to increase after 7 days' EGF treatment. Wall stiffness, evaluated from the stress-strain curves, showed a continuous decrease in circumferential direction during the first 7 days' EGF treatment. The longitudinal stiffness increased during the first 7 days. The stress-strain curves for both circumferential and longitudinal direction tended to shift back to normal 14 days after starting EGF administration.

**CONCLUSION:** EGF can cause significant changes both in the morphology and in the passive mechanical properties of the rat ileum.

Yang J, Zhao JB, Zeng YJ, Gregersen H. Biomechanical properties of ileum after systemic treatment with epithelial growth factor. *World J Gastroenterol* 2003; 9(10): 2278-2283
http://www.wjgnet.com/1007-9327/9/2278.asp

## INTRODUCTION

Epidermal growth factor (EGF) is a peptide growth factor belonging to the EGF family[1]. Throughout the last decade, the pharmacological potential of systemic treatment of EGF has been explored[2]. In the gastrointestinal tract, EGF causes growth, inhibits gastric acid secretion, and leads to changes in electric potentials, enzyme activity and transport of amino acids[3-6]. Due to the pronounced effects on intestinal mucosa, the therapeutic potential of systemic treatment of EGF has been explored in animal experiments. For example it has been shown that EGF accelerates adaptive growth of the small intestine in different species[7-10].

Only few studies have addressed the effect of systemic treatment of EGF on the normal small intestine. In a series of studies, we have examined its effect on the morphological and biomechanical properties of the small intestine. Vinter-Jensen, *et al*[11] used impedance planimetry to examine the tension-strain properties of the intestinal wall after EGF treatment and found that the circumferential wall stiffness decreased during EGF treatment. However, their study had limitations because the circumferential strain was referenced to the no-load state rather than to the zero-stress state and the mechanical properties were only studied for the circumferential direction.

The present study was to investigate the time-dependent effect of systemic treatment with EGF on the passive biomechanical properties of the small intestine. Segments obtained from the ileum were tested in a biaxial mechanical test machine that renders possible simultaneous inflation and longitudinal stretch. Stress and strain were computed and material constants were derived for the circumferential and longitudinal directions.

## MATERIALS AND METHODS

### *Animals and study design*

Thirty-four female Wistar rats (bred at the Department of Pathology, Aalborg Hospital Denmark) weighing 190-200 grams were included in this study. The animals were housed individually in cages on white special span wall bedding (temperature 21 ℃, humidity 55±5 %, dark/light cycle 12-h shift). They were fed on a standard laboratory diet. The study complied with Danish regulations for care and use of laboratory animals.

Twenty-two rats were allocated to four groups and treated for 2 ($n$=6), 4 ($n$=6), 7 ($n$=6), and 14 ($n$=4) days with human recombinant EGF (150 ug/kg/day, Upstate Biotechnology, New York, USA) using osmotic minipumps (ALZET 2001; ALZA, Palo Alto, California, USA). Twelve rats served as controls ($n$=3 for each time period). The body weights of both the control and EGF treated rats were measured at the beginning and the end of the study.

### *Collection of specimens*

After treatment, the rats were anaesthetized (pentobarbital 50 mg· $kg^{-1}$ intraperitoneally) and a midline laparotomy was performed. The calcium antagonist papaverine (60 mg· $kg^{-1}$) was injected into the lower thoracic aorta through a cannula (22 G/25 mm) in order to abolish any contractile activity in the gastrointestinal tract. A 5.5-cm-long segment from ileum was excised after the attainment of muscle relaxation (no visible contraction was observed). The residual contents in the lumen were gently cleared using saline and the *in vitro* weight and length of the segment were measured. Then the segment was placed in cold Krebs solution containing 6 % dextran aerated

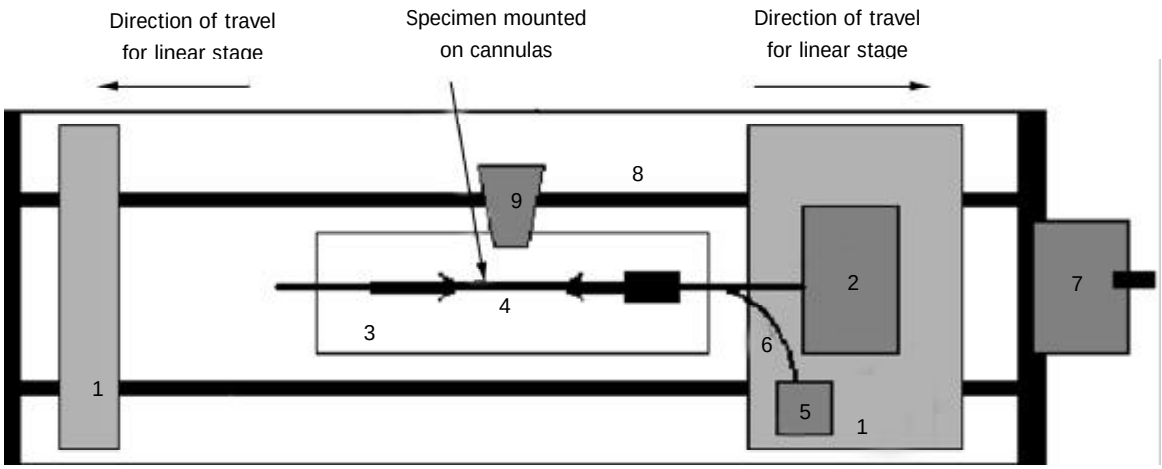



**Figure 1** Legend, biaxial test machine setup. 1: Stage, 2: Force transducer, 3: Organ bath, 4: Specimen, 5: Pressure transducer, 6: Infusion channel, 7: Motor for linear stage, 8: Rails for linear stage, 9: Video camera.

with a gas mixture (95 % $O_2$ and 5 % $CO_2$, pH 7.4). Rings of 1-2 mm in length were cut at the edge of the tissue for no-load and zero-stress state measurements. The remaining part was used for the biaxial test.

### *Stress-strain experiment*

The stress-strain test was conducted on the self-developed biaxial machine (Figure 1), which consists of the force and pressure transducers, the organ bath, motion stage and electronics. The specimen was bathed in the physiological Krebs solution. The two ends of the sample were tied with silk threads on the cannulas and the length between the threads was measured. The length *in vitro* was approximately 30 mm. Then the cannulas were mounted on the rods. The rod on the right side contained a T-connector for changing the pressure in the ileum. The rod on the right was also attached to a force transducer. The rods could be moved towards or apart from each other at controlled rates by two linear stages using a motor. The whole segment was photographed using a video camera (SONY CCD Camera, Japan) for later analysis of length and diameter.

In mechanical testing of living tissues, preconditioning was necessary to obtain repeatable results. This meant that after the specimen was installed in the test machine, the loading and unloading processes were repeated for a number of cycles until the stress-strain relationship became stabilized. The number of cycles required depended on the tissue and method of preparation. In these experiments, we preconditioned the ileum at 6 cm $H_2O$ pressure, 4 stretch cycles to stretch ratio 1.25. The segment was then elongated to stretch ratios of 1.0, 1.08, 1.16, and 1.25. At each stretch level, we applied the pressure to the levels of 0, 2, 4, and 6 cm $H_2O$. The corresponding diameter and gauge length, displacement and axial force at each stress level were monitored.

### *Determination of no-load state and zero-stress state*

The tissue rings were immersed separately in small organ baths containing the aerated Krebs solution. They were photographed in the no-load state and then cut radially on the anti-mesenteric side to obtain their zero-stress state (Figure 2). A 30-min-period was allowed for equilibration after the radial cut and the specimens were photographed again. The selection of this time period was based on pilot experiments.

### *Data analysis*

Morphometric data were obtained from the digitalized images of the segments in the zero-stress, no-load and deformed states. The measurements were done using dedicated software (Sigmascan 4.0, Jandel Scientific, USA). The following data were measured from each specimen at the no-load and zero-stress states (illustrated in Figure 2), which were the circumferential length (*C*), the wall thickness (*h*), the wall area (*A*), and the opening angle at the zero-stress state (α). The subscripts *i*, *o*, *m*, *n*, *z* and *d* refered to the inner (mucosal) surface, outer (serosal) surface, the mid-wall, the no-load state, zero-stress state and deformed condition, respectively. The opening angle α was defined as the angle subtended by two radii drawn from the midpoint of the inner wall to the inner tips of two ends of the specimen. Furthermore, the outer diameter (*D*) and the length ($L_d$) were measured from the images of the deformed segments.

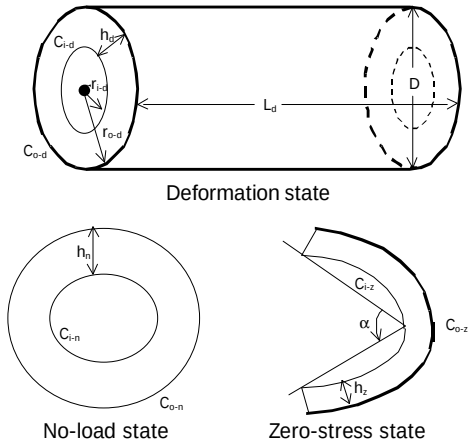



**Figure 2** Legend, illustrations of intestinal small intestinal segment in the no-load, zero-stress and deformed states. C, h, α, denoted the circumference, thickness, and opening angle, respectively. The subscripts *n*, *z*, *d* denoted the no-load state, zero stress state and deformed state, respectively. The subscripts *i*, *o* referred to the inner (mucosal) surface, outer (serosal) surface, respectively.

The measured data were used for computation of the biomechanical parameters. Circumferential residual Green' s strain at the mucosal and serosal surface:

$$E_i = \frac{(\frac{C_{i-n}}{C_{i-z}})^2 - 1}{2} \quad \text{and} \quad E_o = \frac{(\frac{C_{o-n}}{C_{o-z}})^2 - 1}{2} \qquad (1)$$

The stress and strain of the intestine in the deformed state were determined under assumptions that the intestinal wall was thin and homogenous and the intestinal geometry was a circular

tube when pressurized. The circularity assumption had been verified in yet unpublished studies. The simplification we wished to make under the thin wall assumption was that the normal stress in the radial direction, $s_{rr}$, was negligible and that the stresses $s_{qq}$, and $s_{ff}$ were approximately uniform throughout the wall thickness. Here $r$, $q$, $f$ were polar coordinates in radial, circumferential and axial directions, respectively. The calculations were based on knowing the zero-state, no-load state dimensions, the outer diameters and lengths of the specimen at varying pressures, and assuming incompressibility of the intestinal wall. The longitudinal mid-wall stretch ratio, $l_{ff} = \frac{L_d}{L_n}$; the luminal radius, $r_{i-d} = \sqrt{r_{o-d}^2 - \frac{A_n}{p\, l_{ff}}}$; the wall thickness, $h_d = r_{o-d} - r_{i-d}$; the mucosal circumferential length, $C_{i-d} = 2p\, r_{i-d}$; the serosal circumferential length, $C_{o-d} = 2p\, r_{o-d}$; the mid-wall circumferential length, $C_{m-d} = \frac{C_{i-d} + C_{o-d}}{2}$; the mid-wall circumferential length at zero-stress state $C_{m-z} = \frac{C_{i-z} + C_{o-z}}{2}$, the circumferential stretch ratio, $l_{qq} = \frac{C_{m-d}}{C_{m-z}}$.

The longitudinal mid-wall stretch ratio was referenced to the no-load state because tissue strips were not cut for obtaining the zero-stress state in both directions. However, the longitudinal mid-wall length in rat intestine did not differ between the no-load and zero-stress states.

Kirchhoff ' s stress and Green' s strain at a given pressure were computed according to the following equations.

The circumferential and longitudinal Kirchhoff' s stress:

$$S_{qq} = \frac{\Delta \mathrm{Pr}_{i-d}}{(r_{o-d} - r_{i-d})\, l_{qq}^2}, \text{ and } S_{ff} = \frac{F + \Delta P p r_{i-d}^2}{p\, l_{ff}^2 (r_{o-d}{}^2 - r_{i-d}{}^2)} \quad (2)$$

Where ΔP was the transmural pressure difference, $F$ was the force applied on the two ends of intestine.

The circumferential and longitudinal midwall Green' s strain:

$$E_{qq} = \frac{l_{qq}^2 - 1}{2}, \text{ and } \quad E_{ff} = \frac{l_{ff}^2 - 1}{2} \quad (3)$$

The stress and strain under each level of pressure and stretch ratio were calculated. In order to compare the stress-strain relationships among different EGF groups, one dimensional stress-strain curve was fitted, averaged and plotted: Circumferential stress-strain relationship ($P$=0 to $P$=6 $cmH_2O$ ($l_{ff}$=1)) was fitted by the exponential equation: $strain = y_0 + k\exp(stress/t)$. Longitudinal stress-strain relationship ($l_{ff}$=1 to $l_{ff}$=1.25 ($P$=0 $cmH_2O$)) was fitted by another exponential equation because of different curve shapes, $stress = exp(a + b \times strain)$. Where $y_0$, $k$, and $t$, $a$, $b$ were derived from the curve-fit regression done for each of segments. After fitting and averaging, the average curves with error bar could be obtained in different groups.

The exponential strain expression proposed by Fung[12] was applied to a set of test results on EGF treatment ileum. The stress and strain at a fixed stretch ratio of $l_{ff}$=1.16 and at varying pressure ($P$=0, 2, 4, 6 $cmH_2O$) were calculated and fitted to the function.

$$r_0 W^{(2)} = \frac{C}{2}\exp\left[a_1(E_{qq}^2 - E_{qq}^{*2}) + a_2(E_{ff}^2 - E_{ff}^{*2}) + 2a_4(E_{qq}E_{ff} - E_{qq}^* E_{ff}^*)\right] \quad (4)$$

where $W$ was the strain energy per unit mass of the tissue, and $r_0$ was the mass density (mass per unit volume) in the initial zero-stress state. Then $r_0 W^{(2)}$ was the strain energy per unit volume of the tissue at zero-stress state for two dimensions.

From $S_{qq} = \frac{s_{qq}}{l_{qq}^2} = \frac{\partial(r_0 W^{(2)})}{\partial E_{qq}}$ and $S_{ff} = \frac{s_{ff}}{l_{ff}^2} = \frac{\partial(r_0 W^{(2)})}{\partial E_{ff}}$ (5)

We obtained $S_{qq} = C(a_1 E_{qq} + a_4 E_{ff})\exp[F(a,E)]$, and
$S_{ff} = C(a_4 E_{qq} + a_2 E_{ff})\exp[F(a,E)]$ (6)

Where

$$F(a,E) = a_1(E_{qq}^2 - E_{qq}^{*2}) + a_2(E_{ff}^2 - E_{ff}^{*2}) + 2a_4(E_{qq}E_{ff} - E_{qq}^* E_{ff}^*) \quad (7)$$

Where $C$ (with units of stress,) and $a_1$, $a_2$, $a_4$ (dimensionless) were material constants, $E_{\theta\theta}{}^*$, $E_{\phi\phi}{}^*$ were strains corresponding to a selected pair of stresses $S_{\theta\theta}{}^*$, $S_{\phi\phi}{}^*$. The material constants were determined by a modified version of Marquardt' s nonlinear least-squares algorithm.

The meaning of the material constants was discussed in detail by Fung[12]. Constant $a_1$ would affect the curve for $S_{\theta\theta}$ *vs.* $E_{\theta\theta}$, the higher the $a_1$, the curve of $S_{\theta\theta}$ *vs.* $E_{\theta\theta}$ would leave the origin closer to the strain axis, and rise more rapidly, the more non-linear the curve was. Constant $a_2$ would affect the curve for $S_{\phi\phi}$ *vs.* $E_{\phi\phi}$ in a similar way. Constant $a_4$ specified the cross talk between the circumferential and longitudinal directions. The constant $C$ fixed the scale on the stress axis. The larger the value of $a_1$, $a_2$ and $a_4$ was, the smaller $C$ was[12].

### *Statistical analysis*

Data were expressed as mean ±SEM. One-way analysis of variance was used to detect variations between treatment groups (Sigmastat 2.0™, Jandel Scientific, Germany). In case of significance ($P$<0.05), differences were tested between the control group and EGF-treated groups at 2, 4, 7, and 14 days treatment (Dunnet' s Test). If the normality test failed, Kruskal-Wallis one way analysis of variance on ranks was used instead.

## RESULTS

### *Weight and morphological data*

The total body weight did not change (Figure 3A). The ileum weight began to increase after 2 days' EGF treatment (Figure 3B). The wall thickness increased significantly after 4 days' EGF treatment, it did not change further (Figure 3C). A significantly larger wall area was observed in the 4 days' treatment group (Figure 3D). The luminal area did not change significantly (Figure 3E). However, a significantly smaller inner perimeter was observed in the 4 days, and 7 days' EGF treatment groups, but not in the 14 days' group (Figure 3F).

### *Zero-stress state data*

The opening angle increased significantly after 7 days' EGF treatment and continued to increase up to 14 days (Figure 3G). The mucosal residual strain was compressive in all groups and decreased from 4 days of EGF treatment. The serosal residual strain was tensile with relatively higher values after 14 days of EGF treatment (Figure 3H).

### *Biomechanical data*

The stress-strain relationships are shown in Figure 4. In circumferential direction, the curves representing 4 days and 7 days of EGF treatment were shifted to the right, whereas 14 days' EGF treatment curve shifted back towards normal. In longitudinal direction, the stress-strain curves representing 4 days and 7 days of EGF treatment were shifted to the left. Like the circumferential direction, the curves shifted towards normal after 14 days' EGF treatment.

The derived material constants $a_1$, $a_2$, $a_4$, and $C$ from the strain energy function are illustrated in Table 1. Constant $a_1$ and $a_2$ having comparably smaller SEM values were therefore more stable than the other parameters. A significantly smaller $a_1$ value was found in the 7 days' and 14 days' EGF treatment groups and larger $a_2$ values were found in the 2 days' and 7 days' EGF treatment groups. This demonstrated that 7 days'

EGF treatment had the lowest circumferential stiffness and comparatively high longitudinal stiffness, and that the remodeling was most significant in the 7 days' EGF treatment group.

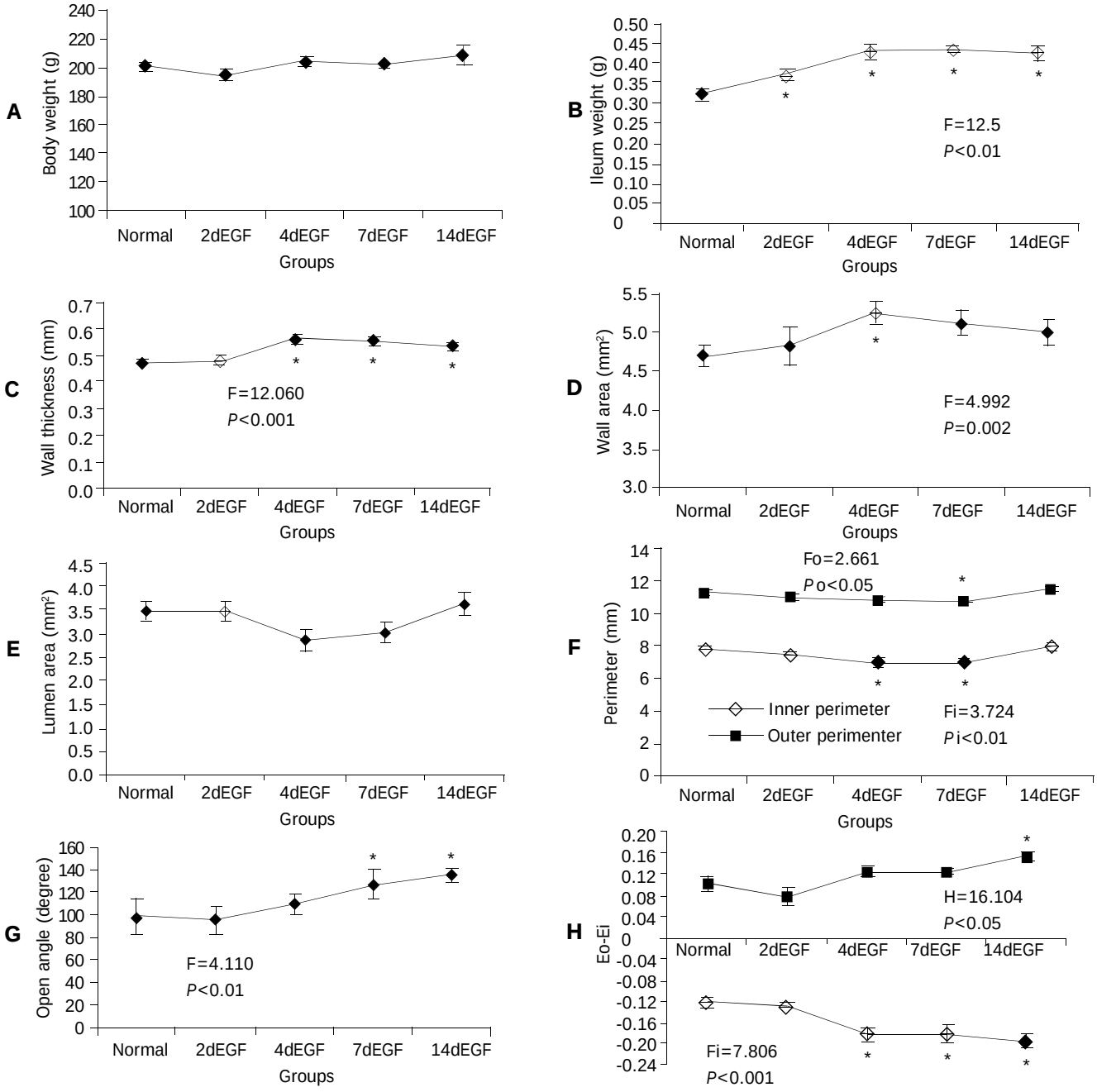



**Figure 3** Legend, morphometric data of ileum in the no-load (A-F) and zero-stress states (G-H). *F* and *P* values were from one-way ANOVA for different treatment groups: If significant difference ($P<0.05$) was found using one-way ANOVA (marked with a), multiple comparisons with normal controls were done using Dunnet's test. Significantly different groups were marked with b.

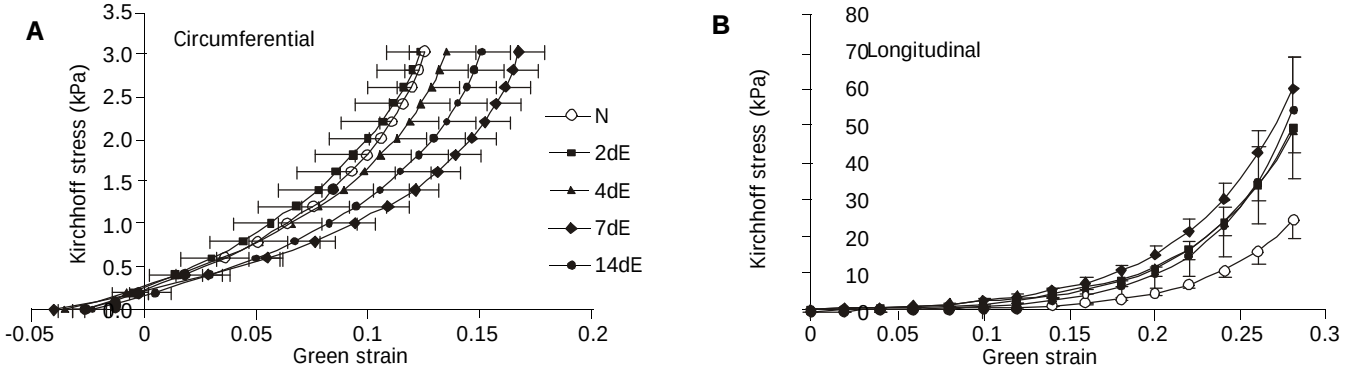



**Figure 4** Legend, intestinal stress-strain curves in the EGF-treated and normal control groups. Translation of the stress-strain curve to the right in the coordinate system implied tissue softening (decreased stiffness) and *vice versa.*

**Table 1** Material constants

| | Normal | 2dEGF | 4dEGF | 7dEGF | 14dEGF |
|---|---|---|---|---|---|
| $a_1$[a] | 25.42±1.91 | 18.19±2.80 | 19.32±3.91 | 11.57±2.04[b] | 13.61±3.00[b] |
| $a_2$[a] | 21.51±3.19 | 36.24±0.95[b] | 24.90±3.72 | 34.74±5.17[b] | 27.51±4.41 |
| $a_4$ | 6.41±1.20 | 12.20±2.98 | 7.13±3.53 | 5.39±1.29 | 4.78±1.15 |
| $C$ (kPa) | 1.13±0.18 | 1.30±0.27 | 2.32±0.97 | 1.75±0.30 | 1.02±0.12 |

A: One-way ANOVA result: $P<0.05$; B: Post analysis (Dunnet´s test) result: significantly different from normal control group ($P<0.05$).

## DISCUSSION

The major findings in the present study were that EGF treatment induced time-dependent morphometric and biomechanical remodeling in the ileum. The ileum weight and wall thickness increased. The biomechanical changes were characterized by increased opening angle, inner and outer residual strains, decreased circumferential wall stiffness and increased longitudinal wall stiffness.

As the function of the GI tract is to a large degree mechanical, it has become increasingly popular to acquire biomechanical information on the GI tract[13-17]. However, many aspects of its biomechanical function are still largely left unexplored. Previous investigations were mainly conducted on uni-axial isolated strip experiments and balloon distension[18,19]. In the isolated strip studies, the structural integrity of the organ wall was not preserved and the strip was not necessarily in its zero-stress state at resting conditions since large residual strains existed in the GI tract[19]. In the balloon distension test, by means of impedance planimetry, the luminal cross-sectional area (CSA) could be measured. CSA as function of pressure provided important information on luminal dimensions during the loading but gave little information about material properties without further mechanical analysis. Furthermore, the tension-strain relationship was only computed for the circumferential direction and the strain was not referenced to the zero stress state[18].

The zero stress state of an organ is the state at which the organ is stress-free, meaning that all external and internal forces are removed. Vaishnav and Vossoughi[20] and Fung[21] independently reported respectively that the no-load state of a blood vessel was not the zero stress state. Recently, residual strains were demonstrated in the esophagus, small intestine, and large intestine[14-16, 22-28] and were found to be closely related to the growth and remodeling[24-30]. Hence the analysis of stress and strain begins with identification of the zero stress state.

This study was to explore how EGF affects geometric and biomechanical remodeling. EGF induced remodeling of the material structure, zero-stress state, and mechanical properties. The changes were quantified in terms of material constants in the constitutive equation. For computation of mechanical parameters we used video image techniques during loading and at no-load and zero-stress states. Assuming the intestine wall to be a membrane, biaxial experiments were done and a two- dimensional pseudostrain energy function was used to express the stress-strain relationship with reference to the zero-stress state. The use of pseudoelasticity to describe biological materials has been justified by Fung *et al*[12]. The vascular study by Liu and Fung[31] found that the zero stress state and the material constants changed with the development of diabetes.

Remodeling of the small intestine was evident after 4 days' EGF treatment with increased wall thickness and decreased luminal area. This was consistent with Vinter-Jensen' s study[11]. They demonstrated that EGF stimulated intestinal wall thickness growth before the surface area growth. According to the histology observation, the growth progress mainly involved the mucosa layer. The speed of growth began to decrease after 4 days' EGF treatment.

The small intestinal residual strains were negative at the mucosal layer and positive at the serosal layer. This implied that the mucosa was under compression in the no-load state and at physiological conditions in the low pressure range, whereas the muscle layers were in tension. As expected, both the residual strain and the opening angle increased in a time-dependent manner. The change of the opening angle was a result of non-uniform tissue remodeling of the organ wall. The opening angle increases when the inner layers had a higher growth rate than the outer layers or when the outer layer' atrophy was more severe than the inner layer[32]. In the present EGF study, the mucosa growth was much faster than that in the other layers, which resulted in the increased opening angle and residual strains. It has been demonstrated that residual stress reduces the stress concentration at the inner wall of the GI tract at no-load and homeostatic states[15, 16, 22]. The residual stress in the resting condition may also serve as a growth-regulation factor[33]. However, additional studies are needed to investigate whether the growth influences the residual stress distribution or *vice versa*.

The biaxial test showed that the wall stiffness changed in response to EGF treatment. After 7 days' EGF treatment, the wall stiffness decreased in circumferential direction and increased in longitudinal direction. The wall stiffness remodeled towards the normal state in both directions after 14 days' EGF treatment. In circumferential direction, the result was consistent with Vinter-Jensen' s report[11]. The different tendency of intestinal wall stiffness change in the two directions demonstrated that EGF-induced tissue remodeling was heterogeneous in the ileum. The elastic modulus of the intestine wall was closely related to the morphological wall composition and the stiffness was mainly dependent on the collagen in the submucosal layer[15, 34]. The submucosal layer did not remodel to the same degree as the mucosal layer. Therefore, the intestinal wall became softer in the circumferential direction after EGF treatment. At this point we could not explain the different remodeling in the two directions. A possible explanation is that the collagen orientation changed its direction during EGF treatment. This study did not intend to evaluate the collagen orientation angle, so this needs to be evaluated in future studies. Another noticeable result was that after 14 days' EGF treatment the curves shifted towards normal in both directions. We hypothesize that the pronounced proliferation of the intestine in the first week is due to direct stimulation by exogenous EGF. However, after one week, a negative feedback mechanism of the organ reduced the response to endogeneous EGF, resulting in reduced growth speed of the intestine. Finally, after two weeks, the remodeling might be determined by the stress in the tissue in accordance with the stress-growth law proposed by Fung[21].

## ACKNOWLEDGEMENT

The recombinant EGF was a generous gift from Professor Esam Z. Dajani, International Drug Development Consultants Corporation, a Division of Mid Gulf U.S.A., Inc., Long Grove, Illinois, U.S.A.

## REFERENCES


1 **Prigent SA**, Lemoine NR. The type 1 (EGFR-related) family of growth factor receptors and their ligands. *Prog Growth Factor Res* 1992; **4**: 1-24

2 **Vinter-Jensen L**. Pharmacological effects of epidermal growth factor (EGF) with focus on the urinary and gastrointestinal tracts. *APMIS* 1999; **93**(Suppl): 1-42

3 **Gregory H**. Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* 1975; **257**: 325-327

4 **Opleta-Madsen K**, Hardin J, Gall DG. Epidermal growth factor upregulates intestinal electrolyte and nutrient transport. *Am J Physiol* 1991; **260**(6Pt 1): 807-814

5 **Ghishan FK,** Kikuchi K, Riedel B. Epidermal growth factor upregulates intestinal Na+ / H+ exchange activity. *Proc Soc Exp Biol Med* 1992; **201**: 289-295

6 **Salloum RM**, Stevens BR, Schultz GS, Souba WW. Regulation of small intestinal glutamine transport by epidermal growth factor. *Surgery* 1993; **113**: 552-559

7 **Dunn JC**, Parungo CP, Fonkalsrud EW, McFadden DW, Ashley SW. Epidermal growth factor selectively enhances functional enterocyte adaptation after massive small bowel resection. *J Surg Res* 1997; **67**: 90-93

8 **Liu CD**, Rongione AJ, Shin MS, Ashley SW, McFadden DW. Epidermal growth factor improves intestinal adaptation during somatostatin administration *in vivo*. *J Surg Res* 1996; **63**: 163-168

9 **Rao R**, Porreca F. Epidermal growth factor protects mouse ildeal mucosa from Triton X-100-induced injury. *Eur J Pharmacol* 1996; **303**: 209-212

10 **Ulshen MH**, Raasch RH. Luminal epidermal growth factor preserves mucosal mass of small bowel in fasting rats. *Clin Sci* 1996; **90**: 427-431

11 **Vinter-Jensen L**, Duch BU, Petersen JA, Ryslev A, Gregersen H. Systemic treatment with epidermal growth factor in the rat. Biomechanical properties of the growing small intestine. *Regul Pept* 1996; **61**: 135-142

12 **Fung YC**, Fronek K, Patitucci P. Pseudoelasticity of arteries and the choice of mathematical expression. *Am J Physiol* 1979; **237**: H620-H631

13 **Gregersen H**, Giversen IM, Rasmussen LM, Tottrup A. Biomechanical wall properties and collagen content in the partially obstructed opossum esophagus. *Gastroenterology* 1992; **103**: 1547-1551

14 **Gregersen H**, Kassab G. Biomechanics of the gastrointestinal tract. *Neurogastroenterol Motil* 1996; **8**: 277-297

15 **Gregersen H**, Kassab GS, Pallencaoe E, Lee C, Chien S, Skalak R, Fung YC. Morphometry and strain distribution in guinea pig duodenum with reference to the zero-stress state. *Am J Physiol* 1997; **273**(4Pt 1): G865-G874

16 **Gregersen H**, Lee TC, Chien S, Skalak R, Fung YC. Strain Distribution in the layered wall of the esophagus. *J Biomech Eng* 1999; **121**: 442-448

17 **Orvar KB**, Gregersen H, Christensen J. Biomechanical characteristics of the human esophagus. *Dig Dis Sci* 1993; **38**: 197-205

18 **Gregersen H**, Vinter-Jensen L, Juhl CO, Dajani EZ. Impedance planimetric characterization of the distal oesophagus in the Goettingen minipig. *J Biomech* 1996; **29**: 63-68

19 **Tottrup A**, Forman A, Uldbjerg N, Funch-Jensen P, Andersson KE. Mechanical properties of isolated human esophageal smooth muscle. *Am J Physiol* 1990; **258**(3Pt 1): G338-G343

20 **Vaishnav RN**, Vossoughi J. Estimation of residual strains in aortic segments. In: Hall CW, eds. Biomedical engineering II. Recent Developments. *New York: Pergamon Press* 1983: 330-333

21 **Fung YC**. What principle governs the stress distribution in living organs? In: Fung YC, Fukada E, Junjian W, eds. Biomechanics in China, Japan and USA. *Science, Beijing, China* 1983: 1-13

22 **Gao C**, Zhao J, Gregersen H. Histomorphometry and strain distribution in pig duodenum with reference to the zero-stress state. *Dig Dis Sci* 2000; **45**: 1500-1508

23 **Lu X**, Gregersen H. Regional distribution of axial strain and circumferential residual strain in the layered rabbit oesophagus. *J Biomech* 2001; **34**: 225-233

24 **Dou Y**, Gregersen S, Zhao J, Zhuang F, Gregersen H. Effect of refeeding after starvation on biomechanical properties in rat small intestine. *Med Eng Phys* 2001; **23**: 557-566

25 **Dou Y**, Gregersen S, Zhao J, Zhuang F, Gregersen H. Morphometric and biomechanical intestinal remodeling induced by fasting in rats. *Dig Dis Sci* 2002; **47**: 1158-1168

26 **Dou Y**, Lu X, Zhao J, Gregersen H. Morphometric and biomechanical remodeling in the intestine after small bowel resection in the rat. *Neurogastroenterol Motil* 2002; **14**: 43-53

27 **Zhao J**, Sha H, Zhou S, Tong X, Zhuang FY, Gregersen H. Remodelling of zero-stress state of small intestine in streptozotocin-induced diabetic rats. Effect of Gliclazide. *Dig Liver Dis* 2002; **34**: 707-716

28 **Zhao JB**, Sha H, Zhuang FY, Gregersen H. Morphological properties and residual strain along the small intestine in rats. *World J Gastroenterol* 2002; **8**: 312-317

29 **Saini A**, Berry C, Greenwald S. Effect of age and sex on residual stress in the aorta. *J Vasc Res* 1995; **32**: 398-405

30 **Fung YC**, Liu SQ. Change of residual strains in arteries due to hypertrophy caused by aortic constriction. *Circ Res* 1989; **65**: 1340-1349

31 **Liu SQ**, Fung YC. Influence of STZ-induced diabetes on zero-stress states of rat pulmonary and systemic arteries. *Diabetes* 1992; **41**: 136-146

32 **Rodriguez EK**, Hoger A, McCulloch AD. Stress-dependent finite growth in soft elastic tissues. *J Biomech* 1994; **27**: 455-467

33 **Fung YC**, Liu SQ. Changes of zero-stress state of rat pulmonary arteries in hypoxic hypertension. *J Appl Physiol* 1991; **70**: 2455-2470

34 **Gregersen H**, Kassab GS, Fung YC. The zero-stress state of the gastrointestinal tract: biomechanical and functional implications. *Dig Dis Sci* 2000; **45**: 2271-2281


**Edited by** Wu XN and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2284-2288
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Effects of *Rheum tanguticum* polysaccharide on TNBS -induced colitis and CD4$^+$T cells in rats

Li Liu, Zhi-Peng Wang, Chang-Tai Xu, Bo-Rong Pan, Qi-Bing Mei, Yin Long, Jia-Yun Liu, Si-Yuan Zhou

**Li Liu, Zhi-Peng Wang, Qi-Bing Mei, Si-Yuan Zhou,** Department of Pharmacology, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China
**Yin Long, Jia-Yun Liu,** Department of Traditional Chinese Medicine, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China
**Chang-Tai Xu,** Editorial Department of Journal of Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China
**Bo-Rong Pan,** Department of Oncology, Xijing Hospital, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China
**Supported by** the National Natural Science Foundation of China, No 30100239
**Correspondence to:** Qi-Bing Mei, Department of Pharmacology, Fourth Military Medical University, Xi' an 710032, Shaanxi Province, China. qbmei@hotmail.com
**Telephone:** +86-29-3374552 **Fax:** +86-29-3374552
**Received:** 2003-03-10 **Accepted:** 2003-06-19

## Abstract

**AIM:** To study the effects of *Rheum tanguticum* polysaccharide$_{-1}$ (RTP$_{-1}$) on ulcerative colitis in rats induced by 2, 4, 6-trinitrophene sulphonic acid (TNBS) and their possible mechanism.

**METHODS:** RTP$_1$ (200 mg·kg$^{-1}$, ig) extracted from *Rheum tanguticum* Maxim. ex Regel was administrated to rats with colitis induced by TNBS for 5 d, 7 d, 10 d and 14 d, respectively. The effects of RTP$_1$ and dexamethasone (DX, 0.2 mg·kg$^{-1}$, ig) were contrastively investigated. The MPO level and SOD activity were determined by chromatometry. The expansion and protein expression of CD4$^+$T lymphocytes isolated from colon mucosae and mesenteric lymph nodes of colitis rats were performed by immunohistochemical analysis and Western-blot methods.

**RESULTS:** Treatments of RTP$_1$ (200 mg·kg$^{-1}$, ig) significantly reduced diarrhea, mortality, colon mass, ulcer areas and MPO level in colon mucosae on days 5, 7, 10 and 14 (5.2±1.4, 5.4±0.7, 5.2±1.8, $P<0.05$. 3.4±0.8, $P<0.01$. 16.1±12.1, $P<0.01$. 31.8±8.6, 17.7±5.3, 12.7±4.1, $P<0.05$). The effects of RTP$_1$ were similar to those noted above in DX group, but there were no immunosupressive effects of DX in RTP$_{-1}$ group, such as body mass loss, thymus and spleen atrophy. The decreased number and down-regulated protein levels of CD4$^+$T cells isolated from the colon of colitis rats treated with RTP$_1$ were found.

**CONCLUSION:** RTP$_1$ shows significantly protective effects but lower side effects on rats with colitis induced by TNBS. The mechanism may be due to the resistance to over expansion of CD4.

Liu L, Wang ZP, Xu CT, Pan BR, Mei QB, Long Y, Liu JY, Zhou SY. Effects of *Rheum tanguticum* polysaccharide on TNBS -induced colitis and CD4$^+$T cells in rats. *World J Gastroenterol* 2003; 9(10):2284-2288
http://www.wjgnet.com/1007-9327/9/2284.asp

## INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Cron' s disease, are complex autoimmune diseases whose etiology and pathogenesis have not been fully elucidated. IBD is related with multiple etiologic theories such as genetics, immunology and environment[1,2]. In recent years, a study of animal models of IBD pathogenesis showed that immune imbalance led to the inflammation in the gastrointestinal tract[3]. A growing number of evidences support an important role for dysregulated CD4+T cells response to the antigens, such as enteric bacterial flora, as a common disease mechanism[4-6]. Previous studies have also demonstrated an increasing number of infiltrating CD4+T cells and anti-CD4 antibodies are thought to be effective[7-9]. The fact showed that an imbalance of excessive CD4/Th1 cell response or inadequate CD4/Th2 cells response was involved in experimental colitis[10]. These studies strongly suggest that immunomodulatory drugs have a bright future in IBD therapy, and CD4 could be a potential therapeutic target of colitis drug research[11]. Many biologic therapies are being evaluated for the treatment of chronic inflammatory bowel diseases[12-15]. However, during recent years, the mainstay therapies for IBD were anti-inflammatory drugs and glucocorticosteroids (GCS)[16,17]. The incidence of IBD is increasing in Asia[18,19], some traditional Chinese medicine therapies, such as moxibustion[20] and decoction, have shown significant therapeutic effectiveness on IBD. Rhubarb which has been used for gastrointestinal disease, is a main ingredient of some decoctions administrated to patients with IBD. Our previous data showed *Rheum tanguticum* polysaccharide (RTP) extracted from *Rheum tanguticum* Maxim. ex Regel, could significantly protect against acute hepatic injury and oxidation injury[21,22]. Although there is no evidence whether IBD patients can benefit from RTP, heparin exhibits significant effects on colitis[23,24] and some plant polysaccharides exhibit immune regulative effects[25,26]. Therefore, we administered RTP$_1$ to colitis rats and investigated its therapeutical effectiveness on colitis rats-induced by TNBS and the expansion and protein level of CD4$^+$ lymphocytes, which were the potential targets of colitis immunoregulatory therapy.

## MATERIALS AND METHODS

### *Animal*

Male Sprague-Dawley rats (220-250 g) obtained from the Animal Center of Fourth Military Medical University were fed on a standard laboratory diet and free to access tap water. The rats were kept in a room at a controlled temperature (22±1 ℃), humidity (65-70 %), and a 12:12-h light-dark cycle. Forty rats were randomly divided into four groups, namely normal, model, RTP (200 mg· kg$^{-1}$, ig) and dexamethasone (DX, 0.20 mg· kg$^{-1}$, ig) groups.

### *Materials*

Sephacryl-S400 was from Pharmacia Biotech AB, dexamethasone (DX) and 2, 4, 6-trinetrophene sulphonic acid (TNBS) were purchased from Sigma, hexadecytrimethyl-ammonium was a product of Fluka. CD4 mAb was a product

of Immunotech Co. SABC immunehistochemical kits were purchased from Boshide Co. Electrophoresis apparatus was purchased from Bio-Rad.

### *Preparation of polysaccharide from Rheum tanguticum Maxim. ex Regel*

*Rhubarb*, identified by professor Ren Yi Northwest University of china, was *Rheum tanguticum* Maxim.ex Regel. *Rheum tanguticum* polysaccharide (RTP) was extracted according to the methods described previously[27]. In brief, *Rheum tanguticum* Maxim (1 kg) was fragmented and boiled 3 times,8 hours each time with absolute ethanol for 24 hours to extract the components dissolved in ethanol. The residue was boiled for another three times (8 h each time) with water to extract polysaccharide. All the water extractions were finally pooled and mixed with a finally concentrated ethanol solution of 75 ml/L to precipitate the polysaccharide-enriched fractions. After proteins removed by freezing-thawing methods, crude *Rheum tanguticum* polysaccharide (RTP) was obtained by dialysis, concentration and lyophilization. In this study, the polysaccharide (uronic acid) content of $RTP_1$ was 97.4 % (27.5 %), and their molecular masses were 60 Kda-80 Kda , and $RTP_1$was administered orally to rats to study the effects and mechanism on colitis.

### *Induction of ulcerative colitis model in rats*

Male Sprague-Dawley rats were fasted for 24 hours before experimentation but with free access to drinking tap water. Colitis model was produced as before[28] (*Gastroenterology* 1989;96:795-811). In brief, the rats (except rats in normal group) were lightly anesthetized with ether. A rubber catheter (OD 2 mm) was inserted rectally into the colon so that the tip was 8 cm proximal to the anus, approximately at the splenic flexure. TNBS dissolved in 40 mL/L in ethanol was instilled into the lumen of colon through the rubber catheter. Saline, RTP and DX were administrated 6 hours after induction of colitis model. The body weight was recorded during the experimental period. The rats were anesthetized with ether and killed by cervical dislocation on d5, d7, d10, d14, respectively. Each was 10 rats. The distal colon was removed and opened by a longitudinal incision, rinsed with saline, and weighed. Mucosal damage was assessed by measurement of ulcerative areas in colon mucosae by a single observer blinded to characteristics or treatment of the animals being studied. Colon samples were then excised, snap-frozen in liquid nitrogen, and stored at -80 ℃ for later assay of myloperoxidase (MPO) and superoxide dismutase (SOD). Additional samples were preserved in formaldehyde for histological and immunohistochemical analysis. Meantime, the spleen and thymus were removed and weighed.

### *Determination of biochemical marker*

The activity of MPO and SOD was determined by chromatometry. In brief, the colon tissue was suspended in 5 g/L hexadecytrimethyl-ammonium (pH6.0, 50 mg of tissue per milliliter) for MPO level and suspended in PBS (pH 7.2, 100 mg per milliliter) for SOD level and then homogenized for 15 s by using a polytron generator in ice bathing. The MPO and SOD levels in colon tissue were determined by using the technique described as before (*Gastroenterology* 1984;84:1344).

### *Immunohistochemical analysis on $CD4^+$ lymphocyte*

Immunohistochemical staining was performed on paraffin embedded sections by using biotinylated anti-CD4 mAb, and then operated according to the procedure for SBC immunohistochemiacal kit.

### *Western-blot analysis on $CD4^+$ lymphocyte*

The rats with colitis induced by TNBS ethanol were killed by cervical dislocation. The lumenal lymphoid node was separated and homogenized in 1 ml lysis buffer in ice bathing for 10 min, then centrifuged at 11 000 g for 5 min at 4 ℃, and 30 μl suspension was moved to lodding buffer 15 ml. The samples were boiled for 10 min and subjected to electrophoresis on 120 g/L SDS-acrylamide gel. The gel was then electroblotted onto a ultrocellulose membrane, and detection of CD4 protein was conducted by using anti-CD4 mAb.

### *Statistical analysis*

Results were expressed as mean ± standard deviation ($\bar{x}\pm s$). Differences between two groups were examined by Student' s *t*-test. A probability (*P* value) of less than 0.05 was considered statistically significant.

## RESULTS

### *Effects of $RTP_1$ on rats with TNBS-induced colitis*

Grossly visible colon wall thicking, inflammation, and ulcers were found in the animals that received TNBS in 400 mL/L ethanol. Treatments of $RTP_1$ (200 $mg \cdot kg^{-1}$ ig) significantly reduced diarrhea, mortality, colon mass and ulcer area. The effect of $RTP_1$ on colitis was similar to that in DX group (Figure 1, Table 1).

**Table 1** Effects of $RTP_1$ (200 $mg \cdot kg^{-1}$, ig) on rats with TNBS -induced colitis ($n$=10, $\bar{x}\pm s$)

| *t* / d | Colon mass index ($mg \cdot g^{-1}$) | | | | Ulcerative area (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal | Model | $RTP_1$ | DX | Normal | Model | $RTP_1$ | DX |
| 5 | 2.8±0.4 | 7.0±1.4[d] | 5.2±1.4[a] | 5.5±0.6[a] | 0.0±0.0 | 35.7±12.9[d] | 16.1±12.1[b] | 26.2±4.1[a] |
| 7 | | 6.9±1.7[d] | 5.4±0.7[a] | 5.6±0.4[a] | | 44.5±12.0[d] | 31.8±8.6[a] | 35.7±2.4[a] |
| 10 | | 7.3±1.5[d] | 5.2±1.8[a] | 5.2±0.6[b] | | 27.5±13.7[d] | 17.7±5.3[a] | 22.1±4.9 |
| 14 | | 5.4±0.9[d] | 3.4±0.8[b] | 4.1±0.3[b] | | 21.8±9.7[d] | 12.7±4.1[a] | 13.5±2.7[a] |

[a]$P$<0.05, [b]$P$<0.01 *vs* Model; [d]$P$<0.01 *vs* Normal.

**Table 2** Effects of $RTP_1$ on SOD and MPO activity of colon in rats with TNBS- induced colitis ($n$=10, $\bar{x}\pm s$)

| *t* / d | SOD ($NU \cdot g^{-1}$) | | | | MPO ($U \cdot g^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal | Model | $RTP_1$ | DX | Normal | Model | $RTP_1$ | DX |
| 5 | 40.9±7.7 | 48.7±8.4[c] | 69.1±16.1[b] | 72.5±13.5[b] | 0.09±0.02 | 9.7±3.2[d] | 2.9±1.9[b] | 4.8 ±1.0[d] |
| 7 | | 54.7±7.8[d] | 66.0±6.8[b] | 67.2±12.1[b] | | 7.7±1.5[d] | 3.1±0.8[b] | 6.1±1.1[e] |
| 10 | | 44.4±7.9 | 63.4±4.8[b] | 60.1±5.4[b] | | 6.0 ±1.9[d] | 4.3±0.9[a] | 4.3±1.1[a] |
| 14 | | 38.8±5.7 | 60.7±10.5[b] | 60.2±6.4 [b] | | 4.1±1.2[d] | 1.8±0.5[b] | 2.9±0.8[e] |

[a]$P$<0.05, [b]$P$<0.01 *vs* Model. [c]$P$<0.05, [d]$P$<0.01 *vs* Normal. [e]$P$<0.01 *vs* $RTP_1$.

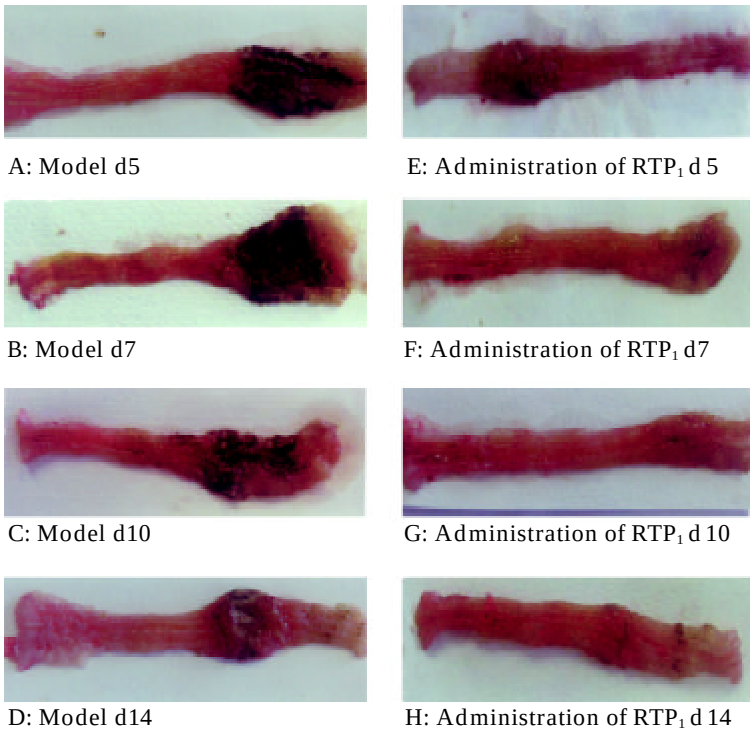



**Figure 1** Photographs of rats colon. A-D. TNBS-induced colitis with gross enlargement of the colon and large ulcers. E-H. TNBS with RTP$_1$, smaller ulcer area and reduced colon mass.

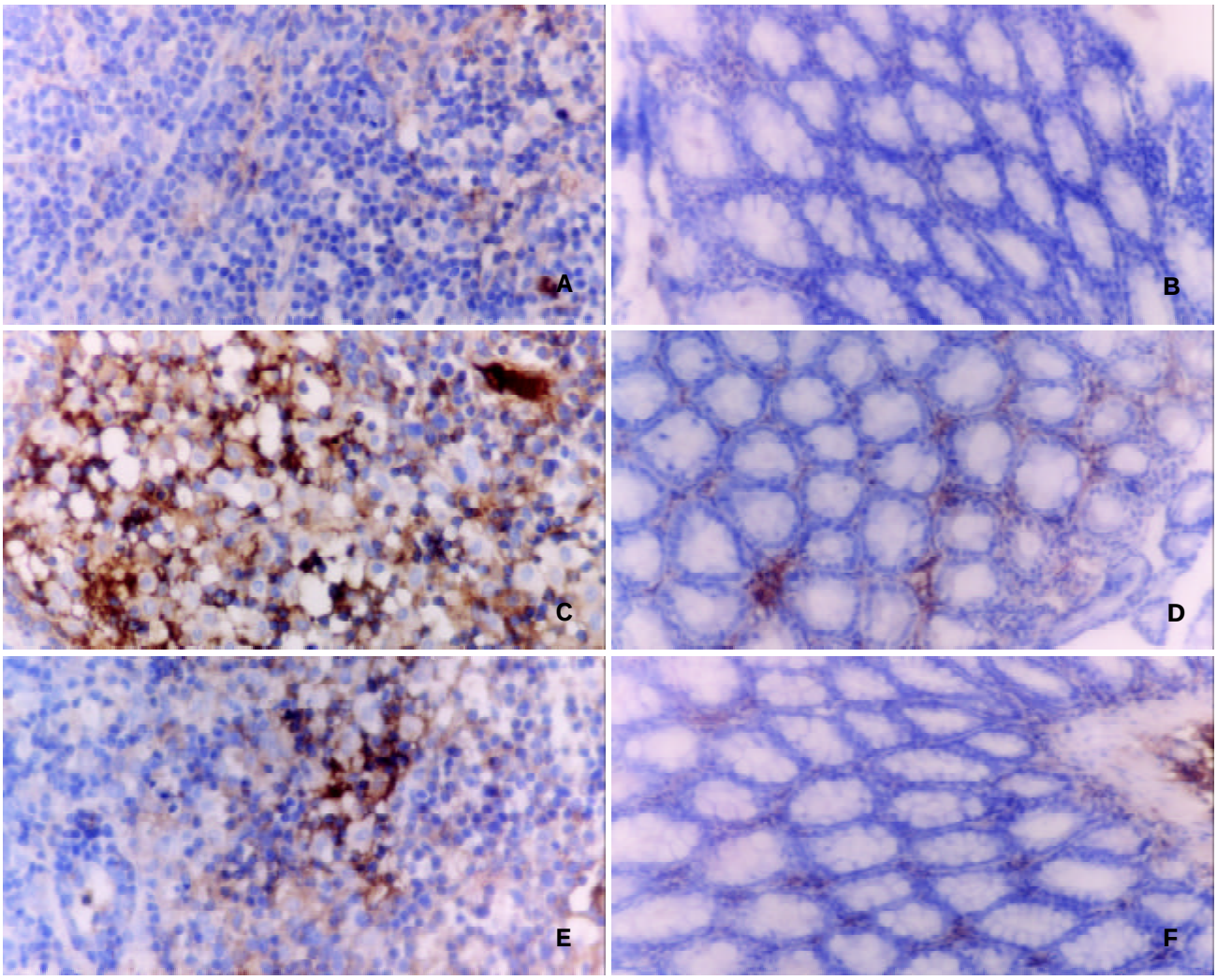



**Figure 3** Immunohistochemical analysis on CD4$^+$ lymphocytes in mesenteric lymphoid nodes and colon tissues SABC×400. A, C, E: Mesenteric lymphoid nodes. B, D, F: Colon tissues.

**Table 3** Effects of $RTP_1$ on thymus, spleen and body mass in rats with TNBS- induced colitis ($n$=10, $\bar{x}\pm s$)

| $t$ / d | Body mass (g) | | | | Spleen index (mg·g$^{-1}$) | | | | Thymus index (mg·g$^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Normal | Model | $RTP_1$ | DX | Normal | Model | $RTP_1$ | DX | Normal | Model | $RTP_1$ | DX |
| 5 | 242±16 | 210±26$^{b}$ | 235±32 | 215±27$^{d}$ | 2.3±0.4 | 3.2±0.4$^{d}$ | 3.0±0.4$^{d}$ | 3.0±0.6$^{d}$ | 2.4±0.03 | 0.9±0.3$^{d}$ | 1.2±0.5$^{d}$ | 0.7±0.2$^{d}$ |
| 7 | 255±23 | 223±39$^{c}$ | 243±24 | 196±21$^{bde}$ | | 3.9±1.0$^{d}$ | 3.6±0.6$^{d}$ | 2.3±0.2 | | 1.1±0.5$^{d}$ | 1.3±0.5$^{d}$ | 0.6±0.3$^{bd}$ |
| 10 | 277±29 | 252±27 | 267±38 | 176±33$^{bde}$ | | 2.6±0.2$^{c}$ | 2.7±0.5 | 2.4±0.2 | | 1.0±0.4$^{d}$ | 1.2±0.4$^{d}$ | 0.4±0.7$^{bd}$ |
| 14 | 278±30 | 258±22$^{c}$ | 272±31 | 165±31$^{bde}$ | | 2.7±0.3$^{c}$ | 2.5±0.6 | 1.8±0.3$^{bd}$ | | 1.2±0.3$^{d}$ | 1.4±0.2$^{d}$ | 0.02±0.01$^{bd}$ |

$^{a}P$<0.05, $^{b}P$<0.01 *vs* Model; $^{c}P$<0.05, $^{d}P$<0.01 *vs* Normal; $^{e}P$<0.05, $^{f}P$<0.01 *vs* $RTP_1$.

### *Effects of $RTP_1$ on SOD and MPO of colon mucosa*

The MPO activity and SOD level in TNBS-induced colitis rats were much higher than those in control. Treatments with $RTP_1$ (200 mg·kg$^{-1}$ ig) significantly increased SOD activity, meanwhile, decreased MPO level in colon mucosae. The effects of DX on MPO and SOD activity of colon mucosae were similar to those of $RTP_1$ (Table 2).

### *Effects of $RTP_1$ on body weight, thymus and spleen index*

During the inflammatory period, the rats with colitis showed marked body mass loss, thymus atrophy and spleen hypertrophy. Administration of $RTP_1$ (200 mg·kg$^{-1}$, ig) significantly prevented body mass loss. But the effects of DX showed farther body mass loss, thymus atrophy compared with control and $RTP_1$, suggesting severe immunosuppression occurred (Table 3).

### *Effects of $RTP_1$ on $CD4^+$T lymphocytes*

Evidences indicated that $CD4^+$T cells played a key role in the pathogenesis of gastrointestinal tissue damage in ulcerative colitis. According to the results of immunohistochemical and Western-blot of $CD4^+$T lymphocytes isolated from colon tissues, there were a great increase of CD4 lymphocytes in the diseased colon and a significant decrease ofter the treatment with $RTP_1$ (200 mg·kg$^{-1}$ ig). (Figures 2, 3).

**Figure 2** Western-blot analysis of $CD4^+$T cells isolated from mesenteric lymphoid node. Left: Saline, Middle: $RTP_1$, Right: TNBS.

## DISCUSSION

Some plant polysaccharides are known to posses immunorgulatory effects and wide bioactivities[29,30]. Rhubarb has traditionally been used as a folk remedy for gastrointestinal diseases for over two thousand years in China. We hypothesized that polysaccharide isolated from rhubarb might exhibit immune regulation on colitis rats. To test this hypothesis, we extracted polysaccharide from a breed of rhubarb, *Rheum tanguticum* Maxim. ex Regel., then administrated RTP to rats with colitis induced by TNBS. The results showed striking therapeutic effectiveness of RTP on the experimental colitis by attenuating the colon weight increase, decreasing the ulcer area formation, which resulted in a rapid recovery of animals from colitis induced by TNBS-enema. There was no significant difference between RTP and DX on colitis. Besides, RTP had no immunosuppressive side effects as DX.

Ulcerative colitis (UC) is considered to have a multifactorial etiology leading to intricate pathogenic mechanism that results in complex clinical manifestations. In recent years, the established animal models of immunology, genetics and biochemistry have promoted the study on pathogenic mechanism of UC[3]. The animal model of colitis induced by TNBS-enema, used in the present study, was considerated the characteristics as excessive Th1/$CD4^+$T lymphocytes response and similar to human IBD[31]. The TNBS-induced animal model, which was duplicated and cheap, was most commonly used to estimate the therapeutic effectiveness of colitis[3]. Therefore, this TNBS/ethanol-induced animal model may be suitable for investigate the therapeutic effectiveness and mechanism of RTP on colitis.

Colonic lesion and inflammation induced by TNBS-ethanol were accompanied by a drastic elevation of MPO activity in colonic tissues. This enzyme was used as an index of quantitative inflammation and a marker of neutrophil infiltration in the tissue (*Gastroenterology* 1989;96:795-811). In the present study, administration of RTP significantly attenuated the increase of colonic MPO activity, indicating its anti-inflammatory effect. SOD is an antioxidant enzyme that is cytoprotective in many tissues, because it scavenges the overproduced superoxide by stimulants. This study showed that the SOD activity in colonic tissue was significantly increased on days 5 and 7, but returned to normal level on days 10 and 14 after TNBS-enema. It is envisaged that the elevated SOD activity may be a protective reaction in the colonic tissue against the overproduction of superoxide during the early active phase of colonic inflammation. Administration of RTP markedly increased SOD activity after TNBS-enema and kept SOD at a higher level till 14 days. This result indicated that the therapeutic effectiveness of RTP on colitis might be at least in part, through its anti-oxidative effect. However, the underlying mechanisms of the therapeutic effectiveness of RTP need further studies. All these findings implicate that RTP has a very impressive therapeutic effectiveness on experimental colitis, and RTP may be the main component of *Rheum Rheum tanguticum* that is commonly used as a classic Chinese drug against inflammation in gut.

An increasing body of evidence suggests that inflammatory bowel disease (IBD) is due to abnormal function of regulatory T cells required for immunological homeostasis in gut-associated lymphoid tissue (GALT)[32]. CD4+T lymphocytes play an important role in the progressive and perpetuated inflammation of colitis[33,34]. In this study, RTP showed significant effects on resistance to the up-expression of $CD4^+$protein levels, suggesting that the mechanism of RTP on colitis is related to the modulation of $CD4^+$T cell dysfunction. $CD4^+$ T cells containing both $CD45RB^{high}$ and $CD45RB^{low}$ populations participatein as a pathogenic subset that in turn leads to inflammatory reactions selectively in the large intestine, indicating that UC can be cured by maintaining the balance between the two subunits of CD4 and the balance between the cytokines nets[35]. Therefore, $CD4^+$ T cell may be a new potential target of therapeutic drugs on UC patients.

## REFERENCES

1 **Alstead E**. Fertility and pregnancy in inflammatory bowel disease. *World J Gastroenterol* 2001; **7**: 455-459

2 **Kirsner JB**. Historical origins of current IBD concepts. *World J Gastroenterol* 2001; **7**: 175-184
3 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002; **20**: 495-549
4 **Yoshida M**, Shirai Y, Watanabe T, Yamori M, Iwakura Y, Chiba T, Kita T, Wakatsuki Y. Differential localization of colitogenic Th1 and Th2 cells monospecific to a microflora-associated antigen in mice. *Gastroenterology* 2002; **123**: 1949-1961
5 **Elson CO**, Cong Y. Understanding immune-microbial homeostasis in intestine. *Immunol Res* 2002; **26**: 87-94
6 **Cong Y**, Brandwein SL, McCabe RP, Lazenby A, Birkenmeier EH, Sundberg JP, Elson CO. $CD4^+$ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H / HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. *J Exp Med* 1998; **187**: 855-864
7 **Monteleone I**, Vavassori P, Biancone L, Monteleone G, Pallone F. Immunoregulation in the gut: success and failures in human disease. *Gut* 2002; **50**(Suppl 3): III60-64
8 **Cottrez F**, Hurst SD, Coffman RL, Groux H. T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol* 2000; **165**: 4848-4853
9 **Dohi T**, Fujihashi K, Kiyono H, Elson CO, McGhee JR. Mice deficient in Th1- and Th2-type cytokines develop distinct forms of hapten-induced colitis. *Gastroenterology* 2000; **119**: 724-733
10 **Mizoguchi A**, Mizoguchi E, Saubermann LJ, Higaki K, Blumberg RS, Bhan AK. Limited CD4 T-cell diversity associated with colitis in T-cell receptor alpha mutant mice requires a T helper 2 environment. *Gastroenterology* 2000; **119**: 983-995
11 **Gad M,** Brimnes J, Claesson MH. $CD4^+$ T regulatory cells from the colonic lamina propria of normal mice inhibit proliferation of enterobacteria-reactive, disease-inducing Th1-cells from scid mice with colitis**.** *Clin Exp Immunol* 2003; **131**: 34-40
12 **Sandborn WJ**, Targan SR. Biologic therapy of inflammatory bowel disease. *Gastroenterology* 2002; **122**: 1592-1608
13 **Van Montfrans C**, Rodriguez Pena MS, Pronk I, Ten Kate FJ, Te Velde AA, Van Deventer SJ. Prevention of colitis by interleukin 10-transduced T lymphocytes in the SCID mice transfer model. *Gastroenterology* 2002; **123**: 1865-1876
14 **Singh B**, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, Stepankova R, Tlaskalova H, Powrie F. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 2001; **182**: 190-200
15 **Rachmilewitz D**, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, Leoni LM, Raz E. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 2002; **122**: 1428-1441
16 **Xu CT**, Pan BR. Current medical therapy for ulcerative colitis. *World J Gastroenterol* 1999; **5**: 64-72
17 **Jani N**, Regueiro MD. Medical therapy for ulcerative colitis. *Gastroenterol Clin North Am* 2002; **31**: 147-166
18 **Jiang XL**, Cui HF. An analysis of 10218 ulcerative colitis cases in China. *World J Gastroenterol* 2002; **8**: 158-161
19 **Salupere R**. Inflammatory bowel disease in Estonia: a prospective epidemiologic study 1993-1998. *World J Gastroenterol* 2001; **7**: 387-388
20 **Wu HG**, Zhou LB, Shi DR, Liu SM, Liu HR, Zhang BM, Chen HP, Zhang LS. Morphological study on colonic ulcerative colitis treated by moxibustion. *World J Gastroenterol* 2000; **6**: 861-865
21 **Liu L**, Li BL, Mei QB. Effects of *tanguticum Maxim* polysaccharide on acute liver injury of mice. *Disi Junyi Daxue Xuebao* 1999; **20**: 549-551
22 **Liu L**, Mei QB, Zhou SY, Li BL. Effects of *tanguticum Maxim* polysaccharide on antioxidation of mice. *Disi Junyi Daxue Xuebao* 2001; **22**: 530-533
23 **Michell NP**, Lalor P, Langman MJ. Heparin therapy for ulcerative colitis? Effects and mechanisms. *Eur J Gastroenterol Hepatol* 2001; **13**: 449-456
24 **Cui HF**, Jiang XL. Treatment of corticosteroid-resistant ulcerative colitis with oral low molecular weight heparin. *World J Gastroenterology* 1999; **5**: 448-450
25 **Strickland FM**. Immune regulation by polysaccharide: implications for skin cancer. *J Photochem Photobiol B* 2001; **63**: 132-140
26 **Qiu Z**, Jones K, Wylie M, Jia Q, Orndorff S. Modified Aloe barbadensis polysaccharide with immunoregulatory activity. *Planta Med* 2000; **66**: 152-156
27 **Cho CH**, Mei QB, Shang P, Lee SS, So HL, Guo X , Li Y. Studyof the gastrointestineal protective effects of polysaccharides from Angelica sinensis in rats. *Planta Med* 2000; **66**: 348-351
28 **Zheng L**, Gao ZQ, Wang SX. A chronic ulcerative colitis model in rats. *World J Gastroenterol* 2000; **6**: 150-152
29 **Gomez-Flores R**, Calderon CL, Scheibel LW, Tamez-Guerra P, Rodriguez-Padilla C, Tamez-Guerra R, Weber RJ. Immuoenhancing properties of Plantago major leaf extract. *Phytother Res* 2000; **14**: 617-622
30 **Han SB,** Park SH, Lee KH, Lee CW, Lee SH, Kim HC, Kim YS, Lee HS, Kim HM. Polysaccharide isolated from the radix of Platycodon grandiflorum selectively activates B cells and macrophages but not T cells. *Int Immunopharmacol* 2001; **1**: 1969-1978
31 **Strober W**, Fuss IJ, Ehrhardt RO, Neurath M, Boirivant M, Ludviksson BR. Mucosal immunoregulation and inflammatory bowel disease: new insights from murine models of inflammation. *Scand J Immunol* 1998; **48**: 453-458
32 **Kweon MN**, Takahashi I, Yamamoto M, Jang MH, Suenobu N, Kiyono H. Development of antigen induced colitis in SCID mice reconstituted with spleen derived memory type $CD4^{(+)}$ CD45RB $^{(+)}$ T cells. *Gut* 2002; **50**: 299-306
33 **Leithäuser F**, Trobonjaca Z, Moller P, Reimann J. Clustering of colonic lamina propria $CD4^{(+)}$ T cells to subepithelial dendritic cell aggregates precedes the development of colitis in a murine adoptive transfer model. *Lab Invest* 2001; **81**: 1339-1349
34 **Xu D**, Liu H, Komai-Koma M, Campbell C, McSharry C, Alexander J, Liew FY. $CD4^{(+)}CD25^{(+)}$ regulatory T cells suppress differentiation and functions of Th1 and Th2 cells, leishmania major infection, and colitis in mice. *J Immunol* 2003; **170**:394-399
35 **Claesson MH**, Bregenholt S, Bonhagen K,Thoma S, Moller P, Grusby MJ, Leithauser F, Nissen MH, Reimann J. Colitis-inducing potency of $CD4^{(+)}$ T cells in immunodeficient, adoptive hosts depends on their state of activation, IL-12 responsiveness. *J Immunol* 1999; **162**: 3702-3710


**Edited by** Zhu L and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2289-2292
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

# Effect of ischemic preconditioning on P-selectin expression in hepatocytes of rats with cirrhotic ischemia-reperfusion injury

Xiang-Dong Cheng, Xian-Chuan Jiang, Yin-Bing Liu, Cheng-Hong Peng, Bin Xu, Shu-You Peng

**Xiang-Dong Cheng**, Department of Hepatobiliary Surgery, Zhejiang Cancer Hospital,Hangzhou 310022, Zhejiang Province, China
**Xian-Chuan Jiang, Yin-Bing Liu, Cheng-Hong Peng, Bin Xu, Shu-You Peng**, Department of Surgery, Second Affiliated Hospital of Zhejiang University, Hangzhou 310006, Zhejiang Province, China
**Supported by** Science and Technology Fund, Department of Health, Zhejiang Province, No.M-9810
**Correspondence to:** Xiang-Dong Cheng, Department of Hepatobiliary Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, China. chengxd516@sohu.com
**Telephone:** +86-571-88144401-507
**Received:** 2003-04-02 **Accepted:** 2003-05-17

## Abstract

**AIM:** To investigate the effects and mechanisms of ischemic preconditioning (IPC) on the ischemia/reperfusion (I/R) injury of liver cirrhosis in rats and the effect of IPC on P-selectin expression in hepatocytes.

**METHODS:** Forty male SD rats with liver cirrhosis were randomly divided into sham operation group (SO group), ischemia/reperfusion group (I/R group), ischemic preconditioning group (IPC group), L-Arginine preconditioning group (APC group), L-NAME preconditioning group (NPC group), eight rats in each group. Hepatocellular viability was assessed by hepatic adenine nucleotide level and energy charge (EC) determined by HPLC, ALT, AST and LDH in serum measured by auto- biochemical analyzer and bile output. The expression of P-selectin in the liver tissue was analyzed by immunohistochemical technique. Leukocyte count in ischemic hepatic lobe was calculated.

**RESULTS:** At 120 min after reperfusion, the level of ATP and EC in IPC and APC groups was higher than that in I/R group significantly. The increases in AST, ALT and LDH were prevented in IPC and APC groups. The livers produced more bile in IPC group than in I/R group during 120 min after reperfusion (0.101±0.027 *versus* 0.066±0.027 ml/g liver, $P$=0.002). There was a significant difference between APC and I/R groups, ($P$=0.001). The leukocyte count in liver tissues significantly increased in I/R group as compared with SO group ($P$<0.05). The increase in the leukocyte count was prevented in IPC group. Administration of L-arginine resulted in the same effects as in IPC group. However, inhibition of NO synthesis (NPC group) held back the beneficial effects of preconditioning. Significant promotion of P-selectin expression in hepatocytes in the I/R group was observed compared with the SO group ($P$<0.01). IPC or L-arginine attenuated P-selectin expression remarkably ($P$<0.01). However, inhibition of NO synthesis enhanced P-selectin expression ($P$<0.01). The degree of P-selectin expression was positively correlated with the leukocyte counts infiltrating in liver ($r$=0.602, $P$=0.000).

**CONCLUSION:** IPC can attenuate the damage induced by I/R in cirrhotic liver and increase the ischemic tolerance of the rats with liver cirrhosis. IPC can abolish I/R induced leukocyte adhesion and infiltration by preventing post-ischemic P-selectin expression in the rats with liver cirrhosis via a NO-initiated pathway.

Cheng XD, Jiang XC, Liu YB, Peng CH, Xu B, Peng SY. Effect of ischemic preconditioning on P-selectin expression in hepatocytes of rats with cirrhotic ischemia-reperfusion injury. *World J Gastroenterol* 2003; 9(10):2289-2292
http://www.wjgnet.com/1007-9327/9/2289.asp

## INTRODUCTION

Ischemic preconditioning (IPC) refers to a phenomenon in which a tissue is rendered resistant to the deleterious effects of prolonged ischemia and reperfusion (I/R) by prior exposure to a short period of vascular occlusion. This phenomenon was first demonstrated in the heart a decade ago[1] and has been the subject of intensive investigation ever since. Although it is clear that activation of adenosine receptors and protein kinase C (PKC) is critical to the development of the beneficial action of IPC, the downstream effectors in the signaling cascade initiated by IPC are uncertain. Akimisn *et al*[2] and Kubes *et al*[3] have demonstrated that IPC prevents intestinal and skeletal muscle I/R injury by inhibiting postischemic leukocyte-endothelial cell interaction. However, identification of the end effectors of the ant adhesive effects of IPC remains unclear. A likely candidate effector molecule that may be targeted by the signaling cascade initiated by IPC is P-selectin, because post-ischemic leukocyte rolling (and thus subsequent stationary adhesion and emigration) is critically dependent on the expression of P-selectin on venular endothelium[4]. IPC has been commonly studied in the heart, but few studies have been performed on cirrhotic liver IPC. This study was aimed to determine the effects and mechanisms of IPC on the I/R injury rats with liver cirrhosis and the effect of IPC on the expression of P-selectin.

## MATERIALS AND METHODS

### *Reproduction of rat cirrhotic liver model*

Sprague-Dawley (SD) Male rats initially weighing 200±20 g were used.

Subcutaneous injection of 60 % $Ccl_4$ (0.3 mg/kg) was made once every 4 days for 8 weeks and 5% ethanol was allowed for 60 days[5].

### *Operative procedure*

At first, ligamentous attachments around the liver were dissected. The common bile duct was then cannulated and bile output was measured. Ischemia was induced in the median and left lateral hepatic lobes by clamping the corresponding hepatic arterial and portal vein, while the blood flowing to the other lobe was left intact. When the assigned period of warm ischemia was completed, the clamp was removed and the pedicles to the non-ischemic lobe were ligated[6].

### *Grouping of animals*

Forty male SD rats with liver cirrhosis were divided into 5

groups randomly, eight rats in each group. Animals in sham Operation group (SO group) were subjected to anesthesia and laparotomy. Animals in ischemia/reperfusion group (I/R group) were subjected to 30 min of left and middle lobe hepatic ischemia, followed by 120 min of reperfusion. Animals in ischemic preconditioning group (IPC group) were same as I/R group, but subjected to 10 min of ischemia and 5 min of reperfusion prior to I/R. Animals in L-arginine preconditioning group (APC group) were same as IPC group, but treated with a continuous intravenous infusion of L-Arginine (10 mg/kg, portal vein) for 5 min before preconditioning. Animals in L-NAME preconditioning group (NPC group) were same as IPC group, but treated with a continuous intravenous infusion of L-NAME (10 mg/kg, portal vein) for 5 min before preconditioning.

The animals were killed after blood samples were collected from the inferior vena cava after 120 min of reperfusion. Liver samples were excised from the anterior edge of the median lobe before ischemia, after the induction of ischemia and 120 min after reperfusion respectively. The specimens were immersed in liquid nitrogen immediately after sampling to measure the tissue concentration of adenine nucleotides, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP) and total adenosine nucleotide. At 120 min after reperfusion, livers were perfused and fixed *in situ* with 4 % para formaldehyde.

### Energy metabolism

ATP and its metabolites, ADP and AMP in the liver tissue were assayed as follows. After the frozen tissue with a mortar and pestle was ground, the powder was mixed with 1 ml 6 % perchloric acid at 4 ℃. The mixture was centrifuged at 3 000 rpm for 10 min and the supernatant was stored at 4 ℃, then 0.5 ml 6 % perchloric acid was added to the precipitate and centrifuged in the same manner. The supernatant was neutralized with 3 mol/l potassium carbonate and centrifuged at 3 000 rpm for 10 min. The final supernatant was used as a sample for ATP and its metabolites. The tissue concentration of ATP and its metabolites was determined by high-performance liquid chromatography ( HPLC). Energy charge (EC) was equal to (ATP+1/2ADP)/(ATP+ADP+AMP)[7].

### Measurement of serum cytosolic enzymes

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured at 4 ℃ using commercially available kits (Horizon, American) by an auto-biochemistry analyzer.

### Measurement of bile output

Bile output from the ischemic liver was measured through a choledochotomy tube placed in the common bile duct.

### P-selectin expression in liver tissues

Immunohistochemical staining for P-selectin protein was performed using SP technique[8]. The immunostaining of P-selectin was visually classified into four groups: no staining present in any tumor cells (-), slight staining in most of the hepatocytes (+), most of the hepatocytes with moderate staining (++), and strong staining in most of the hepatocytes (+++). Two senior pathologists who did not know the clinicopathological data did the classification.

### Histological examination

Liver samples were excised from the anterior edge of the median lobe 120 min after reperfusion. Small portions (0.5 cm×0.5 cm) were fixed immediately in 4 % buffered para formaldehyde (pH 7.2) and embedded in paraffin. These portions were cut into 4 μm thick sections and stained with hematoxylin and eosin (H & E). Leukocyte count in ischemic hepatic lobe could be calculated randomly under microscopy(×400).

### Statistical analysis

The results were expressed as $\bar{x}\pm s$. The one-way NOVA and H test were used for statistical significance of differences between groups. Correlation analysis between two factors was made by Spearman method. $P<0.05$ was considered significant.

## RESULTS

### Change of ATP, ADP, AMP and EC levels in liver after ischemia and reperfusion

At 30 min of hepatic inflow occlusion, the ATP and EC levels in liver tissues were significantly decreased in I/R, IPC, APC and NPC groups ($P<0.05$). At 120 min after reperfusion, the ATP and EC levels in IPC and APC groups were significantly higher than those in I/R group ($P=0.000$, $P=0.001$). But there was no significant difference between NPC and I/R groups ($P>0.05$) (Table 1).

### Change of ALT, AST and LDH in serum

Significant increases of ALT, AST and LDH levels in serum were observed in the group subjected to ischemia and reperfusion (I/R group) in comparison with the control group (SO group). When ischemia was preceded by 10 min of ischemia and 5 min of reperfusion (IPC), the increases of AST, ALT and LDH in serum were prevented ($P=0.000$). Administration of L-Arginine (APC group) resulted in the same effects on ALT, AST and LDH as above ($P=0.001$). However, infusion of L-NAME (NPC group) inhibited the beneficial effects of preconditioning (Table 2).

### Results of bile output and leukocyte count in ischemic hepatic lobe

The livers produced more bile in IPC group than in I/R group during 120 min after reperfusion (0.101±0.027 *versus* 0.066±0.027 ml/g liver, $P=0.002$). There was a significant difference between APC and I/R, NPC and SO groups ($P=0.001$, $P=0.000$) respectively. However, there was no significant

**Table 1** ATP, ADP, AMP and EC levels in liver after ischemia and reperfusion (U/L)

| Groups | *n* | After ischemia | | | | After reperfusion | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ATP | ADP | AMP | EC | ATP | ADP | AMP | EC |
| SO | 8 | 5.4±1.3 | 3.1±0.8 | 1.0±0.2 | 0.7±0.0 | 5.5±0.8 | 3.2±1.0 | 1.0±0.1 | 0.7±0.0 |
| I/R | 8 | 0.5±0.2[b] | 2.3±0.6[a] | 3.5±1.0[b] | 0.3±0.0[b] | 1.5±0.6[b] | 2.3±1.2 | 2.6±1.3[a] | 0.4±0.1[b] |
| IPC | 8 | 0.5±0.1[b] | 2.1±0.5[a] | 3.6±1.5[b] | 0.3±0.1[b] | 4.1±1.6[ac] | 2.3±0.8 | 1.9±0.9 | 0.6±0.1[ac] |
| APC | 8 | 0.5±0.1[b] | 2.2±0.5[a] | 3.4±0.7[b] | 0.3±0.0[b] | 4.0±1.6[ac] | 2.5±1.1 | 2.2±1.2[a] | 0.6±0.1[ac] |
| NPC | 8 | 0.5±0.2[b] | 2.0±0.7[a] | 3.3±0.6[b] | 0.3±0.0[b] | 2.3±1.6[b] | 2.2±0.9[a] | 3.2±1.1[b] | 0.4±0.1[b] |

[a]$P<0.05$, [b]$P<0.01$, *vs* SO group; [c]$P<0.01$, *vs* I/R group.

difference between NPC and I/R groups ($P$>0.05). The leukocyte counts in liver tissue showed a more significant increase in I/R group than in SO group ($P$=0.000). The increase in leukocytes count was inhibited in IPC group ($P$=0.028). Administration of L-arginine resulted in the same effects as in IPC group ($P$=0.020). However, inhibition of NO synthesis (NPC group) prevented the beneficial effects of preconditioning ($P$>0.05) (Table 3).

**Table 2** Results of ALT, AST and LDH in serum after reperfusion (U/L)

| Groups | Cases | ALT | AST | LDH |
|---|---|---|---|---|
| SO | 8 | 300.5±159.2 | 551.1±84.7 | 4 612.3±1 042.8 |
| I/R | 8 | 2 218.8±825.3[a] | 3 043.8±1 198.9[a] | 13 762.5±5 371.9[a] |
| IPC | 8 | 568.8±214.6[b] | 1 315.0±958.9[b] | 6 266.3±2 425.5[b] |
| APC | 8 | 508.8±142.8[b] | 1 108.8±637.2[b] | 5 355.0±1 237.9[b] |
| NPC | 8 | 2 091.3±684.6[a] | 3 083.8±844.5[a] | 11 361.3±4 211.8[a] |

[a]$P$<0.01, *vs* SO group; [b]$P$<0.01, *vs* I/R group.

**Table 3** Results of bile output and leukocyte count in ischemic hepatic lobe

| Groups | Cases | Bile output (ml/g liver) | Leukocyte count (piece/HP) |
|---|---|---|---|
| SO | 8 | 0.15±0.02 | 181.38±59.23 |
| I/R | 8 | 0.07±0.03[a] | 442.38±94.10[a] |
| IPC | 8 | 0.10±0.03[ab] | 353.00±84.11b[a] |
| APC | 8 | 0.10±0.02[ab] | 347.75±51.53[ba] |
| NPC | 8 | 0.07±0.01[a] | 407.88±90.40[a] |

[a]$P$<0.01, *vs* SO group; [b]$P$<0.05, *vs* I/R group.

### *Results of P-selectin expression in liver tissues*

Significant promotion of P-selectin expression in hepatocytes in the I/R group was observed in comparison with the SO group ($P$=0.000). IPC or L-arginine attenuated P-selectin expression significantly ($P$=0.005). However, inhibition of NO synthesis enhanced the expression of P-selectin ($P$=0.001) (Table 4).

**Table 4** Expression of P-selectin in liver tissues

| Groups | Cases | Grade | | | Average rank |
|---|---|---|---|---|---|
| | | (+) | (++) | (+++) | |
| SO | 8 | 7 | 1 | 0 | 10.69 |
| I/R | 8 | 0 | 5 | 3 | 30.50[a] |
| IPC | 8 | 5 | 3 | 0 | 15.06[b] |
| APC | 8 | 4 | 4 | 0 | 17.25[b] |
| NPC | 8 | 0 | 6 | 2 | 29.00[a] |

[a]$P$<0.01, *vs* SO group; [b]$P$<0.05, *vs* I/R group.

### *Correlation between leukocytes infiltration and P-selectin expression in liver tissues*

Leukocytes infiltration was significantly correlated with P-selectin expression in liver tissues. The degree of P-selectin expression was positively correlated with the counts of leukocyte infiltration in liver ($r$=0.602, $P$=0.000).

## DISCUSSION

IPC is a unique phenomenon which attenuates organ injury caused by I/R. This is accomplished through a brief preceding episode of vascular occlusion which renders these tissues resistant to the deleterious effects of prolonged ischemia and reperfusion. The protective effects of IPC have been well documented in the previous studies involving different tissues and organs. These included cardicmuscle[1,9], skeletal muscle[2], small intestines[10] and more recently the liver[11]. Although the mechanism of IPC is still unclear up to now, several potential mediators (nitrogen monoxide, adenosine, oxide radical, bradykinin and so on) have been found to play different roles in different organs[11-14]. Adenosine and protein kinase C (PKC) were critical to the beneficial actions of IPC in the heart[15]. IPC-induced adenosine $A_1$-receptor stimulation during the period of preconditioning ischemia increased phospholipose C (PLC) activity, an event that is coupled by pertussis toxin-sensitive G proteins[12,13]. Activation of PLC induced the formation of diacylglycerol, which in turn promotes the translocation and activation of PKC. Activation of PKC stimulated the activation of ATP-sensitive potassium (Katp) channels, and the beneficial actions of IPC in the heart were induced[15], while adenosine stimulated NO production in IPC to protect against the injury associated with I/R in liver[16]. In the case of the cirrhotic liver, our work revealed that the ATP and EC levels in IPC group were higher than those in I/R group. There was significantly more bile produced by the livers in IPC group too. However, the increase of AST, ALT and LDH release was attenuated, when IPC was performed before ischemia. This fact shows the protective effect of IPC on preventing ischemia-reperfusion damage of cirrhotic liver. In addition, we found that L-arginine administration in hepatic ischemia reperfusion attenuated the injury in a manner similar to that of IPC. Accordingly, inhibition of NO synthesis abolished the beneficial effects of IPC. Thus, our data suggest that NO is one of the potential mediators of the protective effects of IPC.

Akimisn.[2] and Kubes[3] demonstrated that IPC prevented intestinal and skeletal muscle I/R injury by inhibiting postischemic leukocyte-endothelial cell interactions. These observations are important because they indicate that in addition to protecting against the deleterious effects of ischemia and reperfusion, IPC could induce cellular changes that also prevent leukocyte recruitment to ischemic tissues. This might limit the reperfusion component of I/R injury, which was primarily leukocyte dependent in the small intestine and other organs[17,18]. Thus, in addition to $K^+$-ATP channels, IPC appears to target effector molecules that modulate the inflammatory response to I/R. A likely candidate effector molecule that may be targeted by the signaling cascade initiated by IPC is P-selectin, for several researches have shown that I/R injury is a leukocyte-mediated event resulted from a cascade of acute phase reactants causing leukocyte-endothelial cell interactions. The interactions progressed from rolling to saltation to firm adhesion with subsequent tissue infiltration and organ injury[19-24]. The cascade of acute phase reactants was critically dependent on the expression of P-selectin on venular endothelium[4,25]. In our study, significant promotion of the expression of P-selectin in hepatocytes in the I/R group was observed in comparison with the SO group. IPC or L-arginine attenuated P-selectin expression significantly. However, inhibition of NO synthesis enhanced the expression of P-selectin. The increase in the leukocytes count was prevented in IPC group. Administration of L-arginine resulted in the same effects as in IPC group. In the mean time, there was a significant correlation between leukocytes infiltration and P-selectin expression in liver tissues. The degree of P-selectin expression was positively correlated with the counts of leukocyte infiltration in liver.

In summary, IPC can abolish I/R induced leukocyte adhesion and infiltration by preventing postischemic P-selectin expression in rats with liver cirrhosis via a NO-initiated pathway.

## REFERENCES


1 **Murry CE**, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74**: 1124-1136
2 **Akimitsu T**, Gute DC, Korthuis RJ. Ischemic preconditioning attenuates postischemic leukocyte adhesion and emigration. *Am J Physiol* 1996; **271**(5Pt 2): H2052-2059
3 **Kubes P**, Payne D, Ostrovsky L. Preconditioning and adenosine in I/R-induced leukocyte-endothelial cell interactions. *Am J Physiol* 1998; **274**(4Pt 2): H1230-H238
4 **Kurose I**, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR, Granger DN. Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. *Circ Res* 1994; **74**: 336-343
5 **Wu MC**, Yang GS. Reproduction of cirrhotic liver of rat. *Zhonghua Shiyan Waike Zazhi* 1984; **1**: 145-147
6 **Canada AT**, Stein K, Martel D, Watkins WD. Biochemical appraisal of models for hepatic ischemic/reperfusion injury. *Circ Shock* 1992; **36**: 163-168
7 **Shimabukuro T**, Ymamoto Y, Kume M, Kimoto S, Okamoto R, Morimoto T, Yamaoka Y. Induction of heat shock response: effect on the rat liver with carbon tetrachloride-induced fibrosis from ischemia-reperfusion injury. *World J Surg* 1998; **22**: 464-469
8 **Wang D**, Shi JQ, Liu FX. Immunohistochemical detection of proliferating cell nuclear antigen carcinoma. *China Natl J New Gastroenterol* 1997; **3**: 101-103
9 **Yellon DM**, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. *Lancet* 1993; **342**: 276-277
10 **Jerome SN**, Akimitsu T, Gute DC, Korthuis RJ. Ischemic preconditioning attenuates capillary no-reflow induced by prolonged ischemia and reperfusion. *Am J Physiol* 1995; **268**(5Pt 2): H2063-2067
11 **Ferencz A**, Szanto Z, Borsiczky B, Kiss K, Kalmar-Nagy K, Szeberenyi J, Horvath PO, Roth E. The effects of preconditioning on the oxidative stress in small-bowel autotransplantation. *Surgery* 2002; **132**: 877-884
12 **Downey JM**, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: the role of adenosine and protein kinase C. *Ann NY Acad Sci* 1994; **723**: 82-98
13 **Ishida T**, Yarimizu K, Gute DC, Korthuis RJ. Mechanisms of ischemic preconditioning. *Shock* 1997; **8**: 86-94
14 **Laude K,** Beauchamp P, Thuillez C, Richard V. Endothelial protective effects of preconditioning. *Cardiovasc Res* 2002; **55**: 466-473
15 **Gross GJ**, Fryer RM. Sarcolemmal versus mitochondrial ATP-sentitive $K^+$ channels and myocardial preconditioning. *Circ Res* 1999; **84**: 973-979
16 **Peralta C**, Hotter G, Closa D, Gelpi E, Bulbena O, Rosello-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: role of nitric oxide and adenosine. *Hepatology* 1997; **25**: 934-937
17 **Granger DN**, Korthuis RJ. Physiologic mechanisms of postischemic tissue injury. *Annu Rev Physiol* 1995; **57**: 311-332
18 **Gute DC**, Ishida T, Yarimizu K, Korthuis RJ. Inflammatory responses to ischmia and reperfusion in skeletal muscle. *Mol Cell Biochem* 1998; **179**: 169-187
19 **Sanz MJ**, Johnston B, Issekutz A, Kubes P. Endothelin-1 causes P-selectin-dependent leukocyte rolling and adhesion within rat mesenteric microvessels. *Am J Physiol* 1999; **277**(5Pt 2): H1823-H1830
20 **Cotran RS**, Pober JS. Cytokine-endothelial interactions in inflammation, immunity, and vascular injury. *J Am Soc Nephrol* 1990; **1**: 225-235
21 **Bienvenu K**, Granger DN. Molecular determinants of shear rate-dependent leukocyte adhesion in postcapillary venules. *Am J Physiol* 1993; **264**(5Pt 2): H1504-H1508
22 **Springer TA**. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994; **76**: 301-314
23 **Jaeschke H,** Smith CW. Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* 1997; **61**: 647-653
24 **Lefer AM**, Tsao PS, Lefer DJ, Ma XL. Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. *FASEB J* 1991; **5**: 2029-2034
25 **Lefer DJ,** Flynn DM, Anderson DC, Buda AJ. Combined inhibition of P-selectin and ICAM-1 reduces myocardial injury following ischemia and reperfusion. *Am J Physiol* 1996; **271**(6Pt 2): H2421-H2429


**Edited by** Ma JY

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2293-2299
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

# Pan-enteric dysmotility, impaired quality of life and alexithymia in a large group of patients meeting *ROME* II criteria for irritable bowel syndrome

Piero Portincasa, Antonio Moschetta, Giuseppe Baldassarre, Donato F. Altomare, Giuseppe Palasciano

**Piero Portincasa, Antonio Moschetta, Giuseppe Palasciano,** Section of Internal Medicine, Department of Internal Medicine and Public Medicine (DIMIMP), University Medical School, Bari
**Giuseppe Baldassarre,** Division of Geriatrics, Hospital "*Miulli*", Acquaviva delle Fonti, Bari, Italy
**Donato F. Altomare,** Section of Surgery, Department of Emergency and Organ Transplant (DETO), University Medical School, Bari, Italy
**Correspondence to:** Piero Portincasa, MD, PhD, Chair Person of Semeiotica Medica-Section of Internal Medicine, Department of Internal and Public Medicine (DIMIMP), University of Bari Medical School-Policlinico-70124 Bari-Italy. p.portincasa@semeiotica.uniba.it
**Telephone:** +39-80-5478-227 **Fax:** +39-80-5478-232
**Received:** 2003-05-10 **Accepted:** 2003-08-02

## Abstract

**AIM:** Psychological factors, altered motility and sensation disorders of the intestine can be variably associated with irritable bowel syndrome (IBS). Such aspects have not been investigated simultaneously. The aim of this paper was to evaluate gastrointestinal motility and symptoms, psychological spectrum and quality of life in a large group of IBS patients in southern Italy.

**METHODS:** One hundred IBS patients (F:M=73:27, age 48±2 years, mean±SE) fulfilling ROME II criteria matched with 100 healthy subjects (F:M=70:30, 45±2 years). Dyspepsia, bowel habit, alexithymia, psycho-affective profile and quality of life were assessed using specific questionnaires. Basally and postprandially, changes in gallbladder volumes and antral areas after liquid meal and orocaecal transit time (OCTT) were measured respectively by ultrasonography and $H_2$-breath test. Appetite, satiety, fullness, nausea, and epigastric pain/discomfort were monitored using visual-analogue scales.

**RESULTS:** Compared with controls, IBS patients had increased dyspepsia (score 12.6±0.7 *vs* 5.1±0.2, $P<0.0001$), weekly bowel movements (12.3±0.4 *vs* 5.5±0.2, $P<0.00001$, comparable stool shape), alexithymia (score 59.1±1.1 *vs* 40.5±1.0, $P=0.001$), poor quality of life and psycho-affective profile. IBS patients had normal gallbladder emptying, but delayed gastric emptying ($T_{50}$: 35.5±1.0 *vs* 26.1±0.6 min, $P=0.00001$) and OCTT (163.0±5.4 *vs* 96.6±1.8 min, $P=0.00001$). Fullness, nausea, and epigastric pain/discomfort were greater in IBS than in controls.

**CONCLUSION:** ROME II IBS patients have a pan-enteric dysmotility with frequent dyspepsia, associated with psychological morbidity and greatly impaired quality of life. The presence of alexithymia, a stable trait, is a novel finding of potential interest to detect subgroups of IBS patients with different patterns recoveed after therapy.

Portincasa P, Moschetta A, Baldassarre G, Altomare DF, Palasciano G. Pan-enteric dysmotility, impaired quality of life and alexithymia in a large group of patients meeting *ROME* II criteria for irritable bowel syndrome. *World J Gastroenterol* 2003; 9(10):2293-2299

http://www.wjgnet.com/1007-9327/9/2293.asp

## INTRODUCTION

Irritable bowel syndrome (IBS) is a common, chronic biopsychological functional disorder of unknown aetiology, affecting 10-20 % of all individuals any one time. Psychological factors, altered motility and sensation disorders of the intestine can be variably associated[1]. Dysfunctions of the gastrointestinal tract in IBS appear as altered bowel function[2], associated with pain or discomfort, without organic disease[3-5]. Besides lowered visceral perception thresholds involving the rectum[6] and more proximal districts[7], motility defects seem to develop. Thus, IBS patients may complain of a constellation of both gastrointestinal and extra-intestinal symptoms[7], and a significant proportion of patients may have disordered perception[8,9] resulting in more readily feeling of normal intestinal contractions[10]. Psychological factors, such as abnormal illness attitute[11], may also play a role in the pathogenesis of IBS[5], with a significant impact on health-related quality of life (HRQOL)[12]. More recently, revised diagnostic criteria for IBS have been proposed (ROME II criteria)[4]. Moreover, sophisticated questionnaires are now available for studying both quality of life and specific psychological aspects like the alexithymia construct. The latter includes difficulties in identifying and describing feelings, impoverishment of fantasy life and excessive preoccupation with physical symptoms and external events[13].

Such aspects have not been investigated simultaneously in IBS patients. Therefore, the aim of the present study was to evaluate gastrointestinal motility and symptoms, psychological status, including alexithymia and HRQOL in a large group of IBS patients from a referral center in southern Italy.

## MATERIALS AND METHODS

### *Subjects*

A total number of 200 adult subjects were studied prospectively and divided into 2 cohorts: 100 consecutive patients with IBS (27 males and 73 females) and 100 healthy subjects (30 males and 70 females) with their age and body size matched. The two groups were also comparable for years of education (9.3±1.5 years and 9.6±1.3 years in healthy and IBS, respectively). The characteristics of patients are depicted in Table 1. There was no difference in sex ratio, age and body size. The percentage of non-coffee drinkers and smokers was greater in IBS than in healthy subjects ($0.02<P<0.03$). The IBS group was composed of out-patients, with a firm diagnosis through positive identification of the ROME II diagnostic criteria[4]. With the inclusion criteria used in this study, the specificity of IBS diagnosis was about 98 %[14,15]. Mean years since first diagnosis have been 5.9 years with bowel habit alternating between diarrhea and constipation, according to standardized criteria[4]. During the above mentioned period, patients had tried several -albeit poorly effective- therapeutic trials with the help of their family practices. All patients were symptomatic at the time of the study, and lower abdominal discomfort or pain was the main reason for seeking medical advice, and none was on specific therapy for IBS at the time of evaluation. There was no coexistent disease and all patients had normal haematology, biochemistry, urinalysis, together with a normal colonoscopy

or barium enema if aged over 50 years. Excluded were subjects if they had a history of gastrointestinal surgery. Female subjects were excluded if they were pregnant, breast feeding, or hysterectomised and were studied during the first phase of the menstrual cycle or while taking oestrogen/progesterone contraceptive medication. Drugs and cigarette smoking were not allowed for 48 hours prior to the study while alcohol and caffeine containing drinks were stopped 24 hours prior to the study. All subjects smoked less than 5 cigarettes per day and drank below the recommended safe alcohol limit (that is less than 21 units/week).

Because lactose maldigestion-intolerance and IBS may have almost identical symptoms[16], all patients were screened by lactose $H_2$-breath test, which was invariably negative. Healthy volunteers were recruited from staff members and with the help of local family practices. Laboratory investigations were normal (as above) in all healthy subjects and there was a negative toxicology for substances of abuse. All subjects gave informed consent to join the study which was approved by the Ethics Committee of Bari University Hospital.

### Measures

Symptomatology was assessed by focusing on an estimate of the maximum degree of lower abdominal pain or discomfort, and abdominal bloating in the previous 12 months was assessed by visual analogue scales (VAS)[17]. The weekly frequency of bowel movements over a time span of one month was estimated by a self-assessed diary. A specific stool form scale was also used based on a semi-quantitative score[18]. To carefully characterise the association between IBS and functional dyspepsia[19], the presence and severity of dyspeptic symptoms were quantified in two ways: validated semiquantitative score[20]; and self-assessed VAS of upper gastrointestinal perception monitoring appetite, satiety, nausea, abdominal fullness and upper abdominal (epigastric) pain or discomfort[21]. In the latter case, scores were obtained at baseline (*i.e.* time 0) and at 15, 30, 45, 60, 90 and 120 min postprandially. The Middlesex Hospital Questionnaire (MHQ) symptom check list was used to investigate six symptoms: anxiety, phobic behavior, obsessive-compulsive behavior, somatization, depression, and hysteria[22]. Traits of alexithymia were assessed by the Toronto Alexithymia scale based on a 20-item scale (TAS-20)[23] with the validated Italian translation[24,25]. A final score $\geqslant$61 was considered positive for alexithymia, while a score ranging from 50 to 60 was "border line"[25]. The short form check list (SF-36) was used to assess HRQOL in the following 8 domains: general health, physical functioning, role-physical, role-emotion, social functioning, mental health, body pain, and vitality[26,27].

### Motility studies

After an overnight fast, subjects attended the Functional Investigations Unit. Standard criteria recently reviewed by our group[28] were used to study gallbladder and gastric emptying by functional ultrasonography in response to a liquid test meal, consisting of 200 mL solution totalling 13 g (39 %) fat, 10 g (13 %) protein and 35 g (48 %) carbohydrates, calorie content 1 270 kJ, 365 mmol /L (*Nutridrink*®, Nutricia, Milano, Italy), which was consumed within 2 min. Gallbladder emptying was assessed by monitoring fasting and postmeal course of gallbladder volumes. Sagittal and transverse scans of the gallbladder at its largest dimension were obtained at 5-15 minute intervals over 2 hours. Gastric emptying was assessed by monitoring fasting and postmeal course of antral areas[17,29]. Oro-caecal transit time was measured simultaneously with ultrasonographic studies by hydrogen breath test using 10 g lactulose and collecting the breath with a portable equipment (EC60-Gastrolyzer, Bedfont, USA)[30].

### Statistical analysis

All calculations were performed with the *NCSS 2001* statistical software (Kaysville, UT, USA, see http://www.ncss.com/). Results were given as mean ± standard error (SE). Differences in emptying curves were evaluated by two way *ANOVA* repeated-measures followed by Fisher's LSD multiple comparison test. Differences of means between healthy and IBS were evaluated using the Student's *t* test for unpaired data. Linear regression analysis was performed by the method of least square. The chi-square test was used to assess associations between categorical data. A two-tailed probability (*P*) value of less than 0.05 was considered statistically significant[31,32].

## RESULTS

All subjects tolerated the tests well. Table 1 also shows that VAS scores for lower abdominal pain and bloating were significantly higher in IBS patients than in healthy subjects. Whereas the frequency of bowel movements was greater in IBS patients than in healthy subjects (12.3±0.4 *vs* 5.5±0.2 evacuations/wk, *P*=0.00001) (Figure 1A), stool form was similar (Buckley score: 3.9±0.1 *vs* 3.5±0.1 in patients and controls, respectively),(Figure 1B).

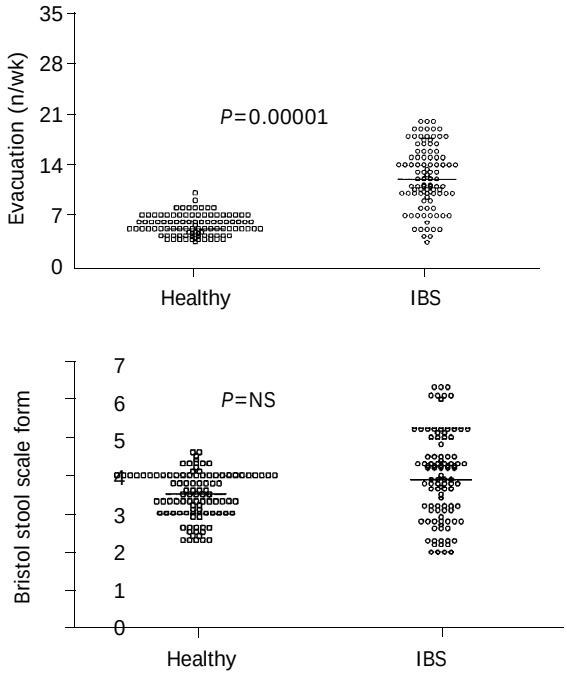



**Figure 1** A: Significantly more bowel movements in IBS patients than in healthy subjects. B: Comparable score for stool form between IBS patients and healthy subjects. Results are expressed as individual data and median (horizontal line).

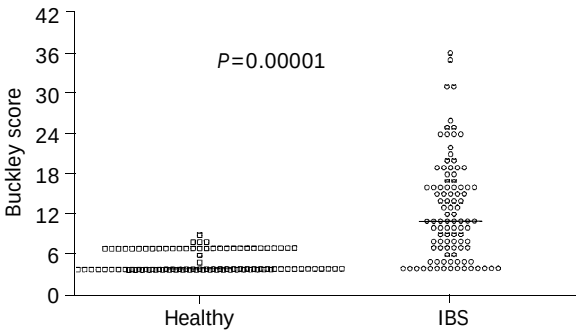



**Figure 2** Significant dyspepsia in IBS patients compared with healthy subjects. Results are expressed as individual data and median.

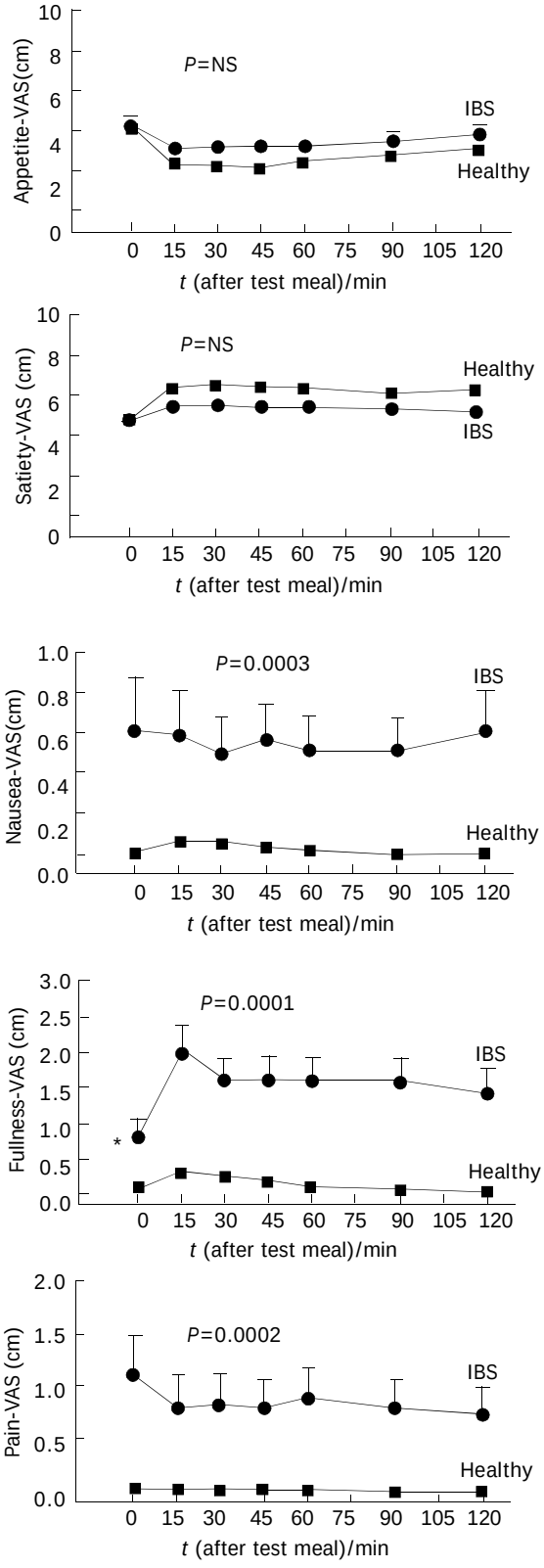



**Figure 3** Time-course of visual analogue scale (VAS) for appetite, satiety, nausea, abdominal fullness and epigastric pain (or discomfort) in healthy subjects. On the X-axis time "0" is before ingestion of test meal. Asterisks indicate significant differences ($0.0001<P<0.001$) of IBS patients *vs* healthy subjects at baseline. Significant differences for nausea, fullness and epigastric pain / discomfort of IBS patients *vs* healthy subjects (area under curve).

Mean score of dyspepsia was greater in patients than in healthy subjects (12.6±0.7 *vs* 5.1±0.2, $P<0.0001$), an abnormal score was found in 75 % and 5 % of patients and healthy subjects, respectively ($P<0.0001$, Figure 2). As expected, the perception of satiety and appetite (both at fasting and as postprandial AUC) showed a strong negative correlation in IBS patients and healthy subjects ($0.91<r<0.92$, $P<0.0001$). There was no difference in appetite and satiety between IBS patients and healthy subjects (either at baseline and postprandially), By contrast, the AUC during 120 min was invariably greater in IBS than in control subjects for nausea (58.0±14.1 *vs*3.9±1.0, $P=0.002$), fullness (133.5±26.1 *vs* 17.1±1.9, $P=0.00002$) and pain/discomfort (75.1±24.2 *vs* 2.5±0.6, $P=0.002$). Time-dependent profiles for these scores are depicted in Figure 3.

**Table 1** Patient characteristics in study group (mean ± SE)

| | IBS | Healthy | *P** |
|---|---|---|---|
| No of subjects | 100 | 100 | |
| Female: Male ratio | 73:27 | 70:30 | NS |
| Age (yr) | 48±2 | 45±2 | NS |
| BMI (Kg / m$^2$) | 23.5±0.4 | 22.9±0.3 | NS |
| Coffee-drinkers No (%) | | | |
| None | 43 (43%) | 24 (24%) | 0.03° |
| <1 per day | 9 (9%) | 22 (22%)° | |
| 1-5 per day | 48 (48%) | 54 (54%)° | |
| Smokers No (%) | 30 (30%) | 15 (15%) | 0.02° |
| Lower abdominal pain (VAS, cm) | 5.8±0.1 | 0.04±0.01 | 0.0001 |
| Abdominal bloating (VAS, cm) | 6.2±0.1 | 0.1±0.02 | 0.0001 |

*Student's *t* test or $\chi^2$ for comparison of proportions, VAS=visual analogue scale.

Results from the MHQ questionnaire are given in Figure 4A which shows that IBS patients had significantly higher scores for anxiety, somatization, phobia and depression than healthy subjects. Results from the TAS 20 questionnaire on alexithymia are given in Figure 4B which shows a significantly increased score in IBS patients (59.1±1.1) compared to healthy subjects (40.5±1.0). An abnormal (*i.e.*>61) score for alexithymia was found in 43 % of patients and in 2 % of healthy subjects. Mean scores of SF-36 are reported in Figure 5 which shows that HRQOL was significantly poorer for all domains in IBS patients compared with healthy subjects. There was no difference between IBS patients and healthy subjects with respect to gallbladder motility, *i.e.* fasting and postprandial volumes and half-emptying times (Table 2). Concerning gastric motility, the mean cross-sectional antral areas at fasting and immediately after the test meal were comparable between IBS patients and healthy subjects (Table 2). However, postprandial minimal antral areas were significantly larger and half-emptying time longer in IBS than in healthy subjects (Table 2, Figure 6). With respect to OCTT, basal levels of $H_2$ were invariably <10 p.p.m. in all subjects (*i.e.* absence of bacterial overgrowth) and did not differ between IBS patients and healthy subjects. OCTT, however, was increased by 62 % in IBS patients compared to healthy subjects (Table 2) with scattered distribution depicted in Figure 7. A value of OCTT above 130 min, representing the upper limit of normal (mean+2SDs), was found in 70 % of patients and in 2 % of healthy subjects ($P=0.000001$).

Overall, the score of dyspepsia was positively correlated with OCTT ($r=0.38$, $P=0.0001$) but not with gastric emptying speed. Also, the dyspepsia score was negatively correlated with all domains of HRQOL ($-0.34< r <-0.43$, $P<0.001$), while there was a positive correlation with somatization ($r=0.32$, $P=0.001$), anxiety ($r=0.29$, $P=0.002$) and alexithymia ($r=0.47$, $P=0.00001$). Frequency of bowel movements increased with anxiety ($r=0.39$,

*P*=0.00001), somatization (*r*=0.34, *P*=0.0004) and alexithymia (*r*=0.52, *P*=0.000001).

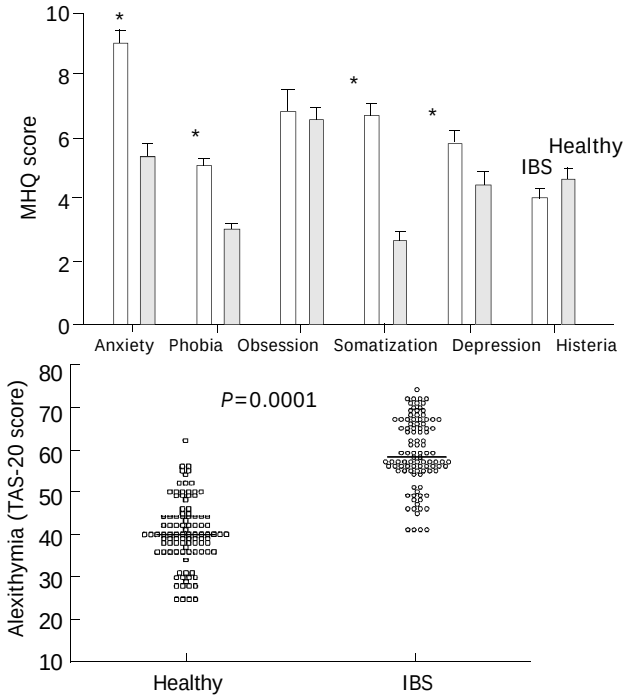



**Figure 4** A: Scores of psychological disorders (mean ± SE, *0.0009<*P*<0.04). B: Scores of alexithymia (individual data and median) in IBS patients and healthy subjects.

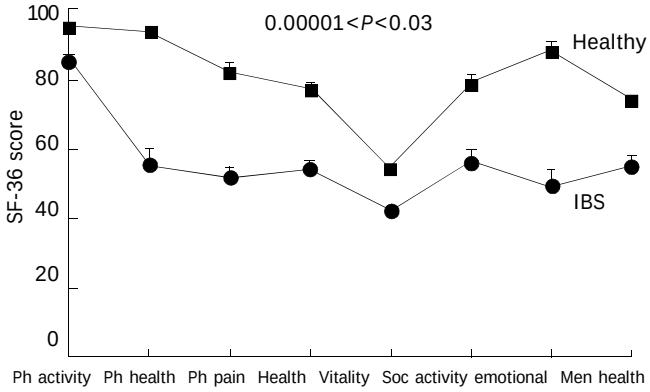



**Figure 5** Profiles of health-related quality of life-HRQOL (SF-36 questionnaire) for IBS patients and healthy subjects. Data are mean±SE. Significantly poorer HRQOL for all 8 scales in IBS patients compared with healthy subjects. Legend: Ph, physical; soc, social.

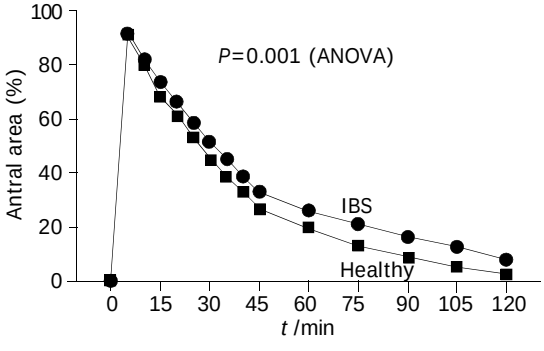



**Figure 6** Time-course of gastric emptying in IBS patients and healthy subjects after ingestion of 200 mL of standard liquid meal. Symbols indicate mean (SE is very small and not visible at each time-point). IBS patients had impaired emptying with larger postprandial antral areas (expressed as % of basal area) than healthy subjects. See also Table 2 for half-emptying time differences.

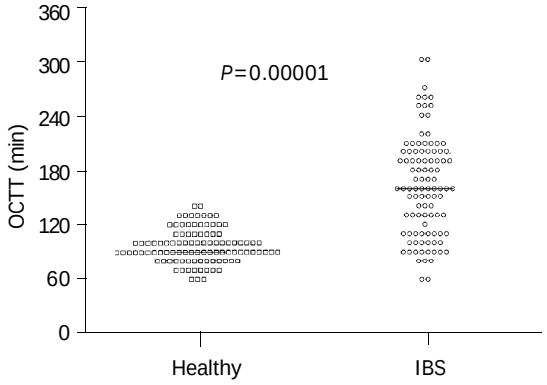



**Figure 7** Significant delay of orocaecal transit time (OCTT) in IBS patients compared with healthy subjects. Results are expressed as individual data and median.

**Table 2** Motility indices in study group (mean ± SE)

| | IBS | Healthy | *P** |
|---|---|---|---|
| No of subjects | 100 | 100 | |
| Gallbladder (volume) | | | |
| Fasting (mL) | 21.4±1.0 | 22.0±0.9 | NS |
| Postprandial residual, mL (%) | 5.3±0.6 (23.4±1.2) | 5.7±0.3 (25.7±0.9) | NS |
| $T_{50}$ (min.) | 20.1±0.9 | 21.4±0.6 | NS |
| Stomach (antral area) | | | |
| Fasting ($cm^2$) | 3.1±0.1 | 3.4±0.1 | NS |
| Postprandial maximal ($cm^2$) | 10.4±0.2 | 11.8±0.2 | 0.001 |
| Postprandial minimal (%) | 6.1±1.0 | 2.8±0.5 | 0.02 |
| $T_{50}$ (min.) | 35.5±1.0 | 26.1±0.6 | 0.00001 |
| Small bowel | | | |
| Orocaecal transit time (min.) | 161.9±5.5 | 96.6±1.8 | 0.00001 |

*Student's *t* test, OCTT=orocaecal transit time, NS=not significant. Indices of gallbladder motility: fasting volume (mean of 3 measurements at -15, -5 and 0 min before test meal, expressed in mL), residual volume (minimum volume measured postprandially, in mL) and half-emptying time ($T_{50}$, time to achieve 50 % decrease of fasting volume). Indices of stomach emptying: fasting antral area (mean of 3 measurements at –15, -5 and 0 min before test meal, expressed in $cm^2$), maximal antral area at 2 min postmeal in $cm^2$, minimal postprandial antral area during the 2-hour observation period (expressed in %, normalized to maximal area after subtracting basal areas: i.e. $100x(A_t\text{-}a)/(A_2\text{-}a)$, where $A_t$=postprandial area at any given time; $a$=basal area; $A_2$=area at 2 min postprandially[37]) and half-emptying time ($T_{50}$).

## DISCUSSION

We used an integrated approach in a large group of IBS patients to investigate gastrointestinal motility patterns in relation to symptoms, quality of life and psychological comorbidity. We believe that the present study may offer a fairly representative picture of IBS characteristics in southern Italy. The ROME II criteria[4] were chosen because they perform well in the clinic and they have greater simplicity than other less recent criteria[1]. Due to the setting where the study was performed (*i.e.* a tertiary referral center), caution must be expressed in interpreting our results, for at least two reasons. Firstly, only a minority of IBS patients were thought to consult physicians[33] and secondly,

characteristics of patients seen in a third referral center miqht differ from those of patients referred to primary or secondary care[25,34]. Nevertheless, IBS patients had multiple and simultaneous gastrointestinal motility defects involving stomach and intestine at a different extent, and were associated with diffuse gastrointestinal symptoms, abnormal psychological status, including alexythimia, and poor quality of life.

Ultrasonography was chosen as a non-invasive and validated technique to assess both gallbladder[35,36] and gastric emptying[17,29,37-40] simultaneously[28]. The protocol we employed, moreover, was further informative due to the simultaneous assessment of small bowel transit by $H_2$-breath test. Such a novel combined procedure allows a one-day (and time-saving) test for studying upper gastrointestinal motility in a clinical setting.

Despite "normal" feeling of appetite and satiety, IBS patients had strikingly abnormal upper gastrointestinal perception for nausea, fullness, epigastric pain/discomfort, both at fasting and postprandially. This was also the case in gastrectomized patients[21], Thus, ingestion of 200 mL of a moderately caloric, isosmotic test meal might prove useful in identifying groups of patients with dyspepsia, as also proposed in different clinical settings[41]. About 30 % of IBS patients included in this study had delayed gastric emptying, as was found in other gastrointestinal functional disorders such as dyspepsia and slow transit constipation[30,42]. Although this study was primarily focusing on patients with lower abdominal symptoms for IBS, we found that the score for dyspepsia was abnormal in about two-thirds of patients, as was also reported by Agreus *et al.* in the Swedish population[43]. Taken together, these findings suggest that dyspepsia and IBS are closely related and develop as a *continuum*[19]. No close correlation existed between delayed gastric emptying and dyspepsia. Indeed, gastric emptying was defective only in a subgroup of dyspeptic patients and this also seemed to be the case in IBS patients. Moreover, both symptoms or gastric half emptying times were poor predictors of gastrointestinal dysmotility in functional dyspepsia[44]. Also, dyspeptic symptoms might originate from an altered fundic receptive relaxation (not measured in the present study) and/ or from disorders of other organs, including duodenum under acidic stress[45].

At variance with an early study in a scant number of IBS patients[46], we found no gross evidence for impaired gallbladder motility. Differences in selection criteria might partly explain the variability. CCK played a key role in postprandial gallbladder contraction, and abnormal sensitivity of the gallbladder smooth muscle to exogenous CCK has been reported in IBS[47]. Apparently, the defect was absent postprandially, since either a high-fat[47] or a low-fat (this study) liquid meal yielded similar gallbladder contractions. Whether or not the trend we showed of faster refilling in IBS points to an abnormality of the smooth muscle in the digestive tract[47], remains to be established. Impaired gallbladder motility has been found in a subgroup of patients with functional dyspepsia[42] and delayed transit constipation[30]. Disturbed motilin and CCK release might be a potential cause of intestinal dysmotility in IBS[48].

This study also showed that OCTT was delayed in IBS. Changes in phase II and phase III components of the migrating motor complex suggested that both local (*i.e.* enteric) and central mechanisms might operate to produce intestinal dysmotility[49]. Delayed OCTT could be independent of colonic transit (as seen in patients with functional dyspepsia[42]) or might be associated with delayed colonic transit (as seen in patients with functional constipation[30]). Although accelerated small bowel and colonic transit have been reported in diarrhea-predominant IBS[50], a similar conclusion could not be drawn from this study, since patients alternated between diarrhea and constipation in their history. IBS patients had rather increased bowel habits, despite delayed OCTT. This finding pointed to a diffuse impairment of visceral sensitivity and perception, involving not only the rectum[6], but also the colon or even more proximal districts such as small bowel and stomach[7,51]. Abnormal motor patterns in the small bowel might be associated with symptoms in patients with IBS (*e.g.* clustered jejunal contractions and propagated giant ileal contractions during abdominal colic[52]). It must be stressed, however, that no motor abnormality in either small or large intestine was pathognomonic for IBS[53]. Whether or not delayed OCTT in IBS could contribute to accumulation of gas in the intestine[54,55] and/or abnormal colonic fermentation in the colon[56], remains to be determined. Interestingly, we found delayed OCTT with increased bowel movements in chronic alcoholic patients during abstinence, associated with dysfunction of autonomic nervous system[57]. This possibility deserves further attention, since a form of subclinical autonomic neuropathy might predispose to a diffuse disorder of smooth muscle,which was suggestive for the multi-organic involvement of the gastrointestinal tract[7].

It is known that visceral hyperalgesia in IBS could exacerbate symptoms due to lactose maldigestion[58]. A recent study found that 24 % of previously diagnosed IBS patients had lactose intolerance[16], this was not the case in the present study where lactose maldigestion or intolerance was an exclusion criterion. It is still controversial whether or not a subset of patients with IBS might be positive for coeliac disease[59,60]. Since the recent British Society of Gastroenterology guidelines estimated that routine antiendomysial antibodies would reveal only 1-2 % of abnormalities[3], systematic screening for coeliac disease in this study was not believed to be cost-effective and was not performed.

It is believed that up to 60 % of IBS patients seen at referral centers might have psychological morbidity[5,61-64]. An abnormal psychological profile in IBS patients emerged also from the present study. Psychological disturbances could influence aspects of bowel habit[1,65]. We found increased bowel movements to be associated with anxiety, somatisation and also with alexithymia. Alexithymia construct is one of the four syndromes in the Diagnostic Criteria for Psychosomatic Research[34]. A stronger positive association was reported between alexithymia and somatoform rather than chronic somatic disorders[25,66,67]. In the present study we employed TAS-20, the most validated questionnaire available so far[23] and found significant alexithymia in over 40 % of IBS patients (increasing to about 80 % if patients with border-line scores were included). These results are in line with those from another although smaller study conducted in a closed geographical area, investigating alexithymia in patients with IBS and other functional gastrointestinal disorders[25]. The finding may have at least two practical implications. Firstly, whether IBS patients are first seen in a gastroenterological or psychiatric setting might determine if patients are classified as suffering mainly from IBS or somatoform disturbances, respectively. Secondly, as alexithymia is seen as a stable feature, it might act as an important prognostic factor related to treatment outcomes in subgroups of IBS patients. If the IBS patients were seen within the broader spectrum of functional gastrointestinal diseases, our findings pointed to an association between alexithymia and tendency to negative affectivity[25,67].

IBS patients showed a significantly poorer HRQOL than healthy subjects. This finding was in accordance with previous studies[12,61] and pointed to related problems, namely absenteeism, the social indirect costs and, ultimately, the need for appropriate treatment of IBS patients. Interestingly, both IBS patients and healthy subjects in this setting had a HRQOL profile remarkably

similar to that derived from subjects across different cultures in the USA and UK[12]. This finding underscored the internal consistency of HRQOL questionnaires. Taken together, our findings confirmed that although IBS was not a life-threatening condition, it could lead to significant impairment of quality of life, at least in the subgroup of patients seen in a tertiary referral center.

In conclusion, these data show that IBS patients have a pan-enteric dysmotility with frequent dyspepsia, associated with psychological morbidity and greatly impaired quality of life. The presence of alexithymia, a stable trait, is a novel finding of potential interest to detect subgroups of IBS patients with different patterns of recovery after therapy.

## REFERENCES


1 **Camilleri M**, Heading RC, Thompson WG. Consensus report: clinical perspectives, mechanisms, diagnosis and management of irritable bowel syndrome. *Aliment Pharmacol Ther* 2002; **16**: 1407-1430
2 **Camilleri M**. Motor function in irritable bowel syndrome. *Can J Gastroenterol* 1999;**13**(Suppl A): 8A-11A
3 **Jones J**, Boorman J, Cann P, Forbes A, Gomborone J, Heaton K, Hungin P, Kumar D, Libby G, Spiller R, Read N, Silk D, Whorwell P. British Society of Gastroenterology guidelines for the management of the irritable bowel syndrome. *Gut* 2000; **47**(Suppl 2): II1-19
4 **Thompson WG**, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. *Gut* 1999; **45**(Suppl 2): II43-II47
5 **Horwitz BJ**, Fisher RS. The irritable bowel syndrome. *N Engl J Med* 2001; **344**: 1846-1850
6 **Mertz H**, Naliboff B, Munakata J, Niazi N, Mayer EA. Altered rectal perception is a biological marker of patients with irritable bowel syndrome. *Gastroenterology* 1995; **109**: 40-52
7 **Whorwell PJ**, McCallum M, Creed FH, Roberts CT. Non-colonic features of irritable bowel syndrome. *Gut* 1986; **27**: 37-40
8 **Houghton LA**, Whorwell PJ. Opening the doors of perception in the irritable bowel syndrome. *Gut* 1997; **41**: 567-568
9 **Azpiroz F**. Hypersensitivity in functional gastrointestinal disorders. *Gut* 2002;**51**(Suppl 1): i25-i28
10 **Naliboff BD**, Munakata J, Fullerton S, Gracely RH, Kodner A, Harraf F, Mayer EA. Evidence for two distinct perceptual alterations in irritable bowel syndrome. *Gut* 1997; **41**: 505-512
11 **Gomborone J**, Dewsnap P, Libby G, Farthing M. Abnormal illness attitudes in patients with irritable bowel syndrome. *J Psychosom Res* 1995; **39**: 227-230
12 **Hahn BA**, Yan S, Strassels S. Impact of irritable bowel syndrome on quality of life and resource use in the United States and United Kingdom. *Digestion* 1999; **60**: 77-81
13 **Kosturek A**, Gregory RJ, Sousou AJ, Trief P. Alexithymia and somatic amplification in chronic pain. *Psychosomatics* 1998; **39**: 399-404
14 **Kruis W**, Thieme C, Weinzierl M, Schussler P, Holl J, Paulus W. A diagnostic score for the irritable bowel syndrome. Its value in the exclusion of organic disease. *Gastroenterology* 1984; **87**: 1-7
15 **Vanner SJ**, Depew WT, Paterson WG, DaCosta LR, Groll AG, Simon JB, Djurfeldt M. Predictive value of the Rome criteria for diagnosing the irritable bowel syndrome. *Am J Gastroenterol* 1999; **94**: 2912-2917
16 **Bohmer CJ**, Tuynman HA. The effect of a lactose-restricted diet in patients with a positive lactose tolerance test, earlier diagnosed as irritable bowel syndrome: a 5-year follow-up study. *Eur J Gastroenterol Hepatol* 2001; **13**: 941-944
17 **Hveem K**, Jones KL, Chatterton BE, Horowitz M. Scintigraphic measurement of gastric emptying and ultrasonographic assessment of antral area: relation to appetite. *Gut* 1996; **38**: 816-821
18 **O'Donnell MR**, Virjee J, Heaton KW. Detection of pseudo diarrhoea by simple assessment of intestinal transit rate. *Br Med J* 1990; **300**: 439-440
19 **Talley NJ**, Fett SL, Zinsmeister AR, Melton LJ, III. Gastrointestinal tract symptoms and self-reported abuse: a population- based study. *Gastroenterology* 1994; **107**: 1040-1049
20 **Buckley MJ**, Scanlon C, McGurgan P, O'Morain C. A validated dyspepsia symptom score. *Ital J Gastroenterol Hepatol* 1997; **29**: 495-500
21 **Portincasa P**, Altomare DF, Moschetta A, Baldassarre G, Di Ciaula A, Venneman NG, Rinaldi M, Vendemiale G, Memeo V, vanBerge-Henegouwen GP, Palasciano G. The effect of acute oral erythromycin on gallbladder motility and on upper gastrointestinal symptoms in gastrectomized patients with and without gallstones: a randomized, placebo-controlled ultrasonographic study. *Am J Gastroenterol* 2000; **95**: 3444-3451
22 **Crown S**, Crisp AH. A short clinical diagnostic self-rating scale for psychoneurotic patients. The Middlesex Hospital Questionnaire (M.H.Q.). *Br J Psychiatry* 1966; **112**: 917-923
23 **Taylor GJ**, Ryan D, Bagby RM. Toward the development of a new self-report alexithymia scale. *Psychother Psychosom* 1985; **44**: 191-199
24 **Bressi C**, Taylor G, Parker J, Bressi S, Brambilla V, Aguglia E, Allegranti I, Bongiorno A, Giberti F, Bucca M, Todarello O, Callegari C, Vender S, Gala C, Invernizzi G. Cross validation of the factor structure of the 20-item Toronto Alexithymia Scale: an Italian multicenter study. *J Psychosom Res* 1996; **41**: 551-559
25 **Porcelli P**, Taylor GJ, Bagby RM, De Carne M. Alexithymia and functional gastrointestinal disorders. A comparison with inflammatory bowel disease. *Psychother Psychosom* 1999; **68**: 263-269
26 **Ware JE**, Snow KK, Kosinski M. SF-36 health survey. Manual and interpretation guide. Boston: The Health Institute: *New England Medical Center* 1993
27 **Stewart AL**, Greenfield S, Hays RD, Wells K, Rogers WH, Berry SD, McGlynn EA, Ware JE Jr. Functional status and well-being of patients with chronic conditions. Results from the Medical Outcomes Study. *JAMA* 1989; **262**: 907-913
28 **Portincasa P**, Colecchia A, Di Ciaula A, Larocca A, Muraca M, Palasciano G, Roda E, Festi D. Standards for diagnosis of gastrointestinal motility disorders. Ultrasonography. *Dig Liver Dis* 2000; **32**: 160-172
29 **Bolondi L**, Bortolotti MSV, Calleti T, Gaiani S, Labo' G. Measurement of gastric emptying by real-time ultrasonography. *Gastroenterology* 1985; **89**: 752-759
30 **Altomare DF**, Portincasa P, Rinaldi M, Di Ciaula A, Martinelli E, Amoruso AC, Palasciano G, Memeo V. Slow-transit constipation: a solitary symptom of a systemic gastrointestinal disease. *Dis Colon Rectum* 1999; **42**: 231-240
31 **Armitage P**, Berry G. Statistical methods in medical research. 3rd ed. Oxford: *Blackwell Science Ltd* 1994
32 **Dawson B**, Trapp RG. Basic & Clinical Biostatistics. 3rd ed. New York: *McGraw-Hill* 2001
33 **Jones R**, Lydeard S. Irritable bowel syndrome in the general population. *BMJ* 1992; **304**: 87-90
34 **Porcelli P**, De Carne M, Fava GA. Assessing somatization in functional gastrointestinal disorders: integration of different criteria. *Psychother Psychosom* 2000; **69**: 198-204
35 **Everson GT**, Braverman DZ, Johnson ML, Kern F Jr. A critical evaluation of real-time ultrasonography for the study of gallbladder volume and contraction. *Gastroenterology* 1980; **79**: 40-46
36 **Portincasa P**, Di Ciaula A, Baldassarre G, Palmieri VO, Gentile A, Cimmino A, Palasciano G. Gallbladder motor function in gallstone patients: sonographic and *in vitro* studies on the role of gallstones, smooth muscle function and gallbladder wall inflammation. *J Hepatol* 1994; **21**: 430-440
37 **Wedmann B**, Schmidt G, Wegener M, Coenen C, Ricken D, Althoff J. Effects of age and gender on fat-induced gallbladder contraction and gastric emptying of a caloric liquid meal: a sonographic study. *Am J Gastroenterol* 1991; **86**: 1765-1770
38 **Ricci R**, Bontempo I, Corazziari E, La Bella A, Torsoli A. Real-time ultrasonography of the gastric antrum. *Gut* 1993; **34**: 173-176
39 **Bergmann JF**, Chassany O, Petit A, Triki R, Caulin C, Segrestaa JM. Correlation between echographic gastric emptying and appetite: influence of psyllium. *Gut* 1992; **33**: 1042-1043
40 **Darwiche G**, Almer LO, Bjorgell O, Cederholm C, Nilsson P. Measurement of gastric emptying by standardized real-time ultrasonography in healthy subjects and diabetic patients. *J Ultrasound Med* 1999; **18**: 673-682
41 **Strid H**, Norstrom M, Sjoberg J, Simren M, Svedlund J, Abrahamsson H, Bjornsson ES. Impact of sex and psychological factors on the water loading test in functional dyspepsia. *Scand J*

*Gastroenterol* 2001; **36**: 725-730
42 **Portincasa P**, Moschetta A, Venneman NG, Palasciano G. Gastrointestinal motility in patients with chronic functional dyspepsia. *Dig Liver Dis (Ital J Gastroenterol) 30 (Suppl. II), A111* 1998
43 **Agreus L**, Svardsudd K, Nyren O, Tibblin G. Irritable bowel syndrome and dyspepsia in the general population: overlap and lack of stability over time. *Gastroenterology* 1995; **109**: 671-680
44 **Wilmer A**, Van Cutsem E, Andrioli A, Tack J, Coremans G, Janssens J. Ambulatory gastrojejunal manometry in severe motility-like dyspepsia: lack of correlation between dysmotility, symptoms, and gastric emptying. *Gut* 1998; **42**: 235-242
45 **Samsom M**, Verhagen MA, vanBerge Henegouwen GP, Smout AJ. Abnormal clearance of exogenous acid and increased acid sensitivity of the proximal duodenum in dyspeptic patients. *Gastroenterology* 1999; **116**: 515-520
46 **Braverman DZ**. Gallbladder contraction in patients with irritable bowel syndrome. *Isr J Med Sci* 1987; **23**: 181-184
47 **Kellow JE**, Miller LJ, Phillips SF, Zinsmeister AR, Charboneau JW. Altered sensitivity of the gallbladder to cholecystokinin octapeptide in irritable bowel syndrome. *Am J Physiol* 1987; **253**: G650-G655
48 **Sjolund K**, Ekman R, Lindgren S, Rehfeld JF. Disturbed motilin and cholecystokinin relese in the irritable bowel syndrome. *Scand J Gastroenterol* 1996; **31**: 1110-1114
49 **Kellow JE**, Eckersley GM, Jones M. Enteric and central contributions to intestinal dysmotility in irritable bowel syndrome. *Dig Dis Sci* 1992; **37**: 168-174
50 **Vassallo M**, Camilleri M, Phillips SF, Brown ML, Chapman NJ, Thomforde GM. Transit through the proximal colon influences stool weight in the irritable bowel syndrome. *Gastroenterology* 1992; **102**: 102-108
51 **Kellow JE**, Phillips SF. Functional disorders of the small intestine. In: Snape WJJ, editor. Pathogenesis of functional bowel disorders. New York: *Plenum Publ Corp* 1989: 171-198
52 **Stivland T**, Camilleri M, Vassallo M, Proano M, Rath D, Brown M, Thomforde G, Pemberton J, Phillips S. Scintigraphic measurement of regional gut transit in idiopathic constipation. *Gastroenterology* 1991; **101**: 107-115
53 **Kellow JE**, Phillips SF. Altered small bowel motility in irritable bowel syndrome is correlated with symptoms. *Gastroenterology* 1987; **92**: 1885-1893
54 **Whorwell PJ**. The problem of gas in irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 1618-1619
55 **Serra J**, Azpiroz F, Malagelada JR. Impaired transit and tolerance of intestinal gas in the irritable bowel syndrome. *Gut* 2001; **48**: 14-19
56 **King TS**, Elia M, Hunter JO. Abnormal colonic fermentation in irritable bowel syndrome. *Lancet* 1998; **352**: 1187-1189
57 **Portincasa P**, Moschetta A, Radicione T, Pugliese S, Castore A, Salerno MT, Palasciano G. Coexistence of diffuse gastrointestinal dysmotility, dyspepsia and autonomic dysfunction in chronic alcoholism. *Gastroenterology* 2001; **120**(Suppl1): A1496
58 **Tolliver BA**, Jackson MS, Jackson KL, Barnett ED, Chastang JF, DiPalma JA. Does lactose maldigestion really play a role in the irritable bowel? *J Clin Gastroenterol* 1996; **23**: 15-17
59 **Sanders DS**, Carter MJ, Hurlstone DP, Pearce A, Ward AM, McAlindon ME, Lobo AJ. Association of adult coeliac disease with irritable bowel syndrome: a case-control study in patients fulfilling ROME II criteria referred to secondary care. *Lancet* 2001; **358**:1504-1508
60 **Hamm LR**, Sorrells SC, Harding JP, Northcutt AR, Heath AT, Kapke GF, Hunt CM, Mangel AW. Additional investigations fail to alter the diagnosis of irritable bowel syndrome in subjects fulfilling the Rome criteria. *Am J Gastroenterol* 1999; **94**: 1279-1282
61 **Whitehead WE**, Burnett CK, Cook EW, III, Taub E. Impact of irritable bowel syndrome on quality of life. *Dig Dis Sci* 1996; **41**: 2248-2253
62 **Drossman DA**, McKee DC, Sandler RS, Mitchell CM, Cramer EM, Lowman BC, Burger AL. Psychosocial factors in the irritable bowel syndrome. A multivariate study of patients and nonpatients with irritable bowel syndrome. *Gastroenterology* 1988; **95**: 701-708
63 **Fullwood A**, Drossman DA. The relationship of psychiatric illness with gastrointestinal disease. *Annu Rev Med* 1995; **46**: 483-496
64 **Whitehead WE**, Bosmajian L, Zonderman AB, Costa PT Jr, Schuster MM. Symptoms of psychologic distress associated with irritable bowel syndrome. Comparison of community and medical clinic samples. *Gastroenterology* 1988; **95**: 709-714
65 **Gorard DA**, Gomborone JE, Libby JW, Farthing MJ. Intestinal transit in anxiety and depression. *Gut* 1996; **39**: 551-555
66 **Bagby RM**, Taylor GJ, Parker JD. Construct validity of the Toronto Alexithymia Scale. *Psychother Psychosom* 1988; **50**: 29-34
67 **Taylor GJ**, Parker JD, Bagby RM, Acklin MW. Alexithymia and somatic complaints in psychiatric out-patients. *J Psychosom Res* 1992; **36**: 417-424

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2300-2307
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

# Association of extraintestinal manifestations of inflammatory bowel disease in a province of western Hungary with disease phenotype: Results of a 25-year follow-up study

Laszlo Lakatos, Tunde Pandur, Gyula David, Zsuzsanna Balogh, Pal Kuronya, Arpad Tollas, Peter Laszlo Lakatos

**Laszlo Lakatos, Tunde Pandur, Gyula David,** 1st Department of Medicine, Csolnoky F. Province Hospital, Veszprem
**Zsuzsanna Balogh,** Department of Medicine, Grof Eszterhazy Hospital, Papa
**Pal Kuronya,** Department of Infectious Diseases, Magyar Imre Hospital, Ajka
**Arpad Tollas,** Department of Medicine, Municipal Hospital, Varpalota
**Peter Laszlo Lakatos,** 1st Department of Medicine, Semmelweis University, Budapest, Hungary
**Correspondence to:** Laszlo Lakatos, MD, 1st Department of Medicine, Csolnoky F. Province Hospital, Korhaz u.1, Veszprem, H-8200 Hungary. laklaci@hotmail.com
**Telephone:** +36-20-911-9339 **Fax:** +36-1-313-0250
**Received:** 2003-06-21 **Accepted:** 2003-08-02

## Abstract

**AIM:** IBD is a systemic disease associated with a large number of extraintestinal manifestations (EIMs). Our aim was to determine the prevalence of EIMs in a large IBD cohort in Veszprem Province in a 25-year follow-up study.

**METHODS:** Eight hundred and seventy-three IBD patients were enrolled (ulcerative colitis/UC/: 619, m/f: 317/302, mean age at presentation: 38.3 years, average disease duration: 11.2 years; Crohn's disease/CD/: 254, m/f: 125/129, mean age at presentation: 32.5 years, average disease duration: 9.2 years). Intestinal, extraintestinal signs and laboratory tests were monitored regularly. Any alteration suggesting an EIMs was investigated by a specialist.

**RESULTS:** A total of 21.3 % of patients with IBD had EIM (UC: 15.0 %, CD: 36.6 %). Age at presentation did not affect the likelihood of EIM. Prevalence of EIMs was higher in women and in CD, ocular complications and primary sclerosing cholangitis (PSC) were more frequent in UC. In UC there was an increased tendency of EIM in patients with a more extensive disease. Joint complications were more frequent in CD (22.4 % *vs* UC 10.2 %, $P<0.01$). In UC positive family history increased the risk of joint complications (OR:3.63). In CD the frequency of type-1 peripheral arthritis was increased in patients with penetrating disease ($P=0.028$). PSC was present in 1.6 % in UC and 0.8 % in CD. Dermatological complications were present in 3.8 % in UC and 10.2 % in CD, the rate of ocular complications was around 3 % in both diseases. Rare complications were glomerulonephritis, autoimmune hemolytic anaemia and celiac disease.

**CONCLUSION:** Prevalence of EIM in Hungarian IBD patients is in concordance with data from Western countries. The high number of EIM supports a role for complex follow-up in these patients.

Lakatos L, Pandur T, David G, Balogh Z, Kuronya P, Tollas A, Lakatos PL. Association of extraintestinal manifestations of inflammatory bowel disease in a province of western Hungary with disease phenotype: Results of a 25-year follow-up study. *World J Gastroenterol* 2003; 9(10):2300-2307
http://www.wjgnet.com/1007-9327/9/2300.asp

## INTRODUCTION

Ulcerative colitis (UC) and Crohn' s disease (CD) are chronic inflammatory diseases of undetermined origin. Inflammatory bowel disease (IBD) is a multifactorial polygenic disease with probable genetic heterogeneity. In this hypothesis, the disease may develop in a genetically predisposed host as a consequence of altered mucosal barrier and disregulated immune response to environmental, in particular enteric antigens, resulting in continuous immune-mediated inflammation[1-4]. IBD predominantly affects the gastrointestinal system but it is associated with a large number of extraintestinal manifestations (EIMs)[5]. Some disorders parallel the activity of the bowel disease but for a number of these conditions, their courses run independently of the course of the intestinal disease[6,7]. Furthermore, there has been some variance in the literature as to whether these EIMs are more associated with CD or UC. In the classical study of Greenstein *et al*.[8] EIMs were classified as colitis associated, small bowel associated and none specific manifestations.

EIMs contribute significantly to morbidity and mortality. Defining specific associations of immune mediated diseases in extraintestinal sites and IBD may be helpful in the better understanding of the pathogenesis of IBD.

The pathogenesis of EIMs is also multifactorial. The role of genetic factors is supported by family and candidate (e.g. certain HLA) gene studies[9-11]. The role of humoral immunity is supported by the higher prevalence of autoantibodies in the presence of EIMs, especially pANCA in primary sclerosing cholangitis (PSC). The immunological and clinical connections between these diseases and IBD have never been fully elucidated.

In this study we aimed to define the prevalence of EIMs in a 25-year follow up study in Hungarian IBD patients. We sought to determine if any of the EIMs was more likely associated with CD or UC, with male or female gender in a follow-up study. Possible associations between EIMs and location and disease behaviour were also investigated.

## MATERIALS AND METHODS

Eight hundred and seventy-three IBD patients followed-up at the Out- and Inpatient Gastroenterology Units of the Csolnoky F. Province Hospital in Veszprem Province were enrolled. This hospital is the secondary referral center for IBD patients in the province.

The data of the 619 UC patients (male/female: 317/302) are summarized in Table 1. The age at presentation varied between 9 and 80 years (average: 38.3 years). Average disease duration was 11.2 years (1-56 years). The location of UC according to the known greatest extent was proctitis in 117,

left sided colitis in 304 (including 171 patients with proctosigmoiditis), subtotal (98) and pancolitis (100) in 198 cases. Two hundred and fifty-four CD patients were included (125 males, 129 females). Average age at presentation was 32.5 years (12-80 years). According to the Vienna classification 192 patients were classified as A1, while 62 as A2. Disease duration was 9.2 years (1-40 years). Location of CD was ileal (L1) in 60, colonic (L2) in 81 and ileocolonic (L3) in 113 cases. Patients with upper GI manifestation had lower GI disease as well and they were classified according to their lower GI disease. According to the disease behavior 83 of our CD patients were defined as non-stricturing non-penetrating, 62 as stricturing and 105 as penetrating. Fifty-eight patients of the 95 penetrating cases had parallel strictures. Patients with indeterminate colitis were excluded.

**Table 1** Clinical data of IBD patients

| | Ulcerative colitis | Crohn's disease |
|---|---|---|
| Number of patients | 619 | 254 |
| Male / female | 317 / 302 | 125 / 129 |
| Mean age at diagnosis | 38.3 yrs (9-80 yrs) | 32.5 yrs (12-80 yrs) |
| Location | Proctitis: 117 | L1: 60 |
| | Left sided colitis: 304 | L2: 81 |
| | Pancolitis: 198 | L3: 113 |
| Behaviour of CD | - | B1: 87 |
| | | B2: 62 |
| | | B3: 105 |

In Crohn's disease (CD). Location: L1: terminal ileum, L2: colonic, L3: ileocolonic, behaviour: B1: non stricturing-non penetrating, B2: stricturing, B3: penetrating.

Patients in remission were followed-up twice per year. Patients who relapsed were followed-up or hospitalised according to the actual disease activity. Special interest was dedicated to the presence of EIM. Screening of EIMs was not performed, therefore the number of EIMs may have been underestimated. Routine follow-up consisted of assessment of patient's complaints, physical examination and laboratory testing.

Any alteration suggesting an EIMs was investigated by a specialist. In this study we did not assess the association between disease activity and the presence of EIM. Major EIMs studied in this report were axial and peripheral arthropathies (including ankylosing spondylitis), aseptic femoral head necrosis, primary sclerosing cholangitis (PSC), small duct cholangitis, autoimmune hepatitis, erythema nodosum, pyoderma gangrenosum, chronic urticaria, acute anterior uveitis, iritis, episcleritis, conjunctivitis, autoimmune hemolytic anaemia (AIHA), immune thrombocytopenic purpura (ITP), celiac disease, myositis, and glomerulonephritis.

Joint involvements were classified as peripheral and/or axial arthropathies. Peripheral arthropathies were divided into two subgroups according to the classification of Orchard *et al*[13]. Type-1 arthritis is an acute self-limiting pauciarticular (less than 5 joints) arthropathy typically affecting large joints. It is associated with other EIMs and its course parallels with the activity of the bowel disease. In contrast, type 2 arthritis is a chronic bilateral, symmetrical polyarticular arthropathy affecting five or more small joints. Its course runs independently of the course of the intestinal disease. Axial arthropathies are divided into sacroileitis and ankylosing spondylitis (SPA). Its incidence is 20-times higher than that in the normal population[14]. Rheumatologists investigated sacroileitis and ankylosing spondylitis cases. Laboratory testing (rheumatoid factor), X-ray and since 1997 MRI examinations were done.

Patients with elevated liver function tests (LFT, aminotransferases, cholestatic enzymes) were followed-up more cautiously. In patients with chronic or progressive elevation of enzyme levels liver biopsy and/or endoscopic retrograde cholangiopancreatography (ERCP) examination was done if patient gave informed consent. The diagnosis of PSC was based on elevated liver function tests, ERCP and consistent histology findings. Small duct PSC was diagnosed if histology suggested PSC, but ERCP could not verify the diagnosis[15,16]. Cholelithiasis, cirrhosis and focal nodular hyperplasia (FNH) were excluded from hepatobiliary manifestations.

In patients with verified thrombosis, blood samples were examined for hypercoagulability including in almost all cases analysis of plasminogen, proteins C and S activity and factor V Leiden mutation.

Patients with glomerulonephritis were followed-up by nephrologists as well. Diagnosis was based on clinical and chemical data and congruent histology findings. Ureteral obstruction was diagnosed by cystoscopy and urography, CT or MRI. If the alteration suggested fistulae in the urinary tract, cytography was also performed.

### *Statistical analysis*

For statistical comparison of the data, Statistica 6.0 (Statsoft Inc, USA) was used. Normality was tested by Shapiro-Wilk's W test. $\chi^2$ test with Yates correction was used to compare groups and odds ratios were calculated.

## RESULTS

The prevalence of major (joint, hepatobiliary, ocular and cutaneous) and all EIMs determined in this study are shown in Table 2. Major EIMs were apparent more frequently in CD than in UC (36.6 % *vs* 15.0 %, $P<0.001$). EIMs were more frequent in patients with a disease duration for more than 10 years in both CD (22.1 % *vs* 48.9 %, $P=0.003$) and UC (22.1 % *vs* 10.4 %, $P<0.001$).

**Table 2** Prevalence of extraintestinal manifestations (EIM) in IBD

| | Total *n* (%) | Disease duration | |
|---|---|---|---|
| | | ⩽ 10 yrs *n* (%) | > 10 yrs *n* (%) |
| IBD | 873 | 511 | 352 |
| Major EIMs | 186 (21.3) | 86 (16.8) | 102 (30.0) |
| All EIM signs | 547 (62.7) | 278 (54.4) | 269 (76.4) |
| Ulcerative colitis | 619 | 357 | 262 |
| Major EIMs | 93 (15.0) | 37 (10.4) | 58 (22.1) |
| All EIM signs | 360 (58.2) | 167 (46.8) | 193 (73.7) |
| Crohn's disease | 254 | 164 | 90 |
| Major EIMs | 93 (36.6) | 49 (29.9) | 44 (48.9) |
| All EIM signs | 187 (73.6) | 111 (67.7) | 76 (84.4) |

The prevalence of EIMs was higher in CD except ocular complications and PSC ($P<0.001$ for joint, hepatobiliary and cutaneous manifestations, Tables 3 and 4). In general, EIMs were more frequent in women except hepatobiliary manifestations and arthropathies in UC patients.

All major EIMs were more prevalent in more extensive UC (Table 5). There was a tendency of increased frequency of joint manifestations in CD patients with colonic involvement (L2 and L3: 23.7 %) compared to patients with only ileal disease (18.3 %, $P$=NS, Table 5). The prevalences of hepatobiliary, ocular and cutaneous manifestations were not different according to disease location.

**Table 3** Age at presentation and prevalence of major extraintestinal manifestations in patients with IBD

| | A1 *n* (%) | A2 *n* (%) |
|---|---|---|
| UC (*n*: 619) | 369 | 250 |
| Joint | 32 (8.7) | 20 (8.0) |
| Hepatobiliary | 49 (13.3) | 28 (11.2) |
| Cutaneous | 17 (4.6) | 7 (2.8) |
| Ocular | 11 (3.0) | 8 (3.0) |
| CD (*n*: 254) | 192 | 62 |
| Joint | 48 (25.0) | 9 (14.5) |
| Hepatobiliary | 48 (25.0) | 9 (14.5) |
| Cutaneous | 20 (10.4) | 6 (9.7) |
| Ocular | 5 (2.6) | 3 (4.8) |

A1: age at presentation <40 yrs, A2: age at presentation ⩾40 yrs.

**Table 4A** Familial IBD and association with extraintestinal manifestations

| | Total | First degree relative | Second degree relative |
|---|---|---|---|
| Ulcerative colitis | 24/619 (3.9 %) | 18 (14 UC+4 CD) (2.9 %) | 6 (5 UC+1 CD) (1.0%) |
| Crohn's disease | 31/254 (12.2 %) | 20 (3 UC+17 CD) (7.9%) | 11 (2 UC+9 CD) (4.3%) |

**Table 4B** Familial IBD and association with extraintestinal manifestations

| | Ulcerative colitis | Familial IBD |
|---|---|---|
| Number of patients | 619 | 24 |
| Joint | 52 (8.4 %) | 6 (25.0 %) |
| Hepatobiliary | 77 (12.4 %) | 3 (12.5 %) |
| Cutaneous | 24 (3.9 %) | 1 (4.2 %) |
| Ocular | 19 (3.0 %) | 2 (8.3 %) |
| | Crohn's disease | Familial IBD |
| Number of patients | 254 | 31 |
| Joint | 57 (22.4 %) | 11 (35.5 %) |
| Hepatobiliary | 57 (22.4 %) | 4 (12.9 %) |
| Cutaneous | 26 (10.2 %) | 2 (6.5 %) |
| Ocular | 8 (3.1%) | 0 |

**Table 5** Prevalence of extraintestinal manifestations in ulcerative colitis and Crohn's disease according to location and disease behaviour

| | Joint *n* (%) | Hepatobiliary *n* (%) | Cutaneous *n* (%) | Ocular *n* (%) |
|---|---|---|---|---|
| Location | | Ulcerative colitis | | |
| Proctitis (*n*=117) | 5 (4.3) | 9 (7.7) | 1 (0.9) | 1 (0.9) |
| Left sided colitis (*n*=304) | 14 (4.6) | 32 (15.7) | 8 (2.6) | 7 (2.3) |
| Pancolitis (*n*=198) | 33 (16.7) | 36 (18.2) | 15 (7.6) | 12 (6.1) |
| Location | | Crohn's disease | | |
| L1 (*n*=60) | 11 (18.3) | 14 (23.3) | 6 (10.0) | 2 (3.3) |
| L2 (*n*=81) | 17 (21.0) | 17 (21.0) | 10 (12.3) | 3 (3.7) |
| L3 (*n*=113) | 29 (25.7) | 26 (23.0) | 10 (8.8) | 3 (2.7) |
| Behaviour | | | | |
| B1 (*n*=87) | 13 (14.9) | 22 (25.3) | 11 (12.6) | 1 (1.1) |
| B2 (*n*=62) | 16 (25.8) | 11 (17.7) | 5 (8.1) | 0 |
| B3 (*n*=105) | 28 (27.6) | 24 (22.9) | 10 (9.5) | 7 (6.7) |

Age at presentation (A1: <40 years, A2: =40 years) did slightly affect the prevalence of EIMs (Table 3). Joint manifestations were more prevalent in CD patients with earlier disease onset (OR: 1.96, 95 % CI: 1.01-4.21). The same tendency was observed for cutaneous manifestations.

Familial disease was seen in 3.9 % of patients with UC and 12.2 % of patients with CD (Tables 4A-B). Joint manifestations were more frequent in UC patients with familial disease (OR: 3.63, 95 % CI: 1.43-9.31) than without. The same tendency was seen in UC patients (OR: 1.9, 95 % CI: 0.87-4.14) and ocular manifestations were found in familial UC cases.

**Table 6A** Joint manifestations in IBD patients

| | Total *n* (%) | Axial arthritis *n* (%) | Type-1 arthritis *n* (%) | Type-2 arthritis *n* (%) |
|---|---|---|---|---|
| Ulcerative colitis | | | | |
| Total (*n*=619) | 52 (8.4) | 20 (3.2) | 17 (2.7) | 13 (2.1) |
| Male (*n*=317) | 24 (7.6) | 10 (3.2) | 7 (2.2) | 7 (2.2) |
| Female (*n*=302) | 28 (9.3)* | 10 (3.4) | 10 (3.3) | 6 (2.0) |
| Crohn's disease | | | | |
| Total (*n*=254) | 57 (22.4) | 26 (10.2) | 29 (11.4) | 8 (3.1) |
| Male (*n*=125) | 23 (18.4) | 11 (8.8) | 12 (9.6) | 3 (2.4) |
| Female (*n*=129) | 34 (26.4) | 15 (11.6) | 17 (13.2) | 5 (3.9) |

*Two female patients with ulcerative colitis had rheumatoid arthritis.

**Table 6B** Joint manifestations in IBD according to location and disease behaviour

| | Total *n* (%) | Axial arthritis *n* (%) | Type-1 arthritis *n* (%) | Type-2 arthritis *n* (%) |
|---|---|---|---|---|
| Location | | Ulcerative colitis | | |
| Proctitis (*n*=117) | 5 (4.3)* | 1 (0.9) | 2 (1.8) | 1 (0.9) |
| Left sided colitis (*n*=304) | 14 (4.6) | 4 (1.3) | 5 (1.6) | 5 (1.6) |
| Pancolitis (*n*=198) | 33 (16.7)* | 15 (7.6) | 10 (5.0) | 7 (3.5) |
| Location | | Crohn's disease | | |
| L1 (*n*=60) | 11 (18.3) | 5 (8.3) | 4 (6.7) | 2 (3.3) |
| L2 (*n*=81) | 17 (21.0) | 7 (8.6) | 10 (12.3) | 3 (3.7) |
| L3 (*n*=113) | 29 (25.7) | 14 (12.4) | 15 (13.3) | 3 (2.7) |
| Behaviour | | | | |
| B1 (*n*=87) | 13 (14.9) | 8 (9.2) | 5 (5.7) | 2 (2.3) |
| B2 (*n*=62) | 16 (25.8) | 8 (12.9) | 6 (9.7) | 3 (4.8) |
| B3 (*n*=105) | 28 (27.6) | 10 (9.5) | 18 (17.1) | 3 (2.9) |

*One patient with proctitis and one with pancolitis had rheumatoid athritis.

Joint manifestations were more frequent in CD than in UC ($P<0.001$, Tables 6A-B.). There was a tendency of increased frequency of joint manifestations in women with CD (26.4 % *vs* 18.4 %, OR: 1.58, 95 % CI: 0.87-2.87). Axial arthritis (10.2 % *vs* 3.2 %, $P=0.0001$) and type 1(11.4 % *vs* 2.7 %, $P=0.0001$) arthritis were more frequent in CD, with equal prevalence of type-2 arthritis. In UC joint manifestations were almost three-fold more frequent in patients with pancolitis compared to proctitis and left sided colitis cases ($P<0.002$ for both, Table 5). In CD a tendency of increased frequency of joint manifestations was observed in patients with colonic involvement (L2 and L3: 23.7 %) or stricturing/penetrating disease (26.3 %) compared to patients with ileal only disease (18.3 %) or non-stricturing non-penetrating disease behavior

(14.9 %, Table 6B). An increased frequency of type 1 arthritis was observed in patients with penetrating compared to non-stricturing non-penetrating disease ($P$=0.028), the same tendency was observed in patients with or without colonic involvement. Type-1 arthritis affected more frequently the joints of the lower extremities (most frequently the knee and ankle), while type-2 arthritis was more common in the joints of the upper extremities.

Hepatobiliary manifestations are summerised in Table 7. PSC was diagnosed in 10 patients with UC and only 2 patients with CD. Small duct PSC was diagnosed in 8 and 6 cases, respectively. Non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) was diagnosed in 9.4 % of UC patients and 19.3 % of CD patients ($P$<0.0001). These patients had unexplained abnormal liver function tests (viral hepatitis, autoimmune, drug or alcohol induced disease, extrahepatic obstruction excluded). Liver biopsy was performed in 22/107 cases, which identified NAFLD or NASH in almost all cases. US proved hepatomegaly in 13/107 (12.1 %). Progression to cirrhosis was not observed in these patients during follow-up.

**Table 7** Hepatobiliary manifestations in IBD patients

| | Total *n* (%) | PSC *n* (%) | Small duct PSC *n* (%) | NAFLD / NASH *n* (%) |
|---|---|---|---|---|
| Ulcerative colitis | | | | |
| Total (*n*=619) | 77* (12.4) | 10 (1.6) | 8 (1.3) | 58 (9.4) |
| Male (*n*=317) | 39 (12.3) | 3 (1.0) | 6 (1.9) | 30 (9.5) |
| Female (*n*=302) | 38* (12.6) | 7 (2.3) | 2 (0.7) | 28 (9.3) |
| Crohn's disease | | | | |
| Total (*n*=254) | 57 (22.4) | 2 (0.8) | 6 (2.4) | 49 (19.3) |
| Male (*n*=125) | 27 (21.6) | 2 (1.6) | 6 (4.8) | 19 (15.2) |
| Female (*n*=129) | 30 (23.3) | 0 | 0 | 30 (23.3) |

*One female patient had autoimmune hepatitis. NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis.

Cutaneous manifestations were seen in 10.2 % of the patients with CD and 3.9 % of the patients with UC (Tables 8A-B). Cutaneous manifestations were more common in women in both UC (male/female: 5.0 %/2.8 %) and CD (13.2 %/7.2 %, OR: 1.95, 95 % CI: 0.85-4.48). Erythema nodosum and pyoderma gangrenosum were the most frequent manifestations. In UC cutaneous manifestations were more frequent in more extensive disease (7.6 % in pancolitis *vs* 2.1 % in proctitis or left sided colitis, $P$=0.002).

Ocular manifestations were apparent in approximately 3.0 % of UC and CD patients (Table 9). The prevalence was more frequent in women in both UC ($P$=0.009, OR: 4.37, 95 % CI: 1.51-12.6) and CD (OR:3.0, 95 % CI=0.67-8). Conjunctivitis, acute anterior uveitis and scleritis were the most frequent manifestations. Ocular manifestations developed mostly during the early years of the disease. In UC more than half of the patients with ocular complication had pancolitis (6.1 % in pancolitis *vs* 1.9 % in left sided colitis or proctitis, $P$=0.01).

Iron deficiency anaemia was seen in 35.8 % of CD patients and in one fourth of UC patients (Tables 10A-C). It was more frequent in women in UC (32.1 % *vs* 19.6 %, $P$<0.001, OR=1.95, 95 % CI: 1.35-2.81). Chronic anaemia was more frequent in patients with CD (9.6 % *vs* 17.7 %, $P$<0.001). The prevalence of macrocytic anaemia was around 4 % in both diseases. It was also observed more frequently in patients with ileocolonic disease than without it ($P$=0.03). The same tendency was observed according to disease behaviour; chronic anaemia tended to be more frequent in patients with stricturing or penetrating disease ($P$=0.06, Table 10C). AIHA developed in four UC patients.

**Table 8A** Cutaneous manifestations in patients with ulcerative colitis (*n*=619)

| | Total *n* (%) | Male *n* (%) | Female *n* (%) |
|---|---|---|---|
| Erythema nodosum | 8 (1.3) | 2 (0.6) | 6 (2.0) |
| Pyoderma gangrenosum | 3 (0.5) | 1 (0.3) | 2 (0.6) |
| Chronic urticaria | 6 (1.0) | 2 (0.6) | 4 (1.3) |
| Psoriasis | 3 (0.5) | 2 (0.6) | 1 (0.3) |
| Aphthous stomatitis | 3 (0.5) | 1 (0.3) | 2 (0.6) |
| Herpes zoster | 2 (0.3) | 1 (0.3) | 1 (0.3) |
| Cellulitis | 2 (0.3) | 0 | 2 (0.6) |
| Recurrent dermatitis | 2 (0.3) | 1 (0.3) | 1 (0.3) |
| Lichen ruber planus | 1 (0.2) | 1 (0.3) | 0 |
| Total | 24 (3.9) | 9 (2.8) | 15 (5.0) |

**Table 8B** Cutaneous manifestations in patients with Crohn's disese (*n*=254)

| | Total *n* (%) | Male *n* (%) | Female *n* (%) |
|---|---|---|---|
| Erythema nodosum | 14 (5.5) | 4 (3.1) | 10 (7.8) |
| Pyoderma gangrenosum | 4 (1.6) | 3 (2.4) | 1 (0.8) |
| Erythema exsudativum multiforme | 2 (0.8) | 1 (0.8) | 1 (0.8) |
| Erythroderma | 2 (0.8) | 1 (0.8) | 1 (0.8) |
| Stevens Johnson syndrome | 1 (0.4) | 0 | 1 (0.8) |
| Psoriasis | 1 (0.4) | 0 | 1 (0.8) |
| Eczema | 1 (0.4) | 0 | 1 (0.8) |
| Recurrent dermatitis | 1 (0.4) | 0 | 1 (0.8) |
| Total | 26 (10.2) | 9 (7.2) | 17 (13.2) |

**Table 9** Ocular manifestations in IBD patients

| | Total *n* (%) | Anterior uveitis *n* (%) | Conjunctivitis *n* (%) | Scleritis *n* (%) |
|---|---|---|---|---|
| Ulcerative colitis | | | | |
| Total (*n*=619) | 20* (3.2) | 6 (1.0) | 9 (1.5) | 4 (0.7) |
| Male (*n*=317) | 4 (1.3) | 1 (0.3) | 3 (1.0) | 1 (0.3) |
| Female (*n*=302) | 16 (5.3) | 5 (1.7) | 6 (2.0) | 3 (1.0) |
| Crohn's disease | | | | |
| Total (*n*=254) | 8 (3.1) | 4 (1.6) | 4 (1.6) | 1 (0.4) |
| Male (*n*=125) | 2 (1.6) | 1 (0.8) | 0 | 1 (0.8) |
| Female (*n*=129) | 6 (4.7) | 3 (2.3) | 4 (3.1) | 0 |

*One orbital pseudotumor was observed in a young female UC patient.

**Table 10A** Hematological manifestations in patients with ulcerative colitis

| | Total *n* (%) | Male *n* (%) | Female *n* (%) |
|---|---|---|---|
| Iron deficieny anaemia | 159 (25.9) | 62 (19.6) | 97 (32.1) |
| Chronic anaemia | 59 (9.6) | 34 (10.7) | 25 (8.3) |
| Macrocytic anaemia | 24 (3.9) | 15 (4.7) | 9 (3.0) |
| AIHA | 4 (0.6) | 2 (0.6) | 2 (0.6) |
| Non-Hodgkin lymphoma | 1 (0.2) | 1 (0.3) | 0 |
| CML | 1 (0.2) | 1 (0.3) | 0 |
| Chronic myeloproliferative disease | 1 (0.2) | 0 | 1 (0.3) |
| Leukemoid reaction | 1 (0.2) | 0 | 1 (0.3) |
| Methaemoglobinaemia | 1 (0.2) | 0 | 1 (0.3) |
| Total | 260 (42.0) | 90 (28.4) | 170 (56.3) |

AIHA: autoimmune hemolytic anaemia, CML: chronic myeloid leukaemia.

**Table 10B** Hematological manifestations in patients with Crohn's disease

| | Total *n* (%) | Male *n* (%) | Female *n* (%) |
|---|---|---|---|
| Iron deficieny anaemia | 91 (35.8) | 40 (32.0) | 51 (39.5) |
| Chronic anaemia | 45 (17.7) | 23 (18.4) | 22 (17.1) |
| Macrocytic anaemia | 11 (4.3) | 6 (4.8) | 5 (3.9) |
| Chronic myeloproliferative disease | 1 (0.4) | 0 | 1 (0.8) |
| Leukopenia | 1 (0.4) | 0 | 1 (0.8) |
| ITP | 1 (0.4) | 0 | 1 (0.8) |
| Total | 150 (59.1) | 69 (55.2) | 81 (62.8) |

ITP: immune thrombocytopenic purpura

**Table 10C** Association between hematological complications and location and disease behaviour in Crohn's disease patients

| | Iron deficieny anaemia *n* (%) | Chronic anaemia *n* (%) | Macrocytic anaemia *n* (%) |
|---|---|---|---|
| Location | | | |
| L1 (*n*=60) | 17 (28.3) | 9 (15.0) | 3 (5.0) |
| L2 (*n*=81) | 22 (27.2) | 9 (11.1) | 2 (2.5) |
| L3 (*n*=113) | 42 (37.2) | 27 (23.9) | 6 (5.3) |
| Behaviour | | | |
| B1 (*n*=87) | 27 (31.0) | 10 (11.4) | 2 (2.3) |
| B2 (*n*=62) | 23 (37.1) | 12 (19.4) | 3 (4.8) |
| B3 (*n*=105) | 41 (39.0) | 23 (21.9) | 6 (5.7) |

Thromboembolic complication was observed in 11 CD and 8 UC patients (Table 11.). Male predominance was observed in UC, while it was more frequent in women with CD, 1/15 (6.3 %) patient was positive for factor V Leiden mutation.

4.5 % of all IBD patients had multiple major extraintestinal diseases, three-fold more frequent in patients with CD than in patients with UC (9.1 % *vs* 3.1 %, $P<0.001$, Table 12.). Rare complications are summarized in Table 13. A relatively high number of glomerulonephritis was worth mentioning.

**Table 11** Thromboembolic complications in patients with IBD

| | Ulcerative colitis | Crohn's disease |
|---|---|---|
| Number of patients (%) | 11/619 (1.8) | 8/254 (3.1) |
| Male/female | 9/2 | 2/6 |
| Location | Proctitis: 0 | L1: 3 |
| | Left sided colitis: 6 | L2: 1 |
| | Pancolitis: 5 | L3: 4 |
| Behaviour of CD | - | B1: 0 |
| | | B2: 2 |
| | | B3: 6 |
| Place of thromboembolism | | |
| Lower extremity thrombosis | 8 | 4 |
| Pulmonary embolism | 1 | 0 |
| Lower extremity thrombosis complicated by pulmonary embolism | 2 | 3 |
| Splenic vein thrombosis | 0 | 1 |

**Table 12** Prevalence of multiple extraintestinal diseases

| | Total *n* | Ulcerative colitis *n* | Crohn's disease *n* |
|---|---|---|---|
| Two | 27 | 14 | 13 |
| Three | 14 | 5 | 9 |
| Four | 1 | 0 | 1 |
| Total | 42/873 (4.5%) | 19/619 (3.1%) | 23/254 (9.3%) |

**Table 13** Extraintestinal manifestations in IBD affecting other organ systems

| | Total *n* (%) | Ulcerative colitis *n* (%) | Crohn's disease *n* (%) |
|---|---|---|---|
| Glomerulonephritis | 3 (0.4) | 1 (0.2) | 2 (0.8) |
| Asthma bronchiale | 7 (0.8) | 4 (0.6) | 3 (1.2) |
| Chronic pancreatitis | 4 (0.5) | 3 (0.5) | 1 (0.4) |
| Acute pancreatitis | 2 (0.2) | 1 (0.2) | 1 (0.4) |
| Celiac disease | 2 (0.2) | 1 (0.2) | 1 (0.4) |
| Thyreoiditis | 2 (0.2) | 2 (0.3) | 0 |
| SLE | 2 (0.2) | 1 (0.2) | 1 (0.4) |

SLE: systemic lupus erythematodes.

## DISCUSSION

It is difficult to define the true prevalence of EIMs in IBD. If one was counting only major EIMs with common immunogenetic background the prevalence was about 20-25 %[1,5,8,17,18]. However, if all possible secondary systemic effects and/or complications of therapy are also included, then almost all patients will have "extra-intestinal manifestations". Prevalence may vary depending on the actual geographic area, IBD population, location and duration of the disease, medication and diagnostic accuracy.

Only few large cohort follow-up data are available on the prevalence of EIMs in IBD. The study of Greenstein *et al*. was one of the first reports[8]. Farmer *et al*.[19] reported a prevalence of 16.7 % during a 13-year follow-up study. EIMs were more prevalent in patients with colonic involvement or previous operations. In the Swedish epidemiology study Monsen *et al*.[20] excluded arthralgia, stomatitis and episcleritis from the EIMs. More recently, Jiang and Cui[21] analyzed the data of 10218 ulcerative colitis cases in China. The frequency of EIMs was 6.1 %, however no further data were available about the type of EIM. Bernstein *et al*.[22] investigated the prevalence of five "major" EIMs (iritis/uveis, PSC, pyoderma gangrenosum, erythema nodosum and ankylosing spondylitis) with the help of the University of Manitoba IBD Database in IBD patients with a disease history of at least 10 years. They found a single EIM prevalence of 6.2 %, which was one of the lowest rates reported. However, peripheral arthropathies were excluded from their study. There is undoubtedly a debate with the diagnosis of peripheral arthopathy, as it is sometimes difficult to distinguish arthropathy from arthralgia, especially in retrospective studies. In contrast, it is one of the most typical EIMs, observed in a high frequency of the patients. Excluding peripheral arthropathy from EIMs in one study and including it in another make the data difficult to compare. The reported overall EIM prevalence of 21.3 % in our study is in concordance with previous studies. The overall prevalence of EIMs was higher in patients with a longer disease history, but age at presentation did not affect the prevalence of EIMs.

There were gender predilections. EIMs were more frequent in femal pateints compared to males, which may support the hypothesis that autoimmune diseases are more common in female subjects.

The role of genetic factors has been implicated in the pathogenesis of IBD and EIMs[8-11]. In our study we investigated the familial occurrence in IBD. Positive family history was four times more frequent in CD compared to UC. The frequencies of joint and ocular manifestations were higher in patients with familial UC. Others have suggested high concordance of the occurrence of EIMs in affected siblings with IBD[23].

Previous studies have suggested multiple extraintestinal diseases[8,22] with certain genetic associations[10,23]. We found multiple extraintestinal diseases in 4.5 % of IBD patients, more

common in patients with CD. The most frequent associations were co-existing ocular, cutaneous and joint (type 1 arthritis) manifestations. The Canadian study reported multiple extraintestinl disease in only 0.3 % of all patients[22], however the rate of multiple EIMs was comparable to our results in most studies[17, 19].

### *Relation of EIMs with type, location and behaviour of IBD*

**Type of IBD** Overall EIMs were more frequent in patients with CD than in patients with UC, in concordance with previous studies[8,24] with the exception of ocular manifestations and PSC. These two EIMs were equally prevalent in CD and UC (both occurred approximately in 3 % of the patiens) in concordance with previous data[10,16,25].

**Location of IBD** In UC EIMs were thought to be more prevalent in extensive disease[5], but there were some contradiction in the literature[13]. In our study the rate of EIMs increased with the increasing extent of the disease. In CD it was generally accepted, that some EIMs were associated with colonic (e.g. peripheral arthropathy) others with small bowel (e.g. cholelithiasis) location[8,19]. We also found that type-1 arthritis was twice as frequent in patients with colonic or ileocolonic disease compared to patients with only ileal disease. The difference was smaller compared to patients with axial arthritis and disappeared in patients type 2 arthritis. The prevalences of cutaneous, ocular and hepatobiliary manifestations were not associated with location.

**Behaviour of CD** We did not find data in the literature about the relation of EIMs and the behaviour of CD, according to the Vienna classification. Earlier studies have suggested that EIMs tended to associate with perianal (=penetrating) disease[26]. We found a higher rate of joint complications in stricturing and penetrating disease than in non-stricturing non penetrating form. All but one ocular disease developed in patients with penetrating disease, but the rate of other complications was similar in the three groups.

### *Extraintestinal manifestations according to affected organs*

**Arthritis is the most common EIM in IBD** IBD related arthropaties were originally classified as axial and peripheral arthritides. Orchard *et al*. subdivided the peripheral disease into type 1 (large joint, pauciarticular, parallels with the course of IBD) and type-2 (small joint, polyarticular, the course independent of IBD) arthritides[13]. In our study joint manifestations were more frequently seen in patients with CD, first of all with the higher prevalence of axial and type-1 arthritis in these patients. Type-1 arthritis was more frequent in patients with stenosing and penetrating disease compared to patients with non-stenosing non-penetrating disease behaviour. In patients with penetrating disease bacterial infections could explain the high prevalence, the reason for the high prevalence in patients with stenosing disease is not clear, perhaps it has a genetic background.

**Hepatobiliary complications** PSC was diagnosed in 1.6 % in UC and even more infrequently in CD. When small duct PSC was included, the overall prevalence was 2.9-3.2 % in both diseases, in concordance with the majority of previous reports (2.4-11 %)[15,16,27]. PSC was a precancerosus condition with increased risk for cholangiocarcinoma and colorectal cancer[28]. In this study 3 patients with PSC and one patient with small duct cholangitis developed colorectal cancer and one PSC patient developed cholangiocarcinoma, which supports a role for more intensive follow-up in these subgroups of patients.

The cause of elevated LFT was found to be steatosis and/or steatohepatitis, reported in 6.3 % in UC and in 4 % in CD in previous studies[29,30]. An Italian study found that 12 % of the 474 asymptomatic IBD patients had hepatobiliary disease[31]. In non-selected or operated UC patients the prevalence of steatosis could be as high as 15-45 %[16,32,33]. In our study the rate of NAFLD was twice as high in CD than in UC, otherwise our data are in concordance with the data of previous studies. Several cutaneous diseases were diagnosed, 14 were classified as EIM. Cutaneous manifestations were more frequent in CD and in female patients. The prevalence of erythema nodosum and pyoderma gangrenosum was in the range previously reported[10,34,35] with very few psoriasis cases.

Ocular complications were equally frequent in both diseases with increased prevalence in women. Anterior uveitis was found at a frequency as previously reported[8,10,22]. We found one orbital pseudotumour in a young woman with severe ulcerative pancolitis. Ocular manifestations occurred mostly in the early few years after diagnosis. Pancolitis patients with UC were more liable to develop ocular manifestations compared to other locations. Ocular manifestations did frequently occur together with other (joint or cutaneous) extraintestinal diseases.

**Haematological complications** Iron deficiency anaemia is the most frequent hematological manifestation. In most of the cases its course ran parallel to the course of the intestinal disease. "Chronic anaemia" that occurs in inflammatory conditions and tumours was mostly apparent in severe, refractory cases and resolved only slowly after remission. Macrocytic anaemia was more frequent in patients with ileal and penetrating/stricturing disease than in patients with colonic involvement or non-stricturing non-penetrating disease. It was less frequent since preventive folate supplementation has been introduced in patients receiving sulfasalazine treatment.

Autoimmune haemolytic anaemia (AIHA) rarely complicates IBD, the reported prevalence rates were between 0.2 % and 1.7 %. Our observed rate (0.6 %) correlated with the previous data. In concordance with other studies AIHA occurred in patients with extensive colitis. In most of the cases AIHA could be treated successfully with steroids and/or immunosuppressive drugs[36]. In our study one patient required splenectomy, and in another case haemolysis subsided only after colectomy.

Increased risk of lymphoma has been reported in IBD, especially in patients receiving immunosupressives, but data were conflicting[37,38]. Our data do not support the notion of increased risk in these patients. In our series one 76-year old male UC patient with left sided colitis developed a high grade B-cell lymphoma in the rectum, but he did not receive immunosuppressive therapy. ITP might be true EIM, methaemoglobinaemia was associated with high dose sulfasalazine treatment, leukaemoid reaction was seen in a fulminant relapse.

Hypercoagulability was thought to be involved in the pathogenesis of IBD[39-41], and increased risk of thromboembolic complications (1-6 %) has been reported in IBD[42]. The prevalence of prothrombotic inherited and/or acquired coagulation abnormalities in patients with IBD remains controversial. Genetic thrombophilia with deficiencies in some coagulation inhibitors (antithrombin, proteins C and S), acquired thrombophilia due to inflammation, antiphospholipid syndrome and mutations of factor V Leiden and recently other genes involved in thrombogenesis (prothrombin mutation 20210A or factor V fV4070G polymorphism) have been described[39,43]. The most common cause of hypercoagulability status in Europe is resistance to activated protein C (APC). Resistance to APC and mutations that cause APC resistance (mainly factor V Leiden) have been of particular interest[42,43]. Some studies reported increased frequency of Leiden mutation in IBD[42], however others found that Leiden mutation was associated with thromboembolism with (14.3 %) or without IBD (15.5 %) but not with IBD (0 % and controls 3.6 %) itself [39,44]. In our study Leiden mutation was infrequent in IBD patients with thrombosis. The frequency was the same

that as observed in normal Hungarian population without thrombosis (5.26 %)[45]. Most thromboembolic episodes developed in the lower extremities, in some patient with pulmonary embolism as a complication. Thrombosis of the splenic vein occurred in one young female CD patient with ileocolonic disease.

**Other EIMs** Glomerulonephritis is a rare complication in IBD. Only case reports could be found in the literature[46]. Deposition of immune-complexes is thought to be involved in the pathogenesis. Finding 3 patients (1 UC and 2 CD) in this series was an unexpected high number. Histology revealed IgA nephropathy, membranous glomerulonephritis and focal glomerulosclerosis. Ductal changes suggesting chronic pancreatitis were common in IBD[47]. Our two pancreatitis cases developed in patients taking azathioprine.

In conclusion, in IBD a variety of EIMs may occur during the course of the disease affecting several organ systems. In Hungarian IBD patients, major EIMs develop in concordance with previous European data in approximately one fifth of the patients. The high number of EIMs supports a role for complex follow-up in these patients.

## ACKNOWLEDGEMENT

Dr. Zsuzsanna Erdelyi, Dr. Agnes Horvath, Dr Gabor Mester (Veszprem), Dr. Sandor Meszaros (Ajka), Dr. Csaba Molnar (Ajka), Dr. Mihály Balogh (Papa) and Dr. Istvan Szipocs for help in data collection and to Gabriella Demenyi for technical assistance.

## REFERENCES

1 **Podolsky DK**. Inflammatory bowel disease. *NEJM* 2002; **347**: 417-428
2 **Farrell RJ**, Peppercorn MA. Ulcerative colitis. *Lancet* 2002; **359**: 331-340
3 **Lakatos L**. Immunology of inflammatory bowel diseases. *Acta Physiol Hung* 2000; **87**: 355-372
4 **Fiocchi C**. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 1998; **115**: 182-205
5 **Weiss A**, Mayer L. Extraintestinal manifestations of inflammatory bowel disease. In: Allan RN, Rhodes JM, Hanauer SB, eds. Inflammatory bowel diseases. *Churchill Livingstone, New York* 1997: 623-636
6 **Lamers CB**. Treatment of extraintestinal complications of ulcerative colitis. *Eur J Gastroenterol Hepatol* 1997; **9**: 850-853
7 **Ljung T**, Staun M, Grove O, Fausa O, Vatn MH, Hellstrom PM. Pyoderma gangrenosum associated with Crohn disease: effect of TNF-alpha blokade with infliximab. *Scand J Gastroenterol* 2002; **37**: 1108-1110
8 **Greenstein AJ**, Janowitz HD, Sachar DB. The extra-intestinal complications of Crohn's disease and ulcerative colitis: a study of 700 patients. *Medicine* 1976; **55**: 401-412
9 **Orchard TR**. Arthritis associated with inflammatory bowel disease. In: Bayless TM, Hanauer SB, eds. Advanced therapy of inflammatory bowel disease. *Decker inc., Hamilton* 2001: 279-282
10 **Orchard TR**, Chua CN, Ahmad T, Cheng H, Welsh KI, Jewell DP. Uveitis and erythema nodosum in inflammatory bowel disease: clinical features and the role of HLA genes. *Gastroenterology* 2002; **123**: 714-718
11 **Roussomoustakaki M**, Satsangi J, Welsh K, Louis E, Fanning G, Targan S, Landers C, Jewell DP. Genetic markers may predict disease behavior in patients with ulcerative colitis. *Gastroenterology* 1997; **112**: 1845-1853
12 **D'Arienzo A**, Manguso F, Scarpa R, Astarita C, D'Armiento FP, Bennato R, Gargano D, Sanges M, Mazzacca G. Ulcerative colitis, seronegative spondyloarthropathies and allergic diseases: the search for a link. *Scand J Gastroenterol* 2002; **37**: 1156-1163
13 **Orchard TR**, Wordsworth BP, Jewell DP. Peripheral arthropathies in inflammaroty bowel disease: their articular distribution and natural history. *Gut* 1998; **42**: 387-391
14 **Russell AS**. Arthritis, inflammatory bowel disease and histocompatibility antigens. *Ann Int Med* 1977; **86**: 820-821
15 **Lee YM**, Kaplan MM. Management of primary sclerosing cholangitis. *Am J Gastroenterol* 2002; **97**: 528-534
16 **Heikius B**, Niemela S, Lehtola J, Karttunen T, Lahde S. Hepatobiliary and coexisting pancreatic duct abnormalities in patients with inflammatory bowel disease. *Scand J Gastroenterol* 1997; **32**: 153-161
17 **Rankin GB**, Watts HD, Melnyk CS, Kelley ML Jr. The National Cooperative Crohn's Disease Study: Extraintestinal manifestations and perianal complications. *Gastroenterology* 1979; **77**: 914-920
18 **Rankin GB**. Extraintestinal and systecim manifestations of inflammatory bowel disease. *Med Clin North Am* 1990; **74**: 39-50
19 **Farmer RG**, Whelan G, Fazio VW. Long-term follow-up of patients with Crohn's disease. *Gastroenterology* 1985; 88: 1818-1825
20 **Monsen U**, Sorstad J, Hellers G, Johannson C. Extracolonic diagnoses in ulcerative colitis: an epidemiological study. *Am J Gastroenterol* 1990; **85**: 711-716
21 **Jiang XL**, Cui HF. An analysis of 10218 ulcerative colitis cases in China. *World J Gastroenterol* 2002; **8**: 158-161
22 **Bernstein CN**, Blanchard JF, Rawsthorne P, Yu N. The prevalence of extraintestinal diseases in inflammatory bowel disease: A population-based study. *Am J Gastroenterol* 2001; **96**: 1116-1122
23 **Satsangi J**, Grootscholten C, Holt H, Jewell DP. Clinical patterns of familial inflammatory bowel disease. *Gut* 1996; **38**: 738-741
24 **Danzi JT**. Extraintestinal manifestations of idiopathic inflammatory bowel disease. *Arch Int Med* 1988; **148**: 297-302
25 **Lee YM**, Kaplan MM. Management of primary sclerosing cholangitis. *Am J Gastroenterol* 2002; **97**: 528-534
26 **Rankin GB**. Extraintestinal and systemic manifestations of inflammatory bowel disease. *Med Clin North Am* 1990; **74**: 39-50
27 **Olsson R**, Danielsson A, Jarnerot G, Lindstrom E, Loof L, Rolny P, Ryden BO, Tysk C, Wallerstedt S. Prevalence of primery sclerosing cholangitis in patients with ulcerative cholitis. *Gastroenterology* 1991; **100**: 1319-1323
28 **Loftus EV**, Sandborn WJ, Tremaine WJ, Mahoney DW, Zinsmeister AR, Offord KP, Melton LJ 3rd. Risk of colorectal neoplasa in patients with primary sclerosing cholangitis. *Gastroenterology* 1996; **110**: 432-440
29 **Perrett AD**, Higgins G, Johnston HH, Massarella G, Truelove SC, Wrigth R. The liver in ulcerative colitis. *Q J Med* 1971; **40**: 211-238
30 **Perrett AD**, Higgins G, Johnston HH, Massarella G, Truelove SC, Wrigth R. The liver in Crohn's disease. *Q J Med* 1971; **40**: 187-209
31 **Riegler G**, D'Inca R, Sturniolo C, Corrao G, Blancho CDV, Di Leo V, Carratu R, Ingrosso M, Pelli MA, Morini S, Valpiani D, Cantarini D, Usai P, Papi C, Caprilli R. Hepatobiliary alterations in patients with inflammatory bowel disease: A multicenter study. *Scand J Gastroenterol* 1998; **33**: 93-98
32 **Broome U**, Glaumann H, Hultcrantz R. Liver histology and follow-up of 68 patients with ulcerative colitis and normal liver function tests. *Gut* 1990; **31**: 468-472
33 **Mattila J**, Aitola P, Matikainen M. Liver lesions found at colectomy in ulcerative colitis: correlation between histological findings and biochemical parameters. *J Clin Pathol* 1994; **47**: 1019-1021
34 **Noussari HC**, Provost TT, Anhalt GJ. Cutaneous manifestations of inflammatory bowel disease. In: Bayless TM, Hanauer SB, eds. Advanced therapy of inflammatory bowel disease. *Decker inc, Hamilton* 2001: 271-274
35 **Tromm A**, May D, Almus E, Voigt E, Greving I, Schwegler U, Griga T. Cutaneous manifestations in inflammatory bowel disease. *Z Gastroenterol* 2001; **39**: 137-144
36 **Giannadaki E**, Potamianos S, Poussomoustakaki M, Kyriakou D, Fragkiadakis N, Manousos ON. Autoimmune hemolytic anemia and positive Coombs test associated with ulcerative colitis. *Am J Gastroenterol* 1997; **92**: 1872-1874
37 **Lewis JD**, Bilker WB, Brensinger C, Deren JJ, Vaughn DJ, Strom BL. Inflammatory bowel disease is not associated with an increased risk of lymphoma. *Gastroenterology* 2001; **121**: 1080-1087

38 **Lewis JD**, Schwartz JS, Lichtenstein GR. Azathioprine for maintenance of remission in Crohn's disease: benefits outweigh the risk of lymphoma. *Gastroenterology* 2000; **118**: 1018-1024
39 **Guedon C**, Le Cam-Duchez V, Lalaude O, Menard JF, Lerebours E, Borg JY. Prothrombotic inherited abnormalities other than factor V Leiden mutation do not play a role in inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 1448-1454
40 **Meucci G**, Pareti F, Vecchi M, Saibeni S, Bressi C, de Franchis R. Serum von Willebrand factor levels in patients with inflammatory bowel disease are related to systemic inflammation. *Scand J Gastroenterol* 1999; **34**: 287-290
41 **Collins CE**, Cahill MR, Newland AC, Rampton DS. Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology* 1994; **106**: 840-845
42 **Liebman HA**, Kashani N, Sutherland D, McGehee W, Kam AL. The factor V Leiden mutation increases the risk of venous thrombosis in patients with inflammatory bowel disease. *Gastroenterology* 1998; **115**: 830-834
43 **Haslam N**, Standen GR, Probert CS. An investigation of the association of the factor V Leiden mutation and inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1999; **11**: 1289-1291
44 **Grip O**, Svensson PJ, Lindgren S. Inflammatory bowel disease promotes venous thrombosis earlier in life. *Scand J Gastroenterol* 2000; **35**: 619-623
45 **Nagy Z**, Nagy A, Karadi O, Figler M, Rumi G Jr, Suto G, Vincze A, Par A, Mozsik G. Prevalence of the factor V Leiden mutation in human inflammatory bowel disease with different activity. *J Physiol Paris* 2001; **95**: 483-487
46 **Pardi DS**, Tremaine WJ, Sandborn WJ, McCarthy JT. Renal and urologic complications of inflammatory bowel disease. *Am J Gastroenterol* 1998; **93**: 504-514
47 **Barthet M**, Hastier P, Bernard JP, Bordes G, Frederick J, Allio S, Mambrini P, Saint-Paul MC, Delmont JP, Salducci J, Grimaud JC, Sahel J. Chronic pancreatitis and inflammatory bowel disease: true or coincidental association? *Am J Gastroenterol* 1999; **94**: 2141-2148

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2308-2312
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

# Seroreactivity against *Saccharomyces cerevisiae* in patients with Crohn's disease and celiac disease

Zsolt Barta, István Csípõ, Gábor G. Szabó, Gyula Szegedi

**Zsolt Barta, István Csí põ, Gábor G. Szabó, Gyula Szegedi,** 3rd Department of Medicine, University of Debrecen, Móricz Zs. Krt. 22. 4004 Debrecen, Hungary
**Correspondence to:** Zsolt Barta, M.D., 3rd Department of Medicine, University of Debrecen, Móricz Zs. Krt.22. 4004 Debrecen, Hungary. mailto:barta@iiibel.dote.hu
**Telephone:** +36-52-453-337 **Fax:** +36-52-414-969
**Received:** 2003-03-12 **Accepted:** 2003-05-09

## Abstract

**AIM:** To explore whether there was anti-*Saccharomyces cerevisiae* antibodies (ASCA) positivity in our patients with biopsy-confirmed celiac disease.

**METHODS:** A cohort of patients with inflammatory bowel diseases (42 patients with Crohn's disease and 10 patients with ulcerative colitis) and gluten sensitive enteropathy (16 patients) from Debrecen, Hungary were enrolled in the study. The diagnosis was made using the formally accepted criteria. Perinuclear antineutrophil cytoplasmic antibodies (pANCA) and *anti-Saccharomyces cerevisiae* antibodies (ASCA), *antiendomysium* antibodies (EMA), antigliadin antibodies (AGA) and anti human tissue transglutaminase antibodies (tTGA) were investigated.

**RESULTS:** The results showed that ASCA positivity occurred not only in Crohn's disease but also in Celiac disease and in these cases both the IgG and IgA type antibodies were proved.

**CONCLUSION:** It is conceivable that ASCA positivity correlates with the (auto-) immune inflammation of small intestines and it is a specific marker of Crohn's disease.

Barta Z, Csípõ I, Szabó GG, Szegedi G. Seroreactivity against *Saccharomyces cerevisiae* in patients with Crohn's disease and celiac disease. *World J Gastroenterol* 2003; 9(10):2308-2312
http://www.wjgnet.com/1007-9327/9/2308.asp

## INTRODUCTION

Gluten-sensitive enteropathy (GSE) or, as it is more commonly called, celiac disease, is an immune-mediated enteropathic condition (specified as an autoimmune inflammatory disease of the small intestine) that is precipitated by the ingestion of gluten, a component of wheat protein, in genetically susceptible persons. Exclusion of dietary gluten results in healing of the *mucosa*, resolution of the malabsorptive state, and reversal of most, if not all, effects of celiac disease. GSE commonly manifests as "silent" celiac disease (i.e., minimal or no symptoms). Histological examination and serologic tests for antibodies against *endomysium*, transglutaminase, and gliadin identify most patients with the disease[1].

Crohn's disease (CD) and ulcerative colitis (UC) are both classified under the medical rubric of inflammatory bowel disease (IBD). It is currently accepted that the term "IBD" does not encompass just two diseases (CD or UC), but rather a group of diseases, triggered and perpetuated by a variety of diverse genetic, environmental, and immunologic factors that share similar clinical manifestations. They cause life-impaired symptoms, necessitate long-term dependence on powerful drugs, and often result in debilitating surgery and even death. Although the etiology of UC and CD remains unclear, in addition to genetic and other environmental factors (food allergens, *etc*.), microorganisms have been discussed as possibly playing an important role. The gastrointestinal (GI) tract has direct contact with the environment and therefore forms a very important protective barrier within the human's organism. The *gut mucosa* has to stop foreign materials, such as bacteria or antigens, from entering the body and also prevent excessive loss of "body material" into the lumen. If the mucosal barrier is broken, an influx of luminal antigens may result in the perpetuation of intestinal inflammation by chronically stimulating resident and recruited immunocompetent cells of the *lamina propria*. Bacteria present within the intestinal lumen or in the intestinal wall are important in the development of mucosal inflammation. Recent reports in the literature do not suggest that a specific persistent infection causes IBD (i.e. the repeatedly blamed *Mycobacterium paratuberculosis avium*), but indicate that enteric pathogens could cause initial onset of IBD and are associated with reactivation of quiescent disease. CD patients may have a heterogeneous serological response to specific bacteria and bacterial related antigens. The serologic responses seen in CD patients include antibodies to *Saccharomyces cerevisiae, Mycobacteria, Bacteriodes, Listeria,* and *E. coli*. Many of the specific organisms have been proposed to directly or indirectly contribute to the pathogenesis of CD. Despite their self-limited character, these infections initiate a cascade of inflammatory events leading to chronic, relapsing disease in a genetically susceptible host ("hit-and-run" hypothesis). So, epidemiological and microbiologic studies suggest that enteropathogenic microorganisms play a substantial role in the clinical initiation and relapses of IBD. Thus microbiologic screening might be helpful in patients with flares of IBD for optimal medical treatment and as many bacteria cannot be cultured, culture-independent techniques are expected to be of great help in identifying microorganisms in IBD. With more comprehensive knowledge of the intestinal microflora and how the latter interacts with the host's immune system, it may be possible to define specific microbial substances in specific patients that are either present or absent from the normal flora to cause disease. The diagnoses of CD, UC and GSE are based on clinical features and the results of barium X-rays, endoscopy, mucosal biopsy histology, and in some cases operative findings and resected bowel pathology and histology. Serologic disease markers are accepted in GSE but unambiguously in IBD. Serologic tests that could serve as an adjunct to these invasive and expensive diagnostic studies, or possibly replace them, would have clinical utility[2-5]. Antibodies have attracted much interest for the study of the immune response in inflammatory bowel disease (IBD) and are also used as a tool for phenotyping. Antibodies to baker's yeast and brewer's yeast (anti-*Saccharomyces cerevisiae* antibodies or ASCA) directed against cell wall oligomannoside epitopes have been proposed as a serological marker for CD[6,7].

They have a sensitivity of 60 % and a specificity of 80-95 % for differentiating CD from controls[8]. One small study also reported the production of ASCA by a fraction of unaffected relatives of Crohn's patients. The role of ASCA's in CD is completely unknown, but one hypothesis links them to increased intestinal permeability. 50-60 % of patients with CD exhibit an activity-related increase in intestinal permeability, and this increase might be predictive for relapse. A variable proportion of healthy first-degree relatives of CD patients also have abnormal permeability of the small intestine, which lead some authors to postulate a primary defect of the tight junctions as etiologic factor in CD. Increased exposure of the epithelium to common food antigens such as yeasts due to a break in the epithelial barrier may then result in an antibody response. The effect of dietary yeast (*Saccharomyces cerevisiae*) on the activity of stable CD disease was assessed in patients. The patients' mean CD activity index (CDAI) while taking baker's yeast was significantly greater than that during yeast exclusion and patients with elevated yeast antibodies tended to develop a higher CDAI while receiving baker's yeast. These results suggest that dietary yeast may affect the activity of CD[9]. Antinuclear cytoplasmic antibodies (ANCA) with a perinuclear staining pattern have been proposed as a serologic marker for UC. pANCA's were found in 60-70 % of patients with UC. There is no clear association of pANCA with severity of disease but it is predictive of pouchitis after restorative proctocolectomy with ileoanal pouch anastomosis[10]. pANCA's are also found in 15-20 % of patients with CD. These patients present diffuse left-sided colitis with symptoms such as rectal bleeding, urgency, and mucus discharge and this phenotype is labeled "UC-like CD".

*Saccharomyces cerevisiae* (SC) is "ubiquitous" yeast and occurs in diverse places in naturally on plants and in the ground. Mankind used in the past and use now yeast for making miscellaneous foods (i.e. beer, bread) so we can come into contact with it principally with our consumed food products and beverages. It is generally accepted that SC is not a pathogen, but it has been suggested that in case of *fungemia* with SC (in patients with damaged/defective immune system) various organs can be affected and injured. The titer of pANCA does not change with the activity of the disease, does not depend on the therapy or the surgical intervention and but persists after resection of the affected part of the bowels. Similarly, the titer of the ASCA is stable irrespective of the activity of the disease and the drug-therapy. ASCA titers correlated with small bowel involvement and occurred particularly in cases of young adults with CD.

The aim of the study was to determine the prevalence of serological markers for GSE and IBD in patients with celiac disease (16 patients), UC (10 patients) and Crohn's disease (42 patients) and to correlate the presence with the characteristics of these diseases.

## MATERIALS AND METHODS

### *Patients*

The entire cohort of patients with IBD (42 patients with CD and 10 patients with UC) and GSE (16 patients) from Debrecen, Hungary were enrolled in the study. Adult patients of both sexes were included. The diagnosis of CD, UC or GSE was made using the formally accepted criteria. The medical records for these patients have previously been reviewed by investigators and abstracted for patient characteristics. The blood samples for the study were collected between January 2000 and March 2000. Their sera were separated and stored at -70 ℃. Determination of serum values was performed by individuals blinded to the clinical data for the patients in our Regional Immunology Laboratory.

### *Specific antibody measurement*

**Gliadin** Glia-test from Diagnosticum Rt.(Budapest, Hungary) was warmed up to room temperature and the dilution of the diluents solution and the buffer solution was dispensed. For the measurement of specific IgG and IgA, the samples were diluted 1:500 (v/v). Micro standardization strips were incubated with one hundred microlitres of washing buffer, negative control, IgA positive control, IgG positive control, and the samples from the patients, for 30 min at 37 ℃. After the incubation, the plate was washed three times with a Labsystems ELISA washer (Beverly, MA). Both of the anti-IgG and anti-IgA conjugate were diluted 1:200 in diluents solution (100 μL per well divided). Wells were again incubated for 30 min at 37 ℃ and washed three times with two hundred microlitres of washing buffer. Next step was incubation with 100 μL of TM B substrate buffer (protected from light) at room temperature for 30 min. Reaction was stopped with 50 μL 4N sulphuric acid per well. Optical density (OD) was determined at 450 nm. All chemical reagents were reagent grade, from Sigma unless otherwise stated.

**Transglutaminase** Greiner plates (N° 655180) were incubated with transglutaminase, dissolved in PBS at 10 μg per milliliters, 50 μL per well, 16 hours at +4 ℃. Binding sites were blocked by incubating with two hundred microlitres per well of 1 % (w/v) bovine serum albumin (BSA) in PBS for 2 hours at 37 ℃. 100 μL per well of each sample as incubated at room temperature for 2 hours. (For the measurement, the serum samples were diluted 1:100) Anti-human IgA/HRPO conjugate, diluted 1:4 000 (v/v) in diluents solution (1 % BSA-PBS) were incubated (100 μL per well) for 1 hour at room temperature. The color reaction was developed by adding a solution (containing 12.5 ml citrate-phosphate buffer pH 5.0, 4.25 mg OPD, 5 μL of 30 % $H_2O_2$). The enzymatic reaction was stopped after 15 min with 50 μL per well of 4N $H_2SO_4$. Optical density (OD) was determined at 492 nm. After each incubation step, wells were washed two times with PBS containing 0.05 % (v/v) Tween 20 (PBS-T) during 10 minutes. Each sample was analyzed in duplicate. All chemical reagents were reagent grade, from Sigma unless otherwise stated.

**Endomysium** Indirect immunofluorescent method was developed in our laboratory. The substrate (umbilical cord) was incubated in PBS with sera (diluted 1:10 in diluent), washed twice with PBS for 10 min, then incubated with anti-human IgA/FITC and IgG/FITC, washed twice with PBS for 10 min. The preparates were coated with a mixture of glycerin/PBS (1:1) and covered with cover plate. Evaluation was interpreted with fluorescent microscopy. All chemical reagents were reagent grade, from Sigma.

**ASCA IgG and IgA** Medizym® ASCA IgA is an enzyme immunoassay for the quantitative determination of IgA antibodies to *Saccharomyces cerevisiae* in human serum. Autoantibodies of the diluted patient samples and calibrators reacted with mannan (cell surface component of baker's yeast) immobilized on the solid phase of a microtiter plate. Following an incubation period of 60 min at 37 ℃, unbound serum components are removed by a washing step. The bound antibodies react specifically with anti-human-IgA-antibodies conjugated to horseradish peroxidase (HRPO). Within the incubation period of 30 min at 37 ℃, excessive conjugate was separated from the solid-phase immune complexes by the following washing step. Horseradish peroxidase converted the colorless substrate solution of 3, 3', 5, 5' -tetramethylbenzidine (TMB) added into a blue product. This enzyme reaction was stopped by dispensing an acidic solution ($H_2SO_4$) into the wells after 10 min at room temperature turning the solution from blue to yellow. The optical density (OD) of the solution at 450 nm was directly proportional to the amount of specific antibodies bound. The standard curve was established by

plotting the concentrations of the antibodies of the standards (x-axis) and their corresponding OD values (y-axis) were measured. The concentration of antibodies of the specimen was directly read off the standard curve.

**ANCA** The presence of ANCA was first screened by means of a fixed neutrophil enzyme-linked immunosorbent assay (ELISA). Methanol-fixed neutrophils were incubated with control and coded sera at 1:100 dilutions. Neutrophil-bound antibody was labeled by alkaline phosphatase-conjugated goat antihuman IgG. After the addition of p-nitrophenol, specific absorbance was measured at 405 nm. The cutoff for positivity was determined by positive controls from well-defined patients with UC. Indirect immunofluorescent staining was then performed on ANCA ELISA-positive samples to determine whether a predominantly perinuclear (pANCA) or cytoplasmic (cANCA) staining pattern was present. Methanol-fixed neutrophils on glass slides were incubated with the coded sera samples (1:20 dilution). Specific binding was visualized by fluorescence microscopy after the addition of fluorescein-labeled antihuman IgG. The specificity of the perinuclear staining pattern in UC was finally confirmed by its disappearance after DNase treatment of the neutrophils. Results were considered positive when both the ANCA titers were above the cutoff and the indirect immunofluorescence revealed a perinuclear binding of ANCA that disappeared after DNase treatment.

### *Statistical analysis*

The relations were concluded from the evidences with statistical methods and summarized them in tables. Data was presented as percentages or mean values ± standard deviation (SD). The statistical package used in data interpretation (the two-tailed Student's *t*-test, Pierce regression coefficient assay) was Statistica for Windows software (StatSoft, Inc., OK, U.S.A.).

## RESULTS

Results are summarized in (Tables 1-3, Figures 1-4). Twenty-seven patients with CD had only colonic localization (27/42), terminal ileitis was in 7 cases (7/42). Both the small and large bowels were affected in 8 cases (8/42). IgG type ASCA was found in 9 patients (1 colitis, 4 ileitis, 4 duodenitis-ileitis-colitis). IgA type ASCA was positive only in the patients with terminal ileitis (6/10) and in the patients with the duodenitis-ileitis-colitis type CD (10/15). The 27 patients with only colonic localization (27/42) had no IgA type ASCA positivity (Figures 1-2). All together, we detected that the increased IgG and IgA type ASCA titers, were mainly in patients with small bowel type CD (with the exception of one patient with colitis-type form with only IgG type ASCA) and celiac disease (Figure 3-4). IgG and IgA ASCA progressed together. IgA type ASCA values were between 0.35-938.19 U/ml (positive $\geqslant$20 U/ml). The prevalence of ASCA in patients with celiac disease outlined a possible role of anti-*Saccharomyces cerevisiae* antibodies in GSE. These findings outlined a noticeable startling resemblance, suggesting a possible kind of connection between CD and GSE.

**Table 1** ASCA positive patients in different diseases of bowels

| | ASCA IgG+ | ASCA IgA+ | ASCA IgG+IgA+ | IgG *vs* IgA corr.*(r)* |
|---|---|---|---|---|
| All patients (*n*=68) | 5 | 4 | 10 | 0.75 |
| Crohn's disease (*n*=42) | 1 | 2 | 8 | 0.81 |
| Ulcerative colitis (*n*=10) | 2 | 1 | 0 | -0.01 |
| Celiac disease (*n*=16) | 2 | 1 | 2 | 0.50 |
| Controls (*n*=20) | 0 | 0 | 0 | -0.18 |

Notes: It is clear that there was no ASCA positivity in the controls. ASCA positivity was common in patients with Crohn's disease. The ASCA IgG- and IgA-type seropositivity was concomitant.

**Table 2** ASCA positive patients with different forms of Crohn's disease

| Affected section of the bowels | ASCA IgG+ | ASCA IgA+ | ASCA IgG+IgA+ |
|---|---|---|---|
| Only the colon (*n*=27) | 1 | 0 | 0 |
| Large and small bowels both (*n*=8) | 0 | 0 | 4 |
| Only small bowels (*n*=7) | 0 | 2 | 4 |
| Small bowel together (*n*=15) | 0 | 2 | 8 |

Notes: Both the IgA and IgG type ASCA associated to the small bowels. $P<0.0004$, ASCA IgG+ *vs* small bowels; $P<0.0001$ ASCA IgA+ *vs* small bowels.

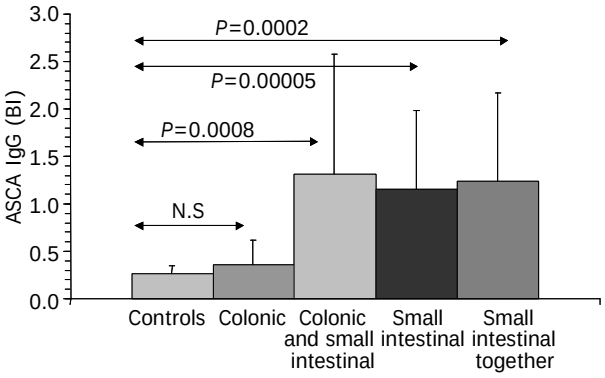



**Figure 1** ASCA IgG values in Crohn's disease.

**Table 3** Summary of ASCA positive cases with Crohn's disease

| Case: | ASCA IgG | ASCA IgA | ASCA IgG+IgA | Age (years) | Sex | Affected sections of gastrointestinal tract |
|---|---|---|---|---|---|---|
| 1. | | + | | 19 | Female | Terminal ileum |
| 2. | | | + | 46 | Male | Terminal ileum |
| 3. | | | + | 37 | Male | Terminal ileum |
| 4. | | | + | 38 | Female | Terminal ileum |
| 5. | + | | | 50 | Female | Colon |
| 6. | | | + | 29 | Male | From the duodenum to the rectum |
| 7. | | + | | 40 | Female | Terminal ileum |
| 8. | | | + | 24 | Male | Terminal ileum + colon |
| 9. | | | + | 49 | Female | From the duodenum to the rectum |
| 10. | | | + | 41 | Female | Terminal ileum |
| 11. | | | + | 27 | Male | Terminal ileum + colon |

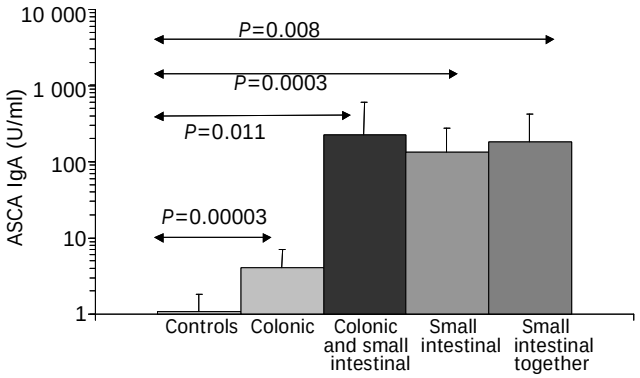



**Figure 2** ASCA IgA values in Crohn's disease.

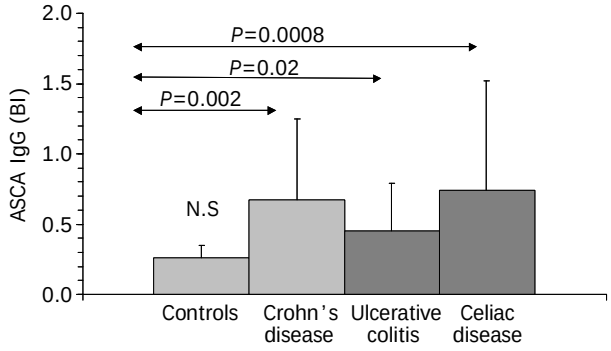



**Figure 3** ASCA IgG values in inflammatory diseases of intestines.

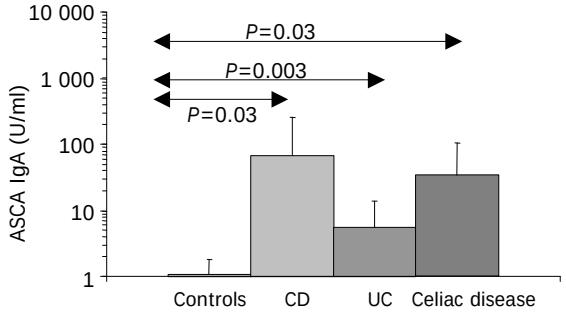



**Figure 4** ASCA IgA values in inflammatory diseases of intestines. [The IgA type ASCA values are between 0.35-938.19 U / ml (positive: IgA ⩾20 U / ml).]

## DISCUSSION

Both celiac disease and Crohn' s disease are characterized by the presence of distinct (auto) antibodies. Based upon our results, theoretically and practically it is thinkable that ASCA positivity is not only a specific marker of Crohn' s disease but correlates with the (auto-) immune inflammation of the small intestines How does it happen?

Macrophages can produce proinflammatory cytokines (i.e. TNF-α) with direct and indirect microbicide activity. Several cell-wall components of *Candida albicans* were investigated in relation with the TNF-alfa secretion of macrophages and confirmed that they all had beta-1,2-oligomannoside[11,12]. The incubation with purified oligomannoside activated macrophages and they secreted TNF-alfa (in dose and molecule-size dependently) *in vitro* and *in vivo*[13,14]. Similar observations were demonstrated with *Saccharomyces cerevisiae*: the oligomannose structures influenced per interaction the cytokine network[15-17]. It is conceivable that miscellaneous *fungus*-oligosaccharides (their signal function in phytopathology is well-known) could play a key role in the regulation of human infections[18]. Oligomannosids of *Saccharomyces cerevisiae* with modification by ASCA can change their immunopathogenicity and trigger a process that results in specific inflammation such as CD.

The human gastrointestinal tract possesses a complex ecosystem, the components of which are multifaceted and metabolically diverse. Although the presence of intestinal microflora certainly contributes to the maintenance of human health, intestinal mucosa has the task, among others, of preventing the passage of commensal microflora and occasional pathogens to other compartments. To carry out such a function, the mucosa has to behave as a physical barrier but it also has to play an active role. Oral tolerance (OT) consists of the oral administration of antigens that could alter the response of the immune system[19]. This is a form of peripheral immune tolerance in which mature lymphocytes in the peripheral lymphoid tissues are rendered non functional or hyporesponsive by prior oral administration of antigens. The mechanisms by which OT is mediated include deletion or anergy and active cellular suppression. The primary factor determining which form of tolerance will be developed after oral administration of antigen is its dosage. Thus, it is thought that low doses of antigen can induce the generation of active suppression, *via* regulatory T cells in the gut-associated lymphoid tissue (GALT), which then migrate to the systemic immune system. These regulatory T cells produce down-regulatory cytokines such as IL-4, IL-10 and TGF-β in a Th2 /Th3 cytokine pattern. Conversely, high dose of antigen favors anergy or clonal deletion. The phenomenon in which regulatory cells, as generated by oral tolerance, are primed in an antigen specific manner, but act in the respective microenvironment in a non-antigen specific manner is called bystander suppression. This phenomenon is of particular interest and can explain the use of oral/mucosal tolerance in T cell mediated autoimmune diseases such as rheumatoid arthritis and some diseases in which the autoantigen remains unknown or where there are reactivities to multiple autoantigens. T helper type 1 (Th1) lymphocytes secrete interleukin (IL)-2, interferon-γ and lymphotoxin-α, and stimulate type 1 immunity, which is characterized by intense phagocytic activity. Conversely, Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13, and stimulate type 2 immunity, which is characterized by high antibody titers. Type 1 and type 2 immunity are not strictly synonymous with cell-mediated and humoral immunity, because Th1 cells also stimulate moderate levels of antibody production, whereas Th2 cells actively suppress phagocytosis. For most infections caused by large eukaryotic pathogens, type 1 immunity is protective, whereas type 2 responses assist with the resolution of cell-mediated inflammation. Severe systemic stress, immunosuppression, or overwhelming microbial inoculation causes the immune system to mount a type 2 response to an infection normally controlled by type 1 immunity. Macrophages also play a crucial role in the mucosal network as they must perform a number of diverse cellular functions that allow them to kill invading micro-organisms and neoplastic cells as well as produce growth factors involved in wound healing. Macrophages that develop these diverse functions arise from a common precursor. By a process of selective adaptation, the common precursor monocyte/ macrophage differentiates into a distinctive macrophage with a different and specific phenotype, characterized by the expression of a specific set of gene products. The local environment plays a critical role in shaping or directing the pattern or pathway of macrophage differentiation: one pathway was believed to play a role in wound repair and characterized by the induction of insulin-like growth factor-1 (IGF-I) and a second pathway was involved in macrophage cytocidal activation and characterized

by the induction of the inducible form of nitric oxide synthase (iNOS)[20,21].

IBDs are a group of diseases due to chronic inflammation of the gastrointestinal tract, but without proved etiology. IBD appears to be resulted from a dysregulated immune response with contributions from environmental, genetic, and bacterial factors. In the last decades, our understanding of the pathogenesis of IBD has greatly expanded but a better insight is needed into the environmental agents responsible for either initiation or perpetuation of IBD. The increasing attention given to the ecosystem of the gut may help define the antigens responsible for immune reactivity and provide opportunities toward application of antigen-specific therapeutic interventions such as induction of tolerance. Further investigation into probiotic agents and their mechanisms is especially appealing as a way to provide alternative therapies to decrease the inflammatory response. Antibodies to an oligomannose epitope of *Saccharomyces cerevisiae* demonstrated in 60-70 % of the patients with Crohn' s disease. The origin and clinicopathological role of ASCA have not been clarified. The sporadic information about ASCA positivity in patients suffering from gluten sensitive enteropathy in the literature suggests another occurrence.

We examined the ASCA' s occurrence in our patients and compared it with the clinical picture of the Crohn' s disease. The results supported the theory that ASCA positivity correlated with small intestinal Crohn' s disease and in these cases both IgG and IgA type antibodies were proved. The relatively high incidence of ASCA in GSE was unexplained but indicated further surveys to elucidate it as it was definitely more than accidental[22]. The antibodies in the sera of the analyzed ASCA positive cases proved a systemic immune response against *Saccharomyces cerevisiae* and suggested the end of the oral tolerance against the yeast' s antigens. The diet restriction (elemental diet, total parenteral nutrition, and fecal diversion) may ameliorate the status of the patients with Crohn' s disease. It can also be speculated that the yeast-free diet as a part of the therapy for the ASCA positive patients can be reasonable, moreover the permanent "forbidding" of the yeast can be an acceptable alternative in case of getting well.

## REFERENCES


1 **Scoglio R,** Di Pasquale G, Pagano G, Lucanto MC, Magazzu G, Sferlazzas C. Is intestinal biopsy always needed for diagnosis of celiac disease? *Am J Gastroenterol* 2003; **98**: 1325-1331

2 **Barnes RM,** Allan S, Taylor-Robinson CH, Finn R, Johnson PM. Serum antibodies reactive with Saccharomyces cerevisiae in inflammatory bowel disease: is IgA antibody a marker for Crohn's disease? *Int Arch Allergy Appl Immunol* 1990; **92**: 9-15

3 **Falchuk KR**, Isselbacher KJ. Circulating antibodies to bovine albumin in ulcerative colitis and Crohn's disease. Characterization of the antibody response. *Gastroenterology* 1976; **70**: 5-8

4 **Hoffenberg EJ**, Fidanza S, Sauaia A. Serologic testing for inflammatory bowel disease. *J Pediatr* 1999; **134**: 447-452

5 **Knoflach P**, Park BH, Cunningham R, Weiser MM, Albini B. Serum antibodies to cow's milk proteins in ulcerative colitis and Crohn's disease. *Gastroenterology* 1987; **92**: 479-485

6 **Main J**, McKenzie H, Yeaman GR. Antibody to Saccharomyces cerevisiae (bakers yeats) in Crohndisease. *BMJ* 1988; **297**: 1105-1106

7 **Giaffer MH**, Clark A, Holdsworth CD. Antibodies to Saccharomyces cerevisiae in patients with Crohns disease and their possible pathogenic importance. *Gut* 1992; **33**: 1071-1075

8 **Quinton JF,** Sendid B, Reumaux D, Duthilleul P, Cortot A, Grandbastien B, Charrier G, Targan SR, Colombel JF, Poulain D. Anti-Saccharomyces cerevisiae mannan antibodies combined with antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease: prevalence and diagnostic role. *Gut* 1998; **42**: 788-791

9 **Barclay GR,** McKenzie H, Pennington J, Parratt D, Pennington CR. The effect of Dietary Yeast on the Activity of Stable Chronic Crohn's Disease. *Scand J Gastroenterol* 1992; **27**: 196-200

10 **Vidrich A,** Lee J, James E, Cobb L, Targan S. Segregation of pANCA antigenic recognition by DNase treatment of neutrophils: ulcerative colitis, type I autoimmune hepatitis, and primary sclerosing cholangitis. *J Clin Immunol* 1995; **15**: 293-299

11 **Domer J**, Elkins K, Ennist D, Baker P. Modulation of immune responses by surface polysaccharides of Candida albicans. *Rev Infect Dis* 1988; **10**: S419-422

12 **Trinel PA,** Plancke Y, Gerold P, Jouault T, Delplace F, Schwarz RT, Strecker G, Poulain D. The Candida albicans phospholipomannan is a family of glycolipids presenting phosphoinositolmannosides with long linear chains of beta-1, 2-linked mannose residues. *J Biol Chem* 1999; **274**: 30520-30526

13 **Janusz MJ,** Austen KF, Czop JK. Isolation of a yeast heptaglucoside that inhibits monocyte phagocytosis of zymosan particles. *J Immunology* 1989; **142**: 959-965

14 **Jouault T,** Lepage G, Bernigaud A, Trinel PA, Fradin C, Wieruszeski JM, Strecker G, Poulain D. Beta-1,2 linked oligomannosides from Candida albicans act as signals for tumor necrosis factor alpha production. *Infect Immun* 1995; **63**: 2378-2381

15 **Heelan BT**, Allan S, Barnes RMR. Identification of a 200-kDa glycoprotein antigen of Saccharomyces cerevisiae. *Immunol Letters* 1991; **28:** 181-186

16 **Sander U,** Kunze I, Broker M, Kunze G. Humoral immune response to a 200-kDa glycoprotein antigen of Saccharomyces cerevisiae is common in man. *Immunology Letters* 1998; **61**: 113-117

17 **Sendid B,** Colombel JF, Jacquinot PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohns disease. *Clin Diagn Lab Immunol* 1996; **2**: 219-226

18 **Ryan C.** Oligosaccharide signals: From plant defense to parasite offense. *Proc Natl Acad Sci U S A* 1994; **91**: 1-2

19 **Strobel S**, Mowat AM. Immune responses to dietary antigens: oral tolerance. *Immunol Today* 1998; **19**: 173-181

20 **Spellberg B**, Edwards JE. Type 1/Type 2 immunity in infectious diseases. *Clin Infect Dis* 2001; **32**: 76-102

21 **Winston BW**, Krein PM, Mowat C, Huang Y. Cytokine-induced macrophage differentiation: a tale of 2 genes. *Clin Invest Med* 1999; **22**: 236-255

22 **Damoiseaux JG,** Bouten B, Linders AM, Austen J, Roozendaal C, Russel MG, Forget PP, Tervaert JW. Diagnostic value of anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies for inflammatory bowel disease: high prevalence in patients with celiac disease. *Clin Immunol* 2002; **22**: 281-288


**Edited by** Xu XQ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2313-1316
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

# Clinical utility, safety and tolerability of capsule endoscopy in urban Southeast Asian population

Tiing-Leong Ang, Kwong-Ming Fock, Tay-Meng Ng, Eng-Kiong Teo, Yi-Lyn Tan

**Tiing-Leong Ang, Kwong-Ming Fock, Tay-Meng Ng, Eng-Kiong Teo, Yi-Lyn Tan,** Division of Gastroenterology, Changi General Hospital, Singapore
**Correspondence to:** Dr. Ang Tiing Leong, Division of Gastroenterology, Department of Medicine, Changi General Hospital, 2 Simei Street 3, 529889 Singapore. tiing_leong_ang@cgh.com.sg
**Telephone:** +65-67888833 **Fax:** +65-67816202
**Received:** 2003-07-17 **Accepted:** 2003-08-02

## Abstract

**AIM:** Capsule endoscopy has demonstrated its clinical utility in the evaluation of small bowel pathology in several Western studies. In this prospective study, we aimed to determine the clinical utility, safety and tolerability of capsule endoscopy in the evaluation of suspected small bowel disease in an urban Southeast Asian population.

**METHODS:** We used the given (M2A) capsule endoscopy system in 16 consecutive patients with suspected small bowel pathology. In 9 patients the indication was obscure gastrointestinal bleeding, while in 6 patients it was to determine the extent of small bowel involvement in Crohn's disease. One patient underwent capsule endoscopy for evaluation of chronic abdominal pain. Patient's tolerability to the procedure was evaluated by standardized questionnaires and all patients were reviewed at one week to ensure that the capsule had been excreted without any adverse events.

**RESULTS:** Abnormal findings were present in 8 patients (50 %). The cause of obscure gastrointestinal bleeding was determined in 5 out of 9 patients. Findings included 2 cases of angiodysplasia, 2 cases of jejunal ulcers and 1 case of both angiodysplasia and jejunal ulcer. One patient had small bowel erosions and foci of erythema of doubtful significance. Ileal lesions were diagnosed in 2 out of 6 patients with Crohn's disease. Capsule endoscopy was well tolerated by all patients. One patient with Crohn's disease had a complication of capsule retention due to terminal ileum stricture. The capsule eventually passed out spontaneously after 1 month.

**CONCLUSION:** Our study, which represented the first Asian series, further confirms the diagnostic utility, safety and tolerability of wireless capsule endoscopy.

Ang TL, Fock KM, Ng TM, Teo EK, Tan YL. Clinical utility, safety and tolerability of capsule endoscopy in urban Southeast Asian population. *World J Gastroenterol* 2003; 9(10):2313-2316
http://www.wjgnet.com/1007-9327/9/2313.asp

## INTRODUCTION

Small bowel imaging is important in the evaluation of obscure gastrointestinal bleeding[1], inflammatory disease of the small bowel[2] and tumours. The main methods of small bowel imaging have been either enteroscopy or small bowel barium studies in the evaluation of luminal pathology. Angiography is a diagnostic option in the context of suspected small intestinal bleeding. Push enteroscopy allows examination of only 80 to 120 cm of the small bowel beyond the ligament of Treitz, while intra-operative enteroscopy requires general anesthesia and laparotomy. Small bowel series and enteroclysis have limited sensitivity, and in particular, could not detect flat lesions such as angiodysplasia[3].

Wireless capsule endoscopy was first reported by Iddan *et al*[4] in 2000, and it represents a major advancement in the imaging of the small intestine. It is able to capture video-images of the mucosal surface of the entire length of the small intestine directly, and has been reported to be virtually pain-free since it is essentially propelled forward through the gastrointestinal tract by peristalsis, without the need for any air-insufflation.

Thus far all published data on the use of capsule endoscopy have been from Western countries, with only 2 case reports on its use from India. One was a case of ileal angiodysplasia[5] while another was that of small intestine tuberculosis[6].

In this prospective study, we aimed to determine the clinical utility, safety and tolerability of capsule endoscopy in the evaluation of suspected small bowel disease in an urban Southeast Asian population.

## MATERIALS AND METHODS

### *Study population*

Consecutive patients with suspected small bowel pathology seen at the Division of Gastroenterology, Changi General Hospital, Singapore, were recruited. Indications for capsule endoscopy included obscure gastrointestinal bleeding, assessment of extent of small intestine involvement in Crohn's disease, evaluation of chronic abdominal pain and assessment for small bowel pathology in the presence of malabsorption. Patients with the following conditions were excluded from the study: pregnant subjects, suspected small bowel obstruction, presence of cardiac pacemaker, subjects expected to undergo MRI examination before elimination of capsule and subjects with swallowing difficulty. The study was approved by the Hospital Ethics Committee. Informed consent was obtained from all patients before commencement of the study.

### *Given diagnostic imaging system*

The given diagnostic imaging system is comprised of the 26 mm by 11 mm M2A® capsule which contains a miniscule color video-camera equipped with a localization feature, a data recorder which is a portable, battery operated external receiving/recording unit that receives data transmitted by the capsule and subsequently allows data downloading, the Rapid® Workstation, a modified personal computer which has been designed for storage, processing and presentation of captured images as well as generation of reports. The technical details have previously been described in an earlier paper[7].

### *M2A® capsule ingestion, data capture and follow-up*

After a 12-hour fast, subjects underwent capsule examination with the M2A®capsule according to the standard procedure. Briefly it involved attaching 8 sensory arrays to the abdomen

based on a standard template. These arrays were then attached to the portable battery powered data recorder. The capsule was swallowed and subjects were advised that fluids were allowed after 2 hours and light snack after 4 hours. Subjects were then allowed to go home. After 8 hours, the subject returned and the equipment was removed and the data were downloaded into the Rapid® Workstation and the images were analyzed using the proprietary software. The subjects were asked to look for the capsule in the feces. They were also reviewed again at 1 week for any complications and to ensure that the capsule had passed out. If excretion of the capsule was not noticed by the subject, an abdominal X-ray would be done.

### *Assessment of tolerability and complications*

All subjects were asked about tolerability of the procedure in the following areas using a standardised questionnaire, which included ease of swallowing, pain or discomfort experienced during the procedure, pain or discomfort experienced after the procedure, overall tolerability of the procedure, and overall convenience of the procedure. The subjects were also evaluated at one week after procedure for any adverse events such as pain, nausea,vomiting and capsule impaction or retention.

### *Capsule image interpretation*

Two gastroenterologists (Drs Tay-Meng Ng and Tiing-Leong Ang) reviewed the capsule images independently and reported on the findings. Questionable findings were discussed. Positive findings were defined as detected abnormalities that were potentially related to the presenting problem.

## RESULTS

### *Demographics*

During the period from mid February to May 2003, 16 consecutive patients with suspected small bowel pathology and no contraindication to capsule endoscopy were recruited. Their mean age was 55.6 years (range: 19 to 82 years) with a male to female ratio of 9:7. Table 1 summarizes the patient profile, indication for capsule endoscopy and the results of the study.

Nine patients had obscure gastrointestinal bleeding, having presented with anaemia and melaena. All underwent both gastroscopy and colonoscopy. These investigations did not reveal significant pathology that could have accounted for the bleeding. Push enteroscopy was done in 2 of these patients, and small bowel series were done in another 4. Apart from small bowel diverticula seen on small bowel series in one patient, the rest of the small bowel imaging were normal.

Six patients were diagnosed to have Crohn' s disease on the basis of characteristic clinical, endoscopic and histologic features. Capsule endoscopy was performed to determine the extent of small bowel involvement in these patients.

One patient had a problem of recurrent abdominal pain and capsule endoscopy was performed to exclude any small intestine pathology after previous endoscopies did not reveal any significant lesions.

### *Findings in capsule endoscopy*

Among the 9 patients with obscure gastrointestinal bleeding, capsule endoscopy was able to diagnose a clinically significant pathology in 5 of them (55.6 %). Two patients had jejunal ulcers (Figure 1). Two patients had angiodysplasia while the last patient had both angiodysplasia and jejunal ulcer. It was felt that the jejunal ulcers could have been related to NSAID usage. A sixth patient had the finding of small bowel erosions and foci of erythema, but the authors felt that these lesions could not have accounted for the gastrointestinal bleeding. Among the 6 patients with Crohn' s disease, capsule endoscopy revealed small bowel pathology in 2 cases. One patient had multiple ileal ulcers and strictures (Figure 2) while the other had erythema in the terminal ileum. These abnormalities were not detected by small bowel barium studies done prior to capsule endoscopy. Overall, abnormalities were present in 50 % (8/16) of the subjects.

### *Patient tolerability and complications*

All the patients rated the procedure as comfortable and very convenient. Despite the size of the capsule being larger than most tablets, all the patients described swallowing as very easy. There was no complaint of pain or discomfort during or after the procedure. However, 2 of the patients commented on the weight of the portable recorder/battery pack.

The capsule was excreted in all the patients without any ill effects. One patient had retention of capsule beyond one week without developing any obstructive symptoms. It took 31 days before the capsule was noted to have passed out spontaneously. This was the patient with Crohn' s disease who had ileal ulcer and stricture. In fact, an attempt was made to retrieve it endoscopically at one week but it was unsuccessful. It was then decided to follow up the patient and wait for spontaneous passage of the capsule as he had remained asymptomatic

**Table 1** Patient characteristics and results of capsule endoscopy

| SN. | Age / sex | Indication for capsule endoscopy | Results of capsule endoscopy |
|---|---|---|---|
| 1 | 66 / male | Extent of small bowel involvement in Crohn's disease | Erythema in terminal ileum |
| 2 | 30 / male | Extent of small bowel involvement in Crohn's disease | Edematous small bowel |
| 3 | 62 / female | Obscure gastrointestinal blood loss | Small bowel angiodysplasia |
| 4 | 39 / male | Extent of small bowel involvement in Crohn's disease | Ulcers and strictures in ileum |
| 5 | 28 / male | Extent of small bowel involvement in Crohn's disease | Normal small bowel |
| 6 | 74 / female | Extent of small bowel involvement in Crohn's disease | Gastric erosions; normal small bowel |
| 7 | 62 / male | Obscure gastrointestinal blood loss | Gastric erosion; normal small bowel |
| 8 | 69 / male | Obscure gastrointestinal blood loss | Jejunal ulcer; small bowel angiodysplasia |
| 9 | 59 / female | Obscure gastrointestinal blood loss | Mild gastritis and duodenitis; normal small bowel |
| 10 | 82 / female | Obscure gastrointestinal blood loss | Gastritis; small bowel angiodysplasia |
| 11 | 54 / female | Extent of small bowel involvement in Crohn's disease | Normal small bowel |
| 12 | 69 / male | Evaluation for small intestine pathology in context of chronic abdominal pain | Normal small bowel |
| 13 | 71 / female | Obscure gastrointestinal blood loss | Jejunal ulcer |
| 14 | 66 / female | Obscure gastrointestinal blood loss | Bleeding jejunal ulcer |
| 15 | 46 / male | Obscure gastrointestinal blood loss | Normal small bowel |
| 16 | 19 / male | Obscure gastrointestinal blood loss | Small bowel erosions and foci of erythema |

throughout, rather then subject him to surgery. For the remaining 15 patients, the capsule was excreted within a week, six of these patients actually did not notice the passage of the capsule, but an abdominal X-ray done at one week proved that the capsule had not been retained.

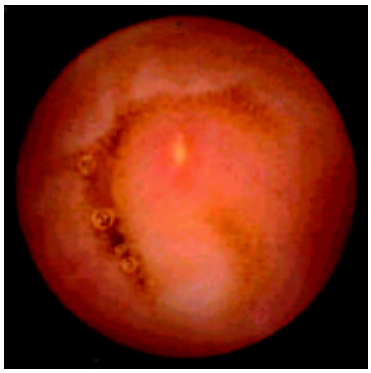


**Figure 1** A case of jejunal ulcer due to NSAIDS.

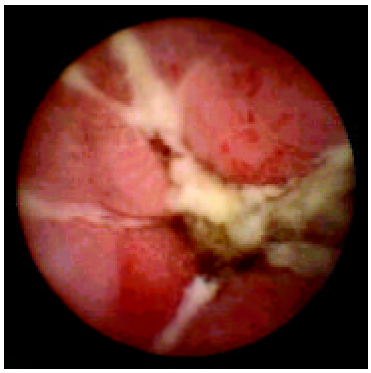


**Figure 2** Terminal ileum ulcer and stricture in a patient with Crohn's disease.

## DISCUSSION

Our study, which represented the first Asian series, further confirmed the diagnostic utility, safety and tolerability of wireless capsule endoscopy. In our series, it was able to reveal significant small intestine pathology in 43.8 % (7/16) of the patients. In particular, for obscure gastrointestinal bleeding, it was able to reveal the cause in 55.6 % of the patients. It was remarkably well tolerated, and apart from one patient with delayed capsule excretion, there were no complications. This complication highlighted the need to be vigilant towards the possibility of intestinal obstruction when considering the use of capsule endoscopy.

In a prospective study of 20 patients, Costamagna *et al*[7] confirmed the superiority of capsule endoscopy over small bowel radiographs in the diagnosis of suspected small bowel disease. Capsule endoscopy was able to detect abnormalities in 17 out of 20 patients, compared to 3 out of 20 using barium studies. Similarly, Scapa[8] in another prospective study found that among 35 patients with suspected small intestine pathology but normal small bowel series, capsule endoscopy was able to detect clinically significant small intestine pathology in 63 % of (22/35) patients.

For the evaluation of obscure gastrointestinal bleeding, our results were comparable to other published data. Scapa[8] was able to find a source of bleeding in 75 % (15/20) of his patients with obscure gastrointestinal bleeding. Lewis[9] had a positive yield of 55 % (11/20) in the evaluation of obscure gastrointestinal bleeding with capsule endoscopy. Ell[10] detected a definite source in 66 % of his patients.

For the diagnosis of Crohn's disease, Fireman[11], Eliakim[12] and Herrerias[13] have demonstrated the clinical utility of capsule endoscopy in diagnosis as well as in assessment of the extent of disease. It was particularly relevant where conventional endoscopic and radiological techniques have not identified pathological findings but clinical suspicion based on symptoms and laboratory tests was strong. In our series, only 33 % of the patients with Crohn's disease had abnormal findings on capsule endoscopy. This probably simply reflected the actual prevalence of small bowel involvement in our series, rather than under-diagnosis, since alternative imagings, such as barium studies when done, did not reveal any small bowel lesions either. In addition it was recognized that about 1/3 of patients with Crohn's disease had ileocolic disease, about 1/3 had colonic disease and about 1/3 had small bowel disease[14]. An important point to note was that one patient actually had ileal stricture that led to delayed excretion of the capsule. In this case, the presence of small bowel stricture was not suspected, as the patient did not have any clinical evidence of small bowel obstruction. It is therefore suggested that if Crohn's disease is a diagnostic consideration, then small bowel series or enema should precede the use of capsule endoscopy in order to minimize the risk of capsule retention. It is also worth noting that despite the stricture, the capsule was able to pass out without any ill effect after 31 days. Therefore in the absence of any signs or symptoms of capsule impaction or obstruction, a conservative approach may be adopted, with the use of high dose steroid to reduce the inflammation and oedema so as to facilitate the passage of the capsule, as occurred in this patient with Crohn's stricture.

Possible complications and limitations exist with any procedure, and capsule endoscopy is no exception, in spite of the high diagnostic yield. The key complication is that of capsule retention proximal to a stricture, and a 5 % retention rate has been reported, with the need for surgery in less than 1 % of patients[15]. Another concern is delayed passage of capsule due to slow transit time, resulting in capsule recording terminating before it has passed to the caecum, and hence incomplete data acquisition. Other limitations included difficulty in determining the exact site of the abnormality in the small bowel, inability to take tissue biopsy, problem of visual clarity due to intestinal fluids and long viewing time of the video, which could take up to 2 hours[7]. It is also costly, with the estimated cost of each capsule being greater than the cost of undergoing colonoscopy in the Singapore context. Thus it is likely that its use would remain selective, being reserved for situations when endoscopy or other imaging modalities have failed to achieve a definite diagnosis. These would include the investigation of obscure gastrointestinal bleeding, and in the context where clinical suspicion for Crohn's disease is strong but the results of all other tests have been equivocal.

In conclusion, our study affirms the diagnostic utility and safety of capsule endoscopy in clinical practice. It has the promise to become a leading method in the evaluation of small bowel pathology, but limitations and the issue of cost need to be addressed.

## ACKNOWLEDGEMENT

Servicom Medical (Singapore) Pte Ltd: For provision of M2A®Capsules (Given Imaging Limited, Yoqneam, Israel)) for the study.

## REFERENCES

1 **Zuckerman GR**, Prakash C, Askin MP, Lewis BS. AGA technical review on the evaluation and management of occult and obscure gastrointestinal bleeding. *Gastroenterology* 2000; **118**: 201-221

2 **Gay GJ**, Delmotte JS. Enteroscopy in small intestinal inflammatory diseases. *Gastrointest Endosc Clin North Am* 1999; **9**: 115-123
3 **Nolan DJ**, Traill ZC. The current role of the barium examination of the small intestine. *Clin Radiol* 1997; **52**: 809-820
4 **Iddan G**, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. *Nature* 2000; **405**: 417
5 **Nagral A**, Nisar P, Nagral S. Capsule endoscopy diagnosis of ileal angiodysplasia. *Indian J Gastroenterol* 2003; **22**: 64-65
6 **Reddy DN**, Sriram PV, Rao GV. Capsule endoscopy appearances of small bowel tuberculosis. *Endoscopy* 2003; **35**: 99
7 **Costamagna G**, Shah SK, Riccioni ME. A prospective trial comparing small bowel radiographs and video capsule endoscopy for suspected small bowel disease. *Gastroenterology* 2002; **123**: 999-1005
8 **Scapa E**, Jacob H, Lewkowicz S. Initial experience of wireless capsule endoscopy for evaluating occult gastrointestinal bleeding and suspected small bowel pathology. *Am J Gastroenterol* 2002; **97**: 2776-2779
9 **Lewis BS**, Swain P. Capsule endoscopy in the evaluation of patients with suspected small intestinal bleeding: results of a pilot study. *Gastrointest Endosc* 2002; **56**: 349-353
10 **Ell C**, Remke S, May A. The first prospective controlled trial comparing wireless capsule endoscopy with push enteroscopy in chronic gastrointeastinal bleeding. *Endoscopy* 2002; **34**: 685-689
11 **Fireman Z**, Mahajna E, Broide E. Diagnosing small bowel Crohn's disease with wireless capsule endoscopy. *Gut* 2003; **52**: 390-392
12 **Eliakim R**, Fischer D, Suissa A. Wireless capsule endoscopy is a superior diagnostic tool in comparision to barium follow-through and computerized tomography in patients with suspected Crohn's disease. *Eur J Gastroenterol Hepatol* 2003; **15**: 363-367
13 **Carucci LR**, Levine MS. Radiographic imaging of inflammatory bowel disease. *Gastroenterol Clin N Am* 2002; **31**: 93-117
14 **Herrerias JM**, Caunedo A, Rodriguez-Tellez M. Capsule endoscopy in patients with suspected Crohn's disease and negative endoscopy. *Endoscopy* 2003; **35**: 564-568
15 **Cave DR**. Wireless video capsule endoscopy. *Clinical Perspectives Gastroenterol* 2002; **5**: 203-207

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2317-2321
World Journal of Gastroenterology
 ISSN 1007-9327

• CLINICAL RESEARCH •

# Budd-Chiari syndrome: Diagnosis with three-dimensional contrast-enhanced magnetic resonance angiography

Jiang Lin, Xiao-Hai Chen, Kang-Rong Zhou, Zu-Wang Chen, Jian-Hua Wang, Zhi-Ping Yan, Ping Wang

**Jiang Lin, Xiao-Hai Chen, Kang-Rong Zhou, Zu-Wang Chen, Jian-Hua Wang, Zhi-Ping Yan, Ping Wang,** Department of Radiology, Affiliated Zhongshan Hospital, Fudan University, Shanghai 200032, China
**Correspondence to:** Dr. Jiang Lin, Department of Radiology, Affiliated Zhongshan Hospital, Fudan University, Shanghai 200032, China. linjiang@zshospital.net
**Telephone:** +86-21-64041990 Ext 2463 **Fax:** +86-21-64038472
**Received:** 2003-04-02 **Accepted:** 2003-05-11

## Abstract

**AIM:** To evaluate the role of three-dimensional contrast-enhanced magnetic resonance angiography (3D CE MRA) in the diagnosis of Budd-Chiari syndrome (BCS).

**METHODS:** Twenty-three patients with BCS underwent 3D CE MRA examination, in which 13 cases were secondary to either hepatocellular carcinoma (11 cases), right adrenal carcinoma (1 case) or thrombophlebitis (1 case) and 10 suffered from primary BCS. The patency of the inferior vena cava (IVC), hepatic and portal veins as well as the presence of intra- and extrahepatic collaterals, liver parenchymal abnormalities and porto-systemic varices were evaluated. Inferior vena cavography was performed in 10 cases. The diagnosis of IVC obstruction by 3D CE MRA was compared with that demonstrated by inferior vena cavography.

**RESULTS:** The major features of BCS could be clearly displayed on 3D CE MRA. Positive hepatic venous signs included tumor thrombosis (9 cases), tumor compression (2 cases), nonvisualization (4 cases) and focal stenosis (2 cases). Positive IVC findings were noted as severe stenosis or occlusion (10 cases), tumor invasion (2 cases), thrombosis (3 cases), thrombophlebitis (1 case) and septum formation (3 cases). Intrahepatic collaterals were shown in 9 patients, 2 of them with "spider web" sign. The displayed extrahepatic collaterals included dilated azygos and hemi-azygos veins (13 cases) and left renal-inferior phrenic-pericardiophrenic veins (2 cases). The occlusion of the left intrahepatic portal veins was found in 2 cases. Porto-systemic varices were detected in 10 patients. Liver parenchymal abnormalities displayed by 3D CE MRA were enlargement of the caudate lobe (7 cases), heterogenous enhancement (18 cases) and complicated tumors (13 cases). Compared with the inferior vena cavography performed in 10 cases, the accuracy of 3D CE MRA was 100 % in the diagnosis of IVC obstruction.

**CONCLUSION:** 3D CE MRA can display the major features of BCS and provide an accurate diagnosis.

Lin J, Chen XH, Zhou KR, Chen ZW, Wang JH, Yan ZP, Wang P. Budd-Chiari syndrome: Diagnosis with three-dimensional contrast-enhanced magnetic resonance angiography. *World J Gastroenterol* 2003; 9(10):2317-2321
http://www.wjgnet.com/1007-9327/9/2317.asp

## INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare disease caused by the obstruction of the hepatic venous outflow or the inferior vena cava (IVC) above the hepatic vein[1]. It often occurs secondary to intrinsic vascular thrombosis, hepatic tumor invasion/compression, or associates with an idiopathic obstructing membrane[1,2]. The clinical signs of ascites, abdominal pain and hepatomegaly are the typical triad of BCS. Since these signs are nonspecific, the clinical diagnosis of this syndrome is difficult. Conventionally, X-ray angiography and/or liver biopsy are used to confirm the diagnosis of BCS with the limitation of invasiveness. Ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) are the noninvasive imaging techniques currently used in the evaluation of the patency of hepatic veins, IVC and portal vein. However, some limitations are also existed in each of these modalities[2-7].

Three-dimensional contrast-enhanced magnetic resonance angiography (3D CE MRA) is a new technique and widely used in the imaging of the arterial system, portal venous system and central venous system[8-16]. However, to our knowledge, few studies have been reported so far concerning its use in the diagnosis of BCS[17]. Therefore, this study was conducted to evaluate the usefulness of this technique in the imaging of BCS and to present various findings of BCS demonstrated on 3D CE MRA.

## MATERIALS AND METHODS

### *Patients*

Twenty-three patients with BCS underwent 3D CE MRA. There were 20 men and 3 women ranging from 26 to 56 years of age (average age 38 years). In 11 patients, BCS was secondary to the invasion and occlusion of the hepatic vein and/or IVC by hepatocellular carcinoma (HCC). In 1 patient, BCS was resulted from tumor invasion of IVC by right adrenal carcinoma. In 1 patient, IVC obstruction was due to superior extension of pelvic thrombophlebitis. The above 13 patients were considered to be secondary BCS. Ten patients had primary BCS with an unknown origin. The diagnosis of BCS was confirmed by inferior vena cavography in 10 cases, percutaneous liver biopsy in 2 cases, and surgery in 5 cases. The diagnosis of the remaining 6 cases was confirmed by combined color Doppler sonography and contrast-enhanced CT.

### *3D CE MRA examination*

All scans were performed using a 1.5T MR imager (Signa, General Electric Medical Systems, Milwaukee, WI.) and a body coil. After the localizing images were obtained, a breathhold T1-weighted fast multiplanar spoiled gradient-echo (FMPSPGR) sequence (repetition time/echo time, 150/4.2 msec; flip angle, 90°; field of view, 360 mm; matrix, 128×256; 18 slices; slice thickness, 7.0 mm; gap, 3.0 mm; one signal acquired) and a respiratory-triggered T2-weighted fast spin-echo sequence (repetition time/echo time, 2800-4200/80 msec; echo train length 8-12, field of view, 360 mm; matrix, 128×256; 18 slices; slice thickness, 7.0 mm; gap, 3.0 mm; 2 signals

acquired) were performed in the liver. For 3D CE MRA, a breath-hold 3D fast spoiled gradient-echo sequence (repetition time/echo time, 5.2-10.2/1.2-1.9 msec; flip angle, 30° or 45°; field of view, 360-480 mm; matrix, 128×256; imaging volume, 75-168 mm; number of partitions, 24-30; one signal acquired; and acquisition time, 19-28 sec) was used.

With T1-weighted and T2-weighted images as reference, the imaging volume of 3D CE MRA was acquired in a coronal plane to cover the hepatic veins, IVC, portal veins and collateral vessels. The imaging volume was determined by a radiologist depending on each patient´s abnormalities, liver size and ability of breathholding.

A gadolinium chelate called gadopentetate-dimeglumine (Magnevist; Schering AG, Berlin, Germany) was used as a contrast material for all examinations, with a concentration of 0.15 mmol per kilogram of body weight. In all cases the contrast material was injected by an experienced MR technician through an antecubital vein with an injection rate of approximately 3 ml/sec. In 2 patients, however, due to poor visualization of IVC after injection of the contrast material into an arm vein, the study was repeated and the contrast agent was injected via a pedal vein for assessment of IVC. Scanning was commenced immediately after the injection and repeated three times with a 6-second delay between each acquisition for patient' s breathing. The first acquisition was the imaging of the arterial phase, the second acquisition was the portal venous phase for demonstration of the portal vein and IVC, while the third acquisition coincided with the image of the late venous phase for visualization of the hepatic veins. Source images of each acquisition were reviewed first, and then these images were reconstructed on a workstation (Advantage windows workstation, General Electric Medical Systems, Milwaukee, WI) to produce projectional images like X-ray angiography. Both maximum intensity projection (MIP) and multiplanar reconstruction (MPR) techniques were employed to analyze the acquired image.

### *Image analysis*

3D CE MRA images were reviewed together by two radiologists unaware of the patients' clinical statuses and other imaging findings. The patency of the hepatic veins, IVC and portal veins was assessed. The presence of intra- and extrahepatic collaterals, liver parenchymal abnormalities and porto-systemic varices were also noted. Inferior vena cavography was performed in 10 cases. The diagnosis of IVC obstruction by 3D CE MRA was compared with that demonstrated by inferior vena cavography.

## RESULTS

### *3D CE MRA findings*

**Hepatic veins** 3D CE MRA demonstrated tumor thrombosis of two or three hepatic veins in each of the 9 patients with HCC (Figure 1). The right and middle hepatic veins were severely compressed and distorted by HCC in 2 patients. Nonvisualization of hepatic veins occurred in 4 patients with primary BCS. Focal stenosis of the right and middle hepatic veins near the caval confluence was detected in 2 patients with primary BCS (Figure 2).

**IVC** Severe stenosis or occlusion of the IVC was found in 10 patients (Figure 3), 3 of them were associated with the external compression or direct invasion of IVC by HCC. Tumor thrombosis was found in 3 cases with HCC (Figure 1). Direct invasion of IVC by right adrenal tumor was observed in 1 case. Thrombosis of IVC was shown in 1 patient with pelvic thrombophlebitis. A septum or obstructing membrane was demonstrated in the IVC in 3 patients (Figure 4). Localized dilatation of the distal IVC and renal veins were shown in 2 cases with IVC obstruction. In 10 patients with inferior vena cavography, the site and extent of IVC obstruction and distribution of collaterals presented by 3D CE MRA were in agreement with those by cavography (Figure 3). The accuracy of 3D CE MRA was 100 % in the diagnosis of IVC obstruction.

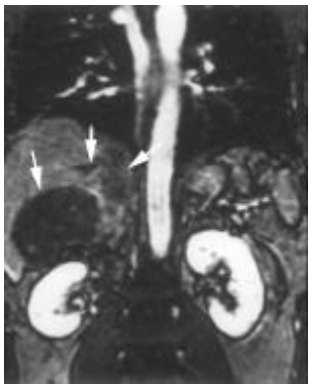


**Figure 1** Source image of three-dimensional contrast-enhanced magnetic resonance angiography shows tumor thrombosis of the right hepatic vein (short arrow) and inferior vena cava (long arrow). A hypointense hepatocellular carcinoma (arrowhead) is shown simultaneously.

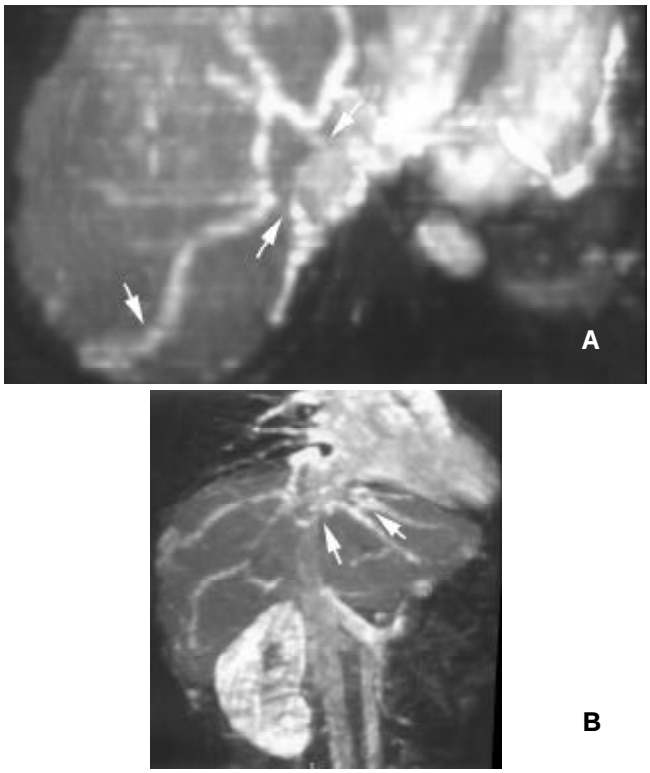



**Figure 2** A: Axial reconstruction of three-dimensional contrast-enhanced magnetic resonance angiography demonstrates focal stenosis of right and middle hepatic veins (arrow heads). Intrahepatic collateral between right hepatic vein and subcapsular vein is also demonstrated (arrow). B: Coronal reconstruction reveals fine collaterals between hepatic veins (arrows), resembling a "spider web".

**Intra-and extrahepatic collaterals** 3D CE MRA revealed fine venous collaterals between the right hepatic veins and subcapsular veins, and between the right, middle and left hepatic veins in 2 patients with focal hepatic vein stenosis, appearing as the typical "spider web" sign (Figure 2B). Large and tortuous veins between enlarged right inferior accessory hepatic veins and right main hepatic veins were identified in 3

patients with IVC occlusion (Figure 3B). Various extrahepatic collaterals were found in the abdominal wall, peritoneal and retroperitoneal areas. Prominent azygos and hemiazygos veins were demonstrated in 13 patients. The left renal-inferior phrenic-pericardiophrenic collaterals were displayed in 2 patients (Figure 5).

**Portal veins and porto-systemic varices** The portal veins were found patent in all observed patients except 2 whose left intrahepatic portal veins were occluded (Figure 6). Gastroesophageal/esophageal varices and spontaneous splenorenal shunts were identified in 8 and 2 patients respectively.

**Liver parenchyma** An enlarged caudate lobe was found in 7 patients. Heterogenous liver enhancement was observed in 18 cases (Figure 6). HCC, which showed hyperintensity in arterial phase and hypointensity in portal venous phase, was found in 13 patients (Figure 1). In 2 of them, the tumor was developed after the primary BCS formation, because these two patients underwent long-term follow-up studies and the initial imaging findings indicated no intrahepatic tumors.

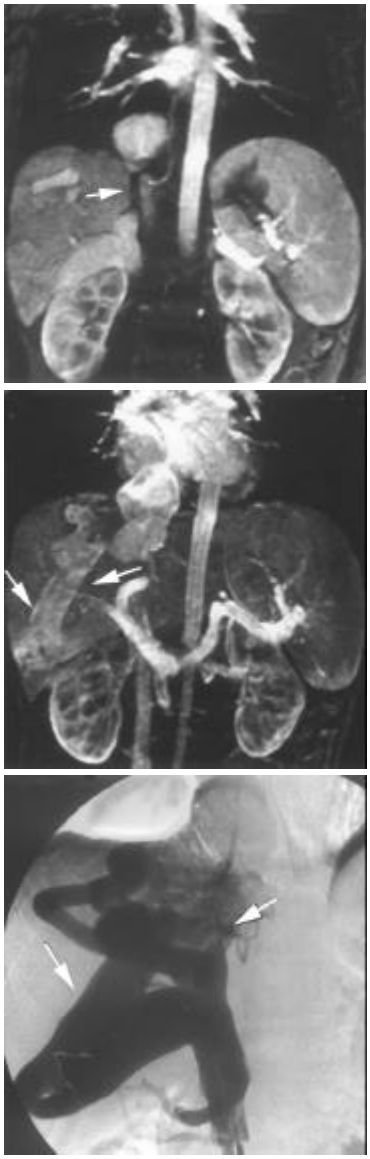



**Figure 3** A: Source image depicts occlusion of the inferior vena cava(arrowhead). B: Maximum intensity projection of three-dimensional contrast-enhanced magnetic resonance angiography depicts tortuous intrahepatic collaterals (arrows) between right inferior accessory hepatic vein and right main hepatic vein. C: Inferior vena cavography confirms the occlusion of the inferior vena cava (arrowhead) and the intrahepatic collaterals (arrow).

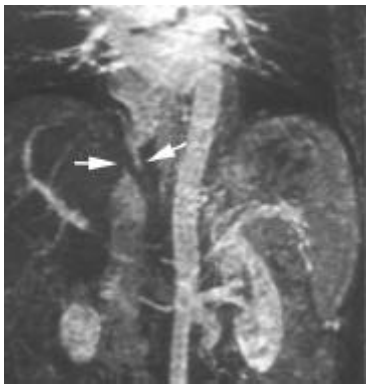


**Figure 4** Three-dimensional contrast-enhanced magnetic resonance angiography identifies a septum (arrowheads) in the inferior vena cava.

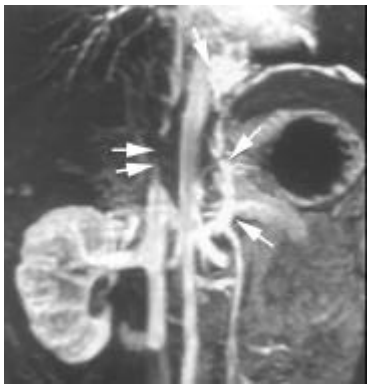


**Figure 5** Three-dimensional contrast-enhanced magnetic resonance angiography detects occlusion of the inferior vena cava(white arrowheads), prominent left renal-inferior phrenic-pericardiophrenic collaterals (arrows) and esophageal varix (black arrowhead).

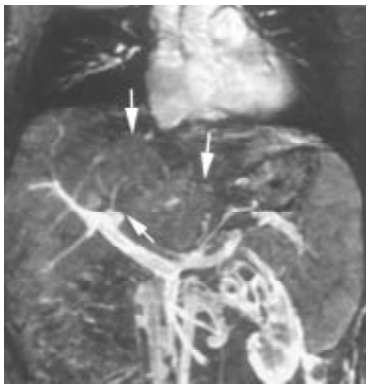


**Figure 6** Three-dimensional contrast-enhanced magnetic resonance angiography demonstrates occlusion of the left portal vein (arrowhead) and heterogeneous enhancement pattern of the liver (arrows).

## DISCUSSION

BCS is difficult to be diagnosed clinically. However, accurate and early diagnosis is important for its treatment. Radiologic assessment is an important step for its diagnosis. Diagnosis by US was noninvasive, relatively inexpensive and readily available, but its accuracy was limited by operator' s experience, poor acoustic window, inaccessible anatomic structure and body forms[1,3,7]. Contrast-enhanced CT was readily available too, but it used ionizing radiation and required intravenous administration of a large amount of iodinated contrast material with the risk of

nephrotoxicity and possible allergic reactions[1,5,6]. X-ray angiography was the reference criteria for the evaluation of BCS, due to its superb spatial resolution[1,3], but it was invasive and uncomfortable. Like CT, it also involved radiation and use of iodinated contrast material. Disadvantages also included high cost, requirement of operator' s expertise (especially for hepatic venography), and associated complications such as hemorrhage. MRI and non-enhanced magnetic resonance angiography (MRA) with time-of-flight (TOF) and phase-contrast (PC) techniques were other non-invasive means for demonstrating the hepatic venous system, but were limited by long acquisition time, motion and flow artifacts, and saturation effects[2,4,18-21].

3D CE MRA is a recently developed, non-invasive, fast and easy to be performed technique, which is capable of depicting vascular anatomy in multiple projections. It involves no radiation. With this technique, the imaging of the hepatic veins, IVC and portal veins is accomplished with only one injection of contrast material and short breath holding. It requires only a peripheral venous injection of a small amount of gadolinium, which is much safer than iodine-based contrast material. By using gadolinium to shorten the T1 value of the blood, it overcomes flow artifacts and saturation effects in TOF and PC. Moreover, it permits assessment of liver parenchyma and extravascular abnormalities during investigation of the vascular system[12,15,17]. So 3D CE MRA has many advantages over the currently more conventional methods for imaging the BCS.

According to our study, 3D CE MRA was able to demonstrate the patency of the hepatic veins, IVC and portal veins. It could show intra- and extrahepatic collaterals as well as porto-systemic varices caused by cirrhosis. 3D CE MRA could distinguish extravascular compression from intravascular thrombosis, detect liver abnormalities and evaluate the extent of the disease. By means of only one examination, 3D CE MRA provided all these crucial information for accurate diagnosis of BCS and for possible surgical or interventional managements, such as porto-caval shunt, liver transplantation, transjugular intrahepatic porto-systemic shunt (TIPSS), percutaneous transluminal angioplasty (PTA) and stent placement[22-27].

Among 13 patients of secondary BCS, tumor thrombosis, external compression and direct invasion of hepatic veins and/or IVC by tumors were the main causes of this disease. Through demonstration of intra- and extrahepatic disorders and the involved blood vessels, 3D CE MRA could disclose the etiology of BCS. For patients with primary BCS, nonvisualization, focal stenosis of the hepatic veins, occlusion or a septum formed in IVC were the most common findings on 3D CE MRA, as have been reported by Miller *et al*[5] with CT and MRI techniques. Compared with vena cavography, 3D CE MRA was 100 % accurate in diagnosis of IVC obstruction. On the basis of this study and Erden' s report[17], 3D CE MRA could replace inferior vena cavography for diagnosing IVC obstruction.

3D CE MRA could evaluate the intra- and extrahepatic collateral pathways. Identification of intrahepatic collateral veins is highly suggestive of BCS[6,17,28]. The intrahepatic collateral veins divert blood away from the stenotic or occluded hepatic vein and into a patent hepatic vein or a systemic vein. According to Cho' s report[6], intrahepatic collaterals were poorly defined on contrast-enhanced CT. In this study, 3D CE MRA found these collateral veins in 2 patients with stenosis of the hepatic veins. On 3D CE MRA, they were identified either by typical "spider web" sign or by large connecting veins between accessory and main hepatic veins. The dilated azygos and hemiazygos veins were the most commonly collateralized extrahepatic routes shown in this study. The infrequent left renal-inferior phrenic-pericardiophrenic collaterals were also seen on 3D CE MRA.

Our study indicated that a strong point of 3D CE MRA was to display the portal venous system simultaneously with hepatic veins and IVC. Obstruction or increased pressure in hepatic veins resulted in portal hypertension and portal flow stasis, which might further cause porto-systemic varices and portal vein thrombosis. Complications of portal hypertension were fatal in more than 50 % of BCS patients[27]. Thus the accurate delineation of the abnormalities of the portal vein, and the anatomic relationship between portal vein, hepatic veins and IVC were very important, particularly when preparing the treatment with porto-caval shunt or TIPSS[22-24,27]. In this study, 3D CE MRA showed the ability to provide all these relevant information.

Hypertrophy of the caudate lobe was found on 3D CE MRA. This was related to independent blood supply and drainage of this lobe. Heterogeneous enhancement pattern observed by 3D CE MRA has been already known from studies on contrast-enhanced CT[1]. It reflected the hemodynamic disturbance in the liver with BCS. 3D CE MRA found 2 patients with primary BCS developed HCC during a long-term follow-up period. The development of HCC in patients with chronic BCS has been reported in the literature[29]. According to that report, many factors, such as chronic viral infection or cirrhosis might play a role in the development of this malignancy.

Our study had two limitations. Firstly, a detailed comparison between 3D CE MRA and X-ray angiography was not performed, because none of our patients underwent hepatic venography and only some had inferior vena cavography. But 3D CE MRA could demonstrate various findings of BCS, which might provide clues to the correct diagnosis. Secondly, the opacification of IVC, after injection of contrast material into an arm vein, was inadequate in 2 patients with severe cirrhosis, portal hypertension and edema. We speculated this was due to the dilution of the contrast material in the porto-systemic collaterals, enlarged spleens and increased extracellular fluid. In these circumstances, the IVC could be enhanced properly by injecting contrast material into a pedal vein.

In conclusion, 3D CE MRA can display various features of BCS and provide an accurate diagnosis.

## REFERENCES


1 **Stanley P**. Budd-Chiari syndrome. *Radiology* 1989; **170**(3Pt 1): 625-627

2 **Kane R**, Eustace S. Diagnosis of Budd-Chiari syndrome: comparison between sonography and MR angiography. *Radiology* 1995; **195**: 117-121

3 **Millener P**, Grant EG, Rose S, Duerinckx A, Schiller VL, Tessler FN, Perrella RR, Ragavendra N. Color Doppler imaging findings in patients with Budd-Chiari syndrome: correlation with venographic findings. *Am J Roentgenol* 1993; **161**: 307-312

4 **Soyer P**, Rabenandrasana A, Barge J, Laissy JP, Zeitoun G, Hay JM, Levesque M. MRI of Budd-Chiari syndrome. *Abdom Imaging* 1994; **19**: 325-329

5 **Miller WJ**, Federle MP, Straub WH, Davis PL. Budd-Chiari syndrome: imaging with pathologic correlation. *Abdom Imaging* 1993; **18**: 329-335

6 **Cho OK**, Koo JH, Kim YS, Rhim HC, Koh BH, Seo HS. Collateral pathways in Budd-Chiari syndrome: CT and venographic correlation. *Am J Roentgenol* 1996; **167**: 1163-1167

7 **Ralls PW**, Johnson MB, Radin DR, Boswell WD Jr, Lee KP, Halls JM. Budd-Chiari syndrome: detection with color Doppler sonography. *Am J Roentgenol* 1992; **159**: 113-116

8 **Leung DA**, Mckinnon GC, Davis CP, Pfammater T, Krestin GP, Debatin JF. Breath-hold, contrast-enhanced, three-dimensional MR angiography. *Radiology* 1996; **200**: 569-571

9 **Kopka L**, Rodenwaldt J, Vosshenrich R, Fischer U, Renner B, Lorf T, Graessner J, Ringe B, Grabbe E. Hepatic blood supply: comparison of optimized dual phase contrast-enhanced three-dimen-

sional MR angiography and digital subtraction angiography. *Radiology* 1999; **211**: 51-58
10 **Zeh H**, Choyke PL, Alexander HR, Bartlett D, Libutti SK, Chang R, Summers RM. Gadolinium-enhanced 3D MRA prior to isolated hepatic perfusion for metastases. *J Comput Assist Tomogr* 1999; **23**: 664-669
11 **Glockner JF**, Forauer AR, Solomon H, Varma CR, Perman WH. Three-dimensional gadolinium-enhanced MR angiography of vascular complications after liver transplantation. *Am J Roentgenol* 2000; **174**: 1447-1453
12 **Hawighorst H**, Schoenberg SO, Knopp MV, Essig M, Miltner P, van Kaick G. Hepatic lesions: morphologic and functional characterization with multiphase breath-hold 3D gadolinium-enhanced MR angiography-initial results. *Radiology* 1999; **210**: 89-96
13 **Saddik D**, Frazer C, Robins P, Reed W, Davis S. Gadolinium-enhanced three-dimensional MR portal venography. *Am J Roentgenol* 1999; **172**: 413-417
14 **Laissy JP**, Trillaud H, Douek P. MR angiography: noninvasive vascular imaging of the abdomen. *Abdom Imaging* 2002; **27**: 488-506
15 **Thornton MJ**, Ryan R, Varghese JC, Farrell MA, Lucey B, Lee MJ. A three-dimensional gadolinium-enhanced MR venography technique for imaging central veins. *Am J Roentgenol* 1999; **173**: 999-1003
16 **Shinde TS**, Lee VS, Rofsky NM, Krinsky GA, Weinreb JC. Three-dimensional gadolinium-enhanced MR venographic evaluation of patency of central veins in the throrax: initial experience. *Radiology* 1999; **213**: 555-560
17 **Erden A**, Erden I, Krayalcin S, Yurdaydin C. Budd-Chiari syndrome: evaluation with multiphase contrast-enhanced three-dimensional MR angiography. *Am J Roentgenol* 2002; **179**: 1287-1292
18 **Colletti PM**, Christopher TO, Terk MR, Boswell MD. Magnetic resonance of the inferior vena cava. *Magn reson imaging* 1992; **10**: 177-185
19 **Ruehm SG**. MR venography. *Eur Radiol* 2003; **13**: 229-230
20 **Butty S**, Hagspiel KD, Leung DA, Angle JF, Spinosa DJ, Matsumoto AH. Body MR venography. *Radiol Clin North Am* 2002; **40**: 899-919
21 **Mohiaddin RH**, Wann SL, Underwood R, Firmin DN, Rees S, Longmore DB. Vena caval flow: assessment with cine MR velocity mapping. *Radiology* 1990; **177**: 537-541
22 **Slakey DP**, Klein AS, Venbrux AC, Cameron JL. Budd-Chiari syndrome: current management options. *Ann Surg* 2001; **233**: 522-527
23 **Perello A**, Garcia-Pagan JC, Gilabert R, Suarez Y, Moitinho E, Cervantes F, Reverter JC, Escorsell A, Bosch J, Rodes J. TIPS is a useful long-term derivative therapy for patients with Budd-Chiari syndrome uncontrolled by medical therapy. *Hepatology* 2002; **35**: 132-139
24 **Schepke M**, Sauerbruch T. Transjugular portosystemic stent shunt in treatment of liver diseasse. *World J Gastroenterol* 2001; **7**: 170-174
25 **Pelage JP**, Denys A, Valla D, Sibert A, Sauvanet A, Belghiti J, Menu Y. Budd-Chiari syndrome due to prothrombotic disorder: mid-term patency and efficacy of endovascular stents. *Eur Radiol* 2003; **13**: 286-293
26 **Suhocki PV**, Trotter JF. Percutaneous hepatic vein reconstruction for Budd-Chiari syndrome. *Am J Roentgenol* 1998; **171**: 189-191
27 **Blum U**, Rossle M, Haag K, Ochs A, Blum HE, Hauenstein KH, Astinet F, Langer M. Budd-Chiari syndrome: technical, hemodynamic, and clinical results of treatment with transjugular intrahepatic portosystemic shunt. *Radiology* 1995; **197**: 805-811
28 **Naganuma H**, Ishida H, Konno K, Komatsuda T, Hamashima Y, Ishida J, Masamune O. Intrahepatic venous collaterals. *Abdom Imaging* 1998; **23**: 166-171
29 **Vilgrain V**, Lewin M, Vons C, Denys A, Valla D, Flejou JF, Belghiti J, Menu Y. Hepatic nodules in Budd-Chiari syndrome: imaging features. *Radiology* 1999; **210**: 443-450

**Edited by** Zhu L

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2322-2324
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Healthy ranges of serum alanine aminotransferase levels in Iranian blood donors

Mehdi Mohamadnejad, Akram Pourshams, Reza Malekzadeh, Ashraf Mohamadkhani, Afsaneh Rajabiani, Ali Ali Asgari, Seyed Meysam Alimohamadi, Hadi Razjooyan, Mansooreh Mamar-Abadi

**Mehdi Mohamadnejad, Akram Pourshams, Reza Malekzadeh, Ashraf Mohamadkhani, Ali Ali Asgari, Seyed Meysam Alimohamadi, Hadi Razjooyan, Mansooreh Mamar-Abadi,** Digestive Disease Research Center, Tehran University of Medical Sciences, Tehran, Iran
**Afsaneh Rajabiani,** Department of Pathology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
**Correspondence to:** Reza Malekzadeh, Digestive Disease Research Center, Tehran University of Medical Sciences, Shariati Hospital, North Kargar Avenue Tehran 14114, Iran. malek@ams.ac.ir
**Telephone:** +98-21-8012992 **Fax:** +98-21-2253635
**Received:** 2003-05-13 **Accepted:** 2003-07-20

## Abstract

**AIM:** The healthy ranges for serum alanine aminotransferase (ALT) levels are less well studied. The aim of this study was to define the upper limit of normal (ULN) for serum ALT levels, and to assess factors associated with serum ALT activity in apparently healthy blood donors.

**METHODS:** A total of 1 939 blood donors were included. ALT measurements were performed for all cases using the same laboratory method. Healthy ranges for ALT levels were computed from the population at the lowest risk for liver disease. Univariate and multivariate analyses were performed to evaluate associations between clinical factors and ALT levels.

**RESULTS:** Serum ALT activity was independently associated with body mass index (BMI) and male gender, but not associated with age. Association of ALT with BMI was more prominent in males than in females. Upper limit of normal for non-overweight women (BMI of less than 25) was 34 U/L, and for non-overweight men was 40 U/L.

**CONCLUSION:** Serum ALT is strongly associated with sex and BMI. The normal range of ALT should be defined for male and female separately.

Mohamadnejad M, Pourshams A, Malekzadeh R, Mohamadkhani A, Rajabiani A, Asgari AA, Alimohamadi SM, Razjooyan H, Mamar-Abadi M. Healthy ranges of serum alanine aminotransferase levels in Iranian blood donors. *World J Gastroenterol* 2003; 9(10):2322-2324
http://www.wjgnet.com/1007-9327/9/2322.asp

## INTRODUCTION

Elevation of aminotransferase level is an important and common finding in different types of parenchymal liver disease. Measurement of serum ALT is one of the most important tests for detection of patients with viral hepatitis or non-alcoholic steatohepatitis (NASH), and the exact definition of upper normal levels of serum ALT activity is an initial and critical step in different screening and follow up studies for chronic liver diseases. Current upper limits of normal for ALT level are set on average, at 40 U/L. This normal range was set in the 1950s and has changed a little since then[1]. Several studies have recently questioned whether previously established values to define normal ALT range are accurate and have suggested that the upper limit of normal should be assessed more accurately and revised accordingly[2,3].

There is no study regarding normal level of ALT in Iranian healthy adults at low risk for chronic liver diseases. This information, in addition to daily clinical practice, is specially important and necessary for different research studies of chronic liver diseases in Iran. The aim of this study was to assess the normal value of ALT in a population at low risk for subclinical chronic liver diseases in the capital city of Tehran and to investigate factors associated with abnormal ALT in this population.

## MATERIALS AND METHODS

### *Study population*

From March 2001 through April 2002, 1 959 apparently healthy blood donors at Tehran Blood Donation Center were randomly recruited into the study. The participants were part of a study for identifying the causes of elevated serum ALT level. After explanation about the objectives of the study and possible necessity for further blood test and follow up, a written informed consent was obtained, and a clinical questionnaire with emphasis on psychosocial and medical history to exclude subjects who were considered the high risk group for blood born infections was completed by a physician interviewer, and serum samples were collected from all consenting subjects. Our study was in accordance with the ethical standards for human experimentation and approved by the Ethical Committee of the Digestive Disease Research Center, Tehran University of Medical Sciences. Body weight and height of all subjects were measured and history of alcohol and drug use was taken.

### *Laboratory methods*

Blood samples were centrifuged within 30 minutes of collection. The biochemistry and virologic tests including hepatitis B s antigen (HBsAg), and hepatitis C virus antibody (HCV Ab), and rapid plasma regain test, and HIV Ab were measured. All tests were performed at Digestive Disease Research Center, Tehran University of Medical Sciences, Tehran, Iran. Analyses of serum ALT levels were performed by using the Hitachi 704 autoanalyser, Tokyo, Japan. The upper limit of normal introduced by manufacturer was 40 U/L for both men and women. Body mass index (BMI) was calculated by dividing the weight (in kg) and the squared height (in meter). We considered a BMI of 24.9 kg/m$^2$ as the upper limit for healthy weight[5].

### *Definitions of ULN ALT value*

Seven methods were used to compare their impact. Method 1: 95$^{th}$ percentile of ALT distribution regardless of the sex. Method 2: 95 $^{th}$ percentile of ALT distribution after separating males and

females. Method 3: a common threshold of 40 IU/L for both males and females proposed by the manufacturer. Method 4: 95th percentile after separating subjects with body mass index (BMI) under the median which was 27.12. Method 5: 95th percentile after separating subjects with body mass index (BMI) under 25 -a threshold proposed for separating abnormal and normal weight-[5]. Method 6: 95th percentile of ALT distribution stratified according to BMI (<25) and sex. Method 7: 95th percentile of ALT distribution in each age decade after separating males and females.

### *Statistical analysis*

Statistical analyses were performed by using the SPSS, version 10.1, software package (SPSS, Inc., Chicago, IL). The 50th (median), and 95th percentiles for ALT level were calculated on the basis of the empirical distribution of the data. We set the upper limit for healthy ALT level to the 95 th percentile, as is commonly done for distribution of a continuous variable in the normal population. The univariate associations between factors and ALT expressed in decimal logarithm were assessed by Pearson's correlation coefficient for quantitative factors and by the Student's *t* test for qualitative factors. Multivariate analyses were used to identify factors independently associated with ALT: linear regression, and logistic regression. *P* values less than 0.05 were considered statistically significant.

## RESULTS

Twenty persons were excluded because of positive HBsAg (10 persons), positive HCV Ab (9 persons), or use of alcohol more than 20 grams daily (1 person). Four persons also consumed less than 20 grams per day of alcohol who were included into the study. Thus a total of 1 939 persons (1 451 males, and 488 females) were included. The characteristics of tested individuals are given in Table 1. Except one subject who took drugs containing female sex hormones, no body had a history of regular drug usage.

**Table 1** Characteristics of 1 939 Blood Donors

| Factor | Mean | SE |
|---|---|---|
| Age (yr) | 37.4 | 0.26 |
| Weight (kg) | 79 | 0.3 |
| Height (cm) | 169.96 | 0.19 |
| BMI (kg/cm$^2$) | 27.35 | 0.09 |
| ALT (U/L) | 19.87 | 0.27 |

Abbreviations: SE: Standard error of the mean.

**Table 2** Correlation of serum ALT with quantitative clinical factors

| Factor | Number | Pearson correlation | *P* value |
|---|---|---|---|
| BMI | 1939 | 0.125 | <0.001 |
| Weight | 1939 | 0.17 | <0.001 |
| Height | 1939 | 0.096 | <0.001 |
| Age | 1939 | 0.027 | 0.23 |

**Table 3** Serum ALT according to sex, and BMI (lower or higher than 25)

| Variable | Count | Mean | SD | *P* Value |
|---|---|---|---|---|
| Female | 488 | 16.4 | 8.8 | <0.001 |
| Male | 1 451 | 21 | 12.3 | |
| All subjects with BMI <25 | 563 | 17.9 | 10.4 | <0.001 |
| All subjects with BMI ⩾25 | 1 376 | 20.7 | 12.1 | |
| Men with BMI <25 | 391 | 19.1 | 10.8 | <0.001 |
| Men with BMI ⩾25 | 1 060 | 21.7 | 12.8 | |
| Women with BMI <25 | 172 | 15.2 | 8.8 | 0.025 |
| Women with BMI ⩾25 | 316 | 17 | 8.7 | |

Abbreviation: SD: Standard deviation.

**Table 4** Prevalence among blood donors with normal and abnormal ALT according to the six definitions

| Subjects | Method 1: 95th percentile of ALT distribution without stratification | Method 2: 95th percentile of ALT distribution stratified according to sex | Method 3: 40 IU/L for both male and female | Method 4: 95th percentile of ALT distribution stratified according to BMI (<27.1) | Method 5: 95th percentile of ALT distribution stratified according to BMI (<25) | Method 6: 95th percentile of ALT distribution stratified according to BMI (<25) and sex |
|---|---|---|---|---|---|---|
| Males | 1451 | 1451 | 1451 | 1451 | 1451 | 1451 |
| Threshold | 40 | 45 | 40 | 39 for BMI⩾27.1 | 39 for BMI <25 | 40 for BMI <25 |
| | | | | 41/ 721 | 20/ 391 | 16/391 |
| | | | | 45 for BMI>27.1 | 43 for BMI ⩾25 | 46 for BMI⩾25 |
| | | | | 40/ 730 | 65/1060 | 48/1060 |
| Normal (%) | 1 363 (94%) | 1378 (95%) | 1363 (94%) | 1370 (94. 5%) | 1366 (94.1%) | 1 387 (95. 6%) |
| Abnormal (%) | 88 (6%) | 73 (5%) | 88 (6%) | 81 (5. 5%) | 85 (5. 9%) | 64 (4.4%) |
| Females | 488 | 488 | 488 | 488 | 488 | 488 |
| Threshold | 40 | 34 | 40 | 39 for BMI⩾27.1 | 39 for BMI <25 | 34 for BMI <25 |
| | | | | 6/249 | 4/ 172 | 8/172 |
| | | | | 45 for BMI >27.1 | 43 for BMI ⩾25 | 34 for BMI⩾25 |
| | | | | 2/ 239 | 2/ 316 | 15/316 |
| Normal (%) | 481 (98. 6%) | 464 (95%) | 481 (98. 6%) | 480 (98.4%) | 482 (98.8%) | 465 (95. 3%) |
| Abnormal (%) | 7 (1.4%) | 24 (5%) | 7 (1.4%) | 8 (1. 6%) | 6 (1. 2%) | 23 (4.7%) |
| All donors | 1939 | 1939 | 1939 | 1939 | 1939 | 1939 |
| Threshold | 40 | 45 for female | 40 | 39 for BMI ⩾27.1 | 39 for BMI <25 | Male: 40 for BMI<25 |
| | | 34 for male | | 45 for BMI>27.1 | 43 for BMI⩾25 | 46 for BMI⩾25 |
| | | | | | | Female: 34 for BMI<25 |
| | | | | | | 34 for BMI⩾25 |

### *Correlation between factors and ALT*

ALT was significantly correlated with BMI, weight, and height, but was not correlated with age (Table 2).

For qualitative factors (Table 3), ALT was higher in males than in females, and ⩾25 than BMI<25 in persons with BMI. Association of ALT with BMI was more prominent in men ($P$<0.001) than in women ($P$=0.025).

Linear regression analysis showed that ALT was independently associated with male sex (Regression coefficient: 4.633, 95 % CI: 3.459-5.808, $P$<0.0001), and BMI (Regression coefficient: 0.362, 95 % CI: 0.237-0.487, $P$<0.0001), but not with height, weight, and age.

Also, logistic regression analysis showed that men were 4.57 times more likely to have elevated ALT (ALT>40) than women (95 % CI, 2.1-9.96, $P$=0.0001). BMI was also independently associated with elevated ALT (OR: 1.07, 95 % CI: 1.03-1.13, $P$=0.004). Age, height, and weight were not found to be related to elevated ALT.

### *Different definitions of abnormal ALT*

The thresholds corresponding to the first six methods to the definition of abnormal ALT are given in Table 4. The threshold to the definition of abnormal ALT according to method 7 is demonstrated in Figure 1.

The threshold for ULN ALT varied from 34 U/L (methods 2 and 6 for females) to 46 U/L (method 6 for males with BMI>25).

The overall median ALT level for the entire study sample was 17 U/L, and the level across the 95$^{th}$ percentile was 40 U/L. In the 1 451 male participants, the median serum ALT level was 18 U/L, and the ALT levels across the 95 th percentile was 45 U/L. The median and 95$^{th}$ percentiles of ALT level in women were 14 U/L and 34 U/L, respectively. When we considered the population at the lowest risk for liver diseases (persons with negative viral markers, use of alcohol less than 20 g/d, normal BMI, and absence of concurrent medication use), the threshold for abnormal ALT was 40 U/L for men, and 34 U/L for women.

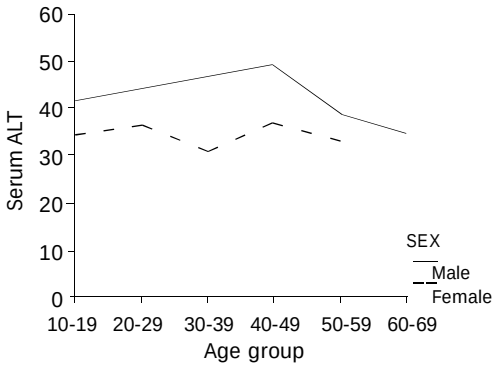



**Figure 1** 95$^{th}$ percentile of ALT distribution in each age decade after separating males and females. Note that males over age 70 (41 subjects), and females over age 60 (8 subjects) were omitted from the chart, because of a small number of them.

## DISCUSSION

This study has identified the factors associated with ALT variability, and determined the thresholds for ALT according to different definitions of ULN ALT. This study further emphasized the findings of previous studies regarding the strong correlation of ALT with sex and BMI[2,3]. This has probably reflected the association between liver steatosis and obesity. The correlation between abnormal ALT and BMI was stronger in men than in women. Additionally, the 95$^{th}$ percentile of ALT in females with BMI <25 was equal to females with BMI ⩾25. This may be due to the fact that the waist to hip ratio (WHR) is higher in men than in women, and non-alcoholic fatty liver disease (NAFLD) is associated with central obesity and higher WHR[5]. WHR was correlated with visceral adipose tissue, which provided a greater supply of potentially hepatotoxic fatty acids to the liver[6].

Our study had some limitations. The study population were apparently healthy blood donors. They could have other unknown factors associated with ALT and they might not exactly reflect the normal general population. Second, the estimation of alcohol consumption was based only on the interview data and might be inaccurate. Third, we could not measure some paraclinical factors associated with ALT such as serum glucose, triglyceride and cholesterol[2,5].

The ULN ALT may differ from different nations and populations and may be influenced by mean BMI and alcohol usage in different societies.

The 95$^{th}$ percentile of ALT in overweight and obese persons may be too high to be defined as the threshold for healthy ranges of ALT. We suggest that even in overweight and obese persons healthy ranges of ALT should be defined as in non-overweight persons (40 U/L and 34 U/L in men and in women respectively). Since higher values may be due to liver steatosis which occurs more frequently in obese persons, and thus may be abnormal. Adjustment of ALT for sex but not for BMI has also been proposed previously[1]. It seems necessary to repeat this type of investigations in a population based sample and in different ethnic and nationals in order to check whether the impact of sex and BMI remain consistent and if it is proved to be so, then the laboratories should set different ranges of ALT for male and female independently.

In conclusion, this study has demonstrated a strong impact of sex and BMI on serum ALT level. Furthermore, if these findings are proved by other studies, then the normal range of ALT according to sex should be defined. This is particularly helpful in follow up and therapy of patients with chronic hepatitis and designing research protocols.

## REFERENCES

1 **Kaplan MM**. Alanine Aminotransferase Levels: What's Normal? *Ann Intern Med* 2002; **137**: 49-51

2 **Prati D**, Taioli E, Zanella A, Della Torre E, Butelli S, Del Vecchio E, Vianello L, Zanuso F, Mozzi F, Milani S, Conte D, Colombo M, Sirchia G. Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med* 2002; **137**: 1-9

3 **Piton A**, Poynard T, Imbert-Bismut F, Khalil L, Delattre J, Pelissier E, Pelissier E, Sansonetti N, Opolon P. Factors associated with serum alanine transaminase activity in healthy subjects: consequences for the definition of normal values, for selection of blood donors, and for patients with chronic hepatitis C. *Hepatology* 1998; **27**: 1213-1219

4 **Willett WC**, Dietz WH, Colditz GA. Guidelines for healthy weight. *N Engl J Med* 1999; **341**: 427-434

5 **Ruhl CE**, Everhart JE. Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the United States. *Gastroenterology* 2003; **124**: 71-79

6 **Falck-Ytter Y**, Younossi ZM, Marchesini G, McCullough AJ. Clinical features and natural history of nonalcoholic steatosis syndromes. *Semin Liver Dis* 2001; **21**: 17-26

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2325-2327
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Adrenomedullin in cirrhotic and non-cirrhotic portal hypertension

V Tahan, E Avsar, C Karaca, E Uslu, F Eren, S Aydin, H Uzun, HO Hamzaoglu, F Besisik, C Kalayci, A Okten, N Tozun

**V Tahan, E Avsar, F Eren, HO Hamzaoglu, C Kalayci, N Tozun,** Gastroenterology Institute, Marmara University
**C Karaca, F Besisik, A Okten,** Gastroenterology Department' Medical Faculty, Istanbul University
**E Uslu, S Aydin, H Uzun,** Biochemistry Department, Cerrahpasa Medical Faculty, Istanbul University
**Correspondence to:** Dr. Veysel Tahan, Alemdag Cad. Yanyol Rifat Bey Sokak Ugur Apt. No=27/19 Kısıklı, Üsküdar, Istanbul/Turkey.
veytahan@yahoo.com
**Telephone:** +90-532-3629602
**Received:** 2003-06-04 **Accepted:** 2003-08-02

## Abstract

**AIM:** Adrenomedullin (ADM) is a potent vasodilator peptide. ADM and nitric oxide (NO) are produced in vascular endothelial cells. Increased ADM level has been linked to hyperdynamic circulation and arterial vasodilatation in cirrhotic portal hypertension (CPH). The role of ADM in non-cirrhotic portal hypertension (NCPH) is unknown. plasma ADM levels were studied in patients with NCPH, compensated and decompensated cirrhosis in order to determine its contribution to portal hypertension (PH) in these groups.

**METHODS:** There were 4 groups of subjects. Group 1 consisted of 27 patients (F/M: 12/15) with NCPH due to portal and/or splenic vein thrombosis (mean age: 41±12 years), group 2 consisted of 14 patients (F/M: 6/8) with compensated (Child-Pugh A) cirrhosis (mean age: 46±4), group 3 consisted of 16 patients (F/M: 6/10) with decompensated (Child-Pugh C) cirrhosis (mean age: 47±12). Fourteen healthy subjects (F/M: 6/8) (mean age: 44±8) were used as controls in Group 4. ADM level was measured by ELISA. NO was determined as nitrite/nitrate level by chemoluminescence.

**RESULTS:** ADM level in Group 1 (236±61.4 pg/mL) was significantly higher than that in group 2 (108.4±28.3 pg/mL) and group 4 (84.1±31.5 pg/mL) (both $P<0.0001$) but was lower than that in Group3 (324±93.7 pg/mL) ($P=0.002$). NO level in group 1 (27±1.4 μmol/L) was significantly higher than that in group 2 (19.8±2.8 μmol/L) and group 4 (16.9±1.6 μmol/L) but was lower than that in Group 3 (39±3.6 μmol/L) (for all three $P<0.0001$). A strong correlation was observed between ADM and NO levels ($r=0.827$, $P<0.0001$).

**CONCLUSION:** Adrenomedullin and NO levels were high in both non-cirrhotic and cirrhotic portal hypertension and were closely correlated, Adrenomedullin and NO levels increased proportionally with the severity of cirrhosis, and were significantly higher than those in patients with NCPH. Portal hypertension plays an important role in the increase of ADM and NO. Parenchymal damage in cirrhosis may contribute to the increase in these parameters.

Tahan V, Avsar E, Karaca C, Uslu E, Eren F, Aydin S, Uzun H, Hamzaoglu HO, Besisik F, Kalayci C, Okten A, Tozun N. Adrenomedullin in cirrhotic and non-cirrhotic portal hypertension. *World J Gastroenterol* 2003; 9(10):2325-2327
http://www.wjgnet.com/1007-9327/9/2325.asp

## INTRODUCTION

Portal hypertension (PH) is a clinical condition characterized by specific hemodynamic abnormalities such as low arterial pressure, high cardiac output, over activity of vasoconstrictor systems and marked decrease in total systemic vascular resistance. Arterial vasodilatation and activation of several vasoactive and neurohumoral systems may play a key role in pathogenesis of sodium and water retention and ascites formation in cirrhosis[1,2]. These changes have been attributed to increased production of vasodilator substances[2,3]. Adrenomedullin (ADM) and nitric oxide (NO) are considered as essential mediators of hyperdynamic state. NO, a potent vasodilator substance synthesized from L-arginin by NO synthase, is increased in cirrhotic patients and experimental models of cirrhosis[3,4]. Specific NO inhibitors have been shown to counteract vasodilatation and hyperdynamic circulation in these groups[4,5]. In addition, circulating levels of potent vasodilating peptides (substance P, CGRP), pulsatile blood flow, and shear stress could contribute to the up regulation of endothelial NO-synthase (e-NOS)[3,6].

ADM is a potent endogenous vasorelaxing factor that was originally isolated from the extracts of human pheochromocytoma[7]. ADM is expressed in adrenal gland, lungs, kidneys, smooth muscle vascular endothelial cells, and splanchnic organs[3,8]. ADM level is increased after stimulation with bacterial endotoxin and cytokines[9]. The renal effects of ADM and part of its cardiovascular effects seem to be mediated by NO[10,11]. Previous studies reported that increased ADM level in cirrhotic patients occurred via increased production or decreased clearance of this substance, the elevation was more prominent in decompensated cirrhotic patients with marked PH[2,4,12,13]. Although ADM level is known to be increased in cirrhotic PH (CPH), there are no data concerning its level in non-cirrhotic PH (NCPH).

To our knowledge this is the first study determining plasma ADM concentrations in NCPH patients. We also aimed to compare the results within the group of compensated and decompensated cirrhotic patients (Child-Pugh A & C) and healthy subjects in order to estimate the contribution of cirrhosis and PH either separately or concomitantly to the elevation of ADM and NO level.

We compared ADM levels in the patients with compensated or decompensated cirrhosis and also in healthy subjects in order to determine whether portal hypertension or cirrhosis led to elevation in ADM and NO levels.

## MATERIALS AND METHODS

### *Patients*

Our study included patients with NCPH, cirrhosis and healthy subjects as control. Cirrhosis was due to hepatitis C infection in all-cirrhotic cases, while in all cases of NCPH the origin was extrahepatic portal venous thrombosis. group 1 included 27 patients (F/M: 12/15 with NCPH due to portal and/or splenic vein thrombosis (mean age: 41±12 years), Group 2 consisted of 14(F/M: 6/8) compensated (Child-Pugh A) cirrhotic patients (mean age: 46±4), group 3 included 16 (F/M: 6/10) decompensated cirrhotic (Child-Pugh C) patients (mean age: 47±12), and group 4 consisted of 14 (F/M: 6/8) healthy subjects (mean age: 44±8) taken as controls.

The diagnosis of PH and cirrhosis was established on the basis of clinical, biochemical and ultrasonographic findings and/or liver biopsy. Patients with active or recent gastrointestinal bleeding, bacterial infection, severe hepatic encephalopathy, cardiopulmonary disease and portal vein thrombosis within the previous ten days were excluded.

### *Study protocol*

All patients and controls received restricted sodium diet (70 mmol/day) for at least 5 days before the study. Diuretics, beta-blockers and other cardioactive drugs were withheld during the 10-day period before the study. At 8.00 a.m. on the day of study, blood samples were drawn after the patients were fasted overnight and then waited for 45 min in supine position. This study was performed in accordance with the Declaration of Helsinki. A written informed consent was obtained from each patient and control subject participating in the study.

### *Biochemical analysis*

Nitric oxide heparinized whole blood was obtained by venipuncture, centrifuged (3 000 rpm, 10 minutes, 0-40 C). Plasma concentration of NO was studied by chemoluminescence method using commercially available colorimetric assay (Roche, Cat No 1746081)[14].

### *Adrenomedullin*

Blood samples for ADM were collected into the vacutainer tubes, which contained EDTA. Blood was transferred from the vacutainer tubes to centrifuge tubes containing aprotinin (0.6 TIU/ml of blood) and gently rocked for several times to inhibit the activity of proteinases. Blood was centrifuged at 1 600 G for 15 min at 4 ℃. Plasma ADM concentration was measured by enzyme immunoassay (Phoenix Pharmaceuticals Inc. Harbor Boulevard, Belmont, California 94002) after extraction through the Sep-pak C-18 column supplied by the manufacturer[15,16].

Plasma in both parameters was immediately frozen and stored at -80 ℃ until assayed.

### *Statistical analysis*

All results were expressed as mean ± standard deviation. Comparisons between the groups were performed by Kruskal-Wallis variance analysis and a *P*- value <0.05 was accepted as statistically significant.

## RESULTS

ADM level in Group 1 (236±61.4 pg/mL) was significantly higher than that in Group 2 (108.4±28.3 pg/mL) and Group 4 (84.1±31.5 pg/mL) (both $P<0.0001$) but was lower than that in Group 3 (324±93.7 pg/mL)($P=0.002$) (Figure 1).

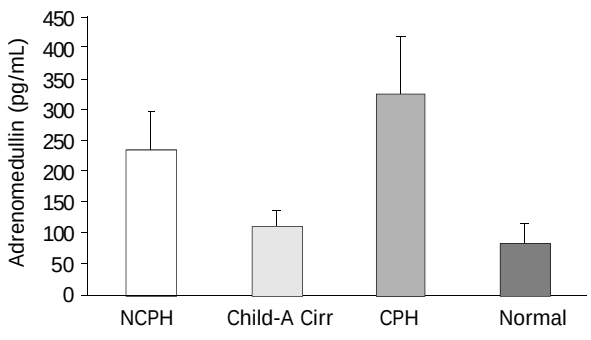



**Figure 1** Adrenomedullin levels in various groups. NCPH: Non-cirrhotic portal hypertension, Cirr: Cirrhosis, CPH: Cirrhotic portal hypertension, Normal: Healthy subjects. NCPH *vs* Child-Pugh A Cirr: $P<0.0001$, NCPH *vs* Healthy Subjets: $P<0.0001$, NCPH *vs* Child-Pugh C CPH: $P=0.002$.

Nitrite/nitrate level in Group 1 (27±1.4 μmol/L) was significantly higher than that in Group 2 (19.8±2.8 μmol/L) and Group 4 (16.9±1.6 μmol/L) but was lower than that in Group 3 (39±3.6 μmol/L) ($P<0.0001$ for all three) (Figure 2). A significant correlation was observed between ADM and NO levels ($r=0.827$, $P<0.0001$) (Figure 3).

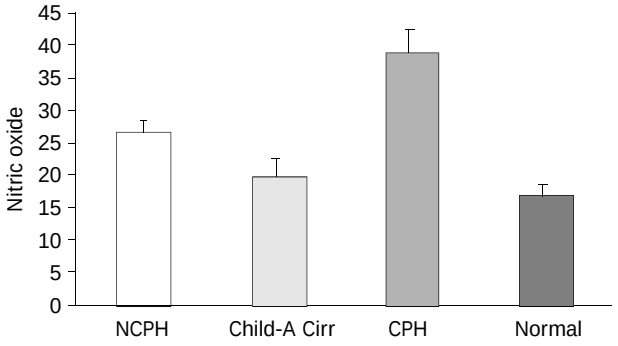



**Figure 2** Nitric oxide levels in various groups. NCPH: Non-cirrhotic portal hypertension, Cirr: Cirrhosis, CPH: Cirrhotic portal hypertension, Normal: Healthy subjects. NCPH *vs* Child-Pugh A Cirr: $P<0.0001$, NCPH *vs* Healthy Subjets: $P<0.0001$, NCPH *vs* Child-Pugh CCPH: $P<0.0001$.

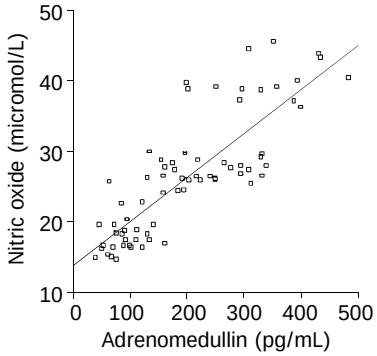



**Figure 3** Correlation between nitric oxide and adrenomedullin A significant correlation was observed between two vasoactive agents ($r=0.827$, $P<0.0001$).

## DISCUSSION

Our study demonstrated increased plasma ADM and NO concentrations in both CPH and NCPH patients, the level of ADM was the highest in the CPH group. Increased ADM level was strongly associated with PH and plasma volume expansion[10,11]. In our series, ADM level was not different between compensated cirrhotic patients and controls but higher in both groups of patients with CPH and NCPH, evidencing the role of PH in the increase of ADM.

Cirrhotic patients showed hemodynamic abnormalities such as arterial hypotension, increased cardiac output and reduced systemic vascular resistance. These changes have been attributed to peripheral vasodilatation. The reduction in the effective blood volume, and the subsequent activation of renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system initiated renal sodium and water retention and ultimately led to ascites formation[11]. Furthermore, excessive production of endogenous vasodilatators played an important role in peripheral vasodilatation. ADM and NO are well known mediators in the pathophysiology of PH. ADM is a potent vasodilatator and natriuretic peptide[4]. ADM concentration was positively correlated with plasma renin activity and aldosterone, which are indicators of RAAS activity[1]. ADM had an indirect effect on hyperdynamic circulation. It might play a role in the

mechanism of initiation of peripheral vasodilatation counterbalanced by the activation of RAAS and sympathetic nervous system and followed by renin and aldosterone increase. Previous studies reported that concentrations of ADM similar to those found in patients with ascites had vasodilatatory effect in the rat mesenteric circulation[2,12]. In addition, administration of ADM to anesthetized rat caused arterial hypotension, increased cardiac output and reduced systemic vascular resistance similar to the circulatory changes observed in cirrhosis[13]. All these changes may account for increased ADM level in patients with ascites and may also be seen in non-cirrhotic PH.

Cytokines were incriminated as to contribute to increased ADM levels via increased levels of TNF-α, interleukin-6 and bacterial endotoxin in advanced liver cirrhosis[18,19]. These factors are the result of parenchymal damage, which is known to stimulate the production of ADM by vascular smooth muscle and endothelial cells. However, a previous study reported that TNF-α and interleukin-6 levels showed very weak correlations with ADM levels[4]. In our study ADM level in CPH patients was significantly higher than that in NCPH patients. We conclude that cytokines are not completely responsible for the significant increase of ADM level in liver cirrhosis but they may contribute to this change.

Increased hepatic outflow resistance is the initial cause of CPH. It stimulates endothelial shear stress. Previous studies reported that ADM mRNA expression in endothelial cells was markedly increased by shear stress[20]. Thus ADM could be increased by the shear stress secondary to hyperdynamic circulation. ADM may also be produced in excess as an outcome of the volume expansion. Increased ADM level was found in cirrhotic chronic renal failure patients[2]. An increased ADM production to balance plasma volume overload might be an additional mechanism accounting for elevated ADM plasma concentration in patients with chronic renal disease.

NO is an important mediator of the hemodynamic alterations of liver cirrhosis. In our study ADM levels correlated with serum nitrite and nitrate levels. It was probably due to the fact that elevated NO levels in patients with advanced liver cirrhosis might further stimulate the production of NO and aggravate the vasodilatation in a vicious cycle[9,10].

In our study, ADM and NO levels in NCPH and advanced cirrhosis were higher than those in healthy controls and compensated cirrhotics. Likewise ADM and NO levels in CPH were higher than those in NCPH. Increased ADM level was closely correlated with NO level.

In conclusion, the results of our study suggest that portal hypertension *per se* is an independent factor for the elevation of ADM and NO in both cirrhotic and non-cirrhotic portal hypertension. Parenchymal destruction at various stages in cirrhosis may further contribute to the effects of these potent vasodilators and lead to a vicious cycle.

## REFERENCES


1 **Groszmann RJ.** Hyperdynamic circulation of liver disease 40 years later: pathophysiology and clinical consequences. *Hepatology* 1994; **20**: 1359-1363

2 **Guevara M,** Gines P, Jimenez W, Sort P, Fernandez-Esparrach G, Escorsell A, Bataller R, Bosch J, Arroyo V, Rivera F, Rodes J. Increased adrenomedullin levels in cirrhosis: relationship with hemodynamic abnormalities and vasoconstrictor systems. *Gastroenterology* 1998; **114**: 336-343

3 **Fernandez-Rodriguez CM,** Prada IR, Prieto J, Montuenga LM, Elssasser T, Quiroga J, Moreiras M, Andrade A, Cuttitta F. Circulating adrenomedullin in cirrhosis: relationship to hyperdynamic circulation. *J Hepatol* 1998; **29**: 250-256

4 **Genesca J**, Gonzalez A, Catalan R, Segura R, Martinez M, Esteban R, Groszmann RJ, Guardia J. Adrenomedullin, a vasodilator peptide implicated in hemodynamic alterations of liver cirrhosis: relationship to nitric oxide. *Dig Dis Sci* 1999; **44**: 372-376

5 **Pizcueta P,** Pique JM, Fernandez M, Bosch J, Rodes J, Whittle BJ, Moncada S. Modulation of the hyperdynamic circulation of cirrhotic rats by nitric oxide inhibition. *Gastroenterology* 1992; **103**: 1909-1915

6 **Fernandez-Rodriguez CM**, Prieto J, Quiroga J, Zozoya JM, Andrade A, Nunez M, Sangro B, Penas J. Plasma levels of substance P in liver cirrhosis: relationship to the activation of vasopressor systems and urinary sodium excretion. *Hepatology* 1995; **21**: 35-40

7 **Kitamura K,** Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 1993; **192**: 553-560

8 **Ichiki Y**, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, Eto T. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett* 1994; **338**: 6-10

9 **Sugo S,** Minamino N, Shoji H, Kangawa K, Kitamura K, Eto T, Matsuo H. Interleukin-1, tumor necrosis factor and lipopolysaccharide additively stimulate production of adrenomedullin in vascular smooth muscle cells. *Biochem Biophys Res Commun* 1995; **207**: 25-32

10 **Ishimitsu T,** Nishikimi T, Saito Y, Kitamura K, Eto T, Kangawa K, Matsuo H, Omae T, Matsuoka H. Plasma levels of adrenomedullin, a newly identified hypotensive peptide, in patients with hypertension and renal failure. *J Clin Invest* 1994; **94**: 2158-2161

11 **Jougasaki M,** Rodeheffer RJ, Redfield MM, Yamamoto K, Wei CM, McKinley LJ, Burnett JC Jr. Cardiac secretion of adrenomedullin in human heart failure. *J Clin Invest* 1996; **97**: 2370-2376

12 **Fabrega E,** Casafont F, Crespo J, de la Pena J, San Miguel G, de las Heras G, Garcia-Unzueta MT, Amado JA, Pons-Romero F. Plasma adrenomedullin levels in patients with hepatic cirrhosis. *Am J Gastroenterol* 1997; **92**: 1901-1904

13 **Kojima H,** Tsujimoto T, Uemura M, Takaya A, Okamoto S, Ueda S, Nishio K, Miyamoto S, Kubo A, Minamino N, Kangawa K, Matsuo H, Fukui H. Significance of increased plasma adrenomedullin concentration in patients with cirrhosis. *J Hepatol* 1998; **28**: 840-846

14 **Green LC,** Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982; **126**: 131-138

15 **Porstmann T**, Kiessig ST. Enzyme immunoassay techniques. An overview. *J Immunol Methods* 1992; **150**: 5-21

16 **Avrameas S**. Amplification systems in immunoenzymatic techniques. *J Immunol Methods* 1992; **150**: 23-32

17 **Schrier RW,** Arroyo V, Bernardi M, Epstein M, Henriksen JH, Rodes J. Peripheral arterial vasodilation hypothesis: a proposal for the initiation of renal sodium and water retention in cirrhosis. *Hepatology* 1988; **8**: 1151-1157

18 **Guarner C,** Soriano G, Tomas A, Bulbena O, Novella MT, Balanzo J, Vilardell F, Mourelle M, Moncada S. Increased serum nitrite and nitrate levels in patients with cirrhosis: relationship to endotoxemia. *Hepatology* 1993; **18**: 1139-1143

19 **Khoruts A,** Stahnke L, McClain CJ, Logan G, Allen JI. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients. *Hepatology* 1991; **13**: 267-276

20 **Chun TH,** Itoh H, Ogawa Y, Tamura N, Takaya K, Igaki T, Yamashita J, Doi K, Inoue M, Masatsugu K, Korenaga R, Ando J, Nakao K. Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. *Hypertension* 1997; **29**: 1296-1302


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2328-2331
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# *Candida* esophagitis: Risk factors in non-HIV population in Pakistan

Javed Yakoob, Wasim Jafri, Shahab Abid, Nadeem Jafri, Muhammad Islam, Saeed Hamid, Hasnain A Shah, Akbar S Hussainy

**Javed Yakoob, Wasim Jafri, Shahab Abid, Nadeem Jafri, Muhammad Islam, Saeed Hamid, Hasnain A Shah, Akbar S Hussainy,** Section of Gastroenterology, Department of Medicine and Pathology, Agha Khan University Hospital, Karachi, Pakistan
**Correspondence to:** Dr. Javed Yakoob, MBBS, PhD. Section of Gastroenterology, Department of Medicine, Agha Khan University Hospital, Stadium Road, Karachi-74800, Pakistan. yakoobjaved@hotmail.com
**Telephone:** +92-21-48594661 **Fax:** +92-21-4934294
**Received:** 2003-06-05 **Accepted:** 2003-08-19

## Abstract

**AIM:** *Candida* esophagitis is a frequent infection in immunocompromised patients. This study was designed to determine its characteristics in non- human immune deficiency virus (HIV) infected patients attending a teaching hospital.

**METHODS:** Clinical records of all patients coded by international classification of diseases 9th revision with clinical modifications' (ICD-9-CM), with *candida* esophagitis diagnosed by esophagogastroduodenoscopy (EGD) and histopathology over a period of 5 years were studied.

**RESULTS:** Fifty-one patients (27 males, 24 females, range 21-77 years old and mean age 52.9 years) fulfilled the criteria (0.34 % of the EGD). The common predisposing factors were carcinoma (OR 3.87, CI 1.00-14.99) and diabetes mellitus (OR 4.39, CI 1.34-14.42). The frequent clinical symptoms were retrosternal discomfort, dysphagia and epigastric abdominal pain with endoscopic appearance of scattered mucosal plaques. Another endoscopic lesion was associated with *candida* esophagitis in 15 % patients.

**CONCLUSION:** Carcinomas, diabetes mellitus, corticosteroid and antibiotic therapy are major risk factors for *candida* esophagitis in Pakistan. It is an easily managed complication that responds to treatment with nystatin.

Yakoob J, Jafri W, Abid S, Jafri N, Islam M, Hamid S, Shah HA, Hussainy AS. *Candida* esophagitis: Risk factors in non-HIV population in Pakistan. *World J Gastroenterol* 2003; 9(10): 2328-2331
http://www.wjgnet.com/1007-9327/9/2328.asp

## INTRODUCTION

Infective esophagitis is a rare disease, affecting mostly immunocompromised patients. *Candida* esophagitis is one of the most common opportunistic infections in patients with impaired immunity and the most common cause of esophageal disease in patients with acquired immune deficiency syndrome (AIDS)[1]. It also occurs in debilitated patients who have received broad-spectrum antibiotics, steroids and immunosuppressants. With the advent of transplantation and AIDS, esophageal infection is now a common medical problem[2]. The common infections involving immunocompromised non-human immunodeficiency virus (HIV) infected patients include *candida* and viral diseases such as *cytomegalovirus* (*CMV*) and *herpes simplex virus* (*HSV*)[2]. Immunocompromised patients who develop esophageal symptoms need to undergo endoscopy to rule out *candida* esophagitis. It is well recognized that *candida* esophagitis may coexist with other esophageal disorders in these patients[3]. The occurrence of multiple simultaneous processes makes definitive endoscopic examination important[4]. Several studies have been carried out in the west for *candida* esophagitis but it has not been studied before in Pakistan. Incidence of AIDS and HIV prevalence are still very low in Pakistan[5]. The purpose of our study was to assess the risk factors associated with *candida* esophagitis in our patients who came from an area with a low incidence of AIDS.

## MATERIALS AND METHODS

### *Patients*

The study was conducted at Agha Khan University Hospital (AKUH) in Karachi, a private Academic Medical Center that offers state of the art medical facilities and is used as a referral center for patients from all over the country for expert opinion and treatment. Karachi is a southern port and the largest metropolitan city in Pakistan with a population of over 11 million. In August 2002 we carried out a retrospective analysis of medical records of all the patients who attended endoscopy unit of gastrointestinal section at the AKUH from January 1997-December 2001 and were diagnosed as *candida* esophagitis. These patients had undergone endoscopy for symptoms arising from the upper gastrointestinal tract over this period and were diagnosed on the basis of endoscopy and histopathology as *candida* esophagitis. Clinical symptoms at the time of presentation, diagnosis, drug treatment dosage and duration preceding the symptoms such as nystatin suspension or fluconazole, past history of oral candidiasis, candida esophagitis, neutrophil and lymphocyte counts from complete blood picture, random blood glucose, hepatitis B, C and HIV serology were noted. A lymphocyte count was described as low when it was less than $1.5\times10^9$ per liter[6]. All endoscopic examinations were performed by staff-members of our hospital's gastroenterology section, using an Olympus videoscope GIF x Q 140. *Candida* esophagitis was diagnosed when characteristic *candidal* plaques were endoscopically identified and pathological confirmation of yeast forms typical for *candida* was found in association with an active esophagitis. Case patients were labeled as group A and the control group as group B which consisted of those patients without a diagnosis of *candida* esophagitis and in whom an endoscopic examination was performed immediately before and after every case patient was examined endoscopically (2 controls per case). Therefore, *candida* esophagitis cases and controls were evaluated by the same medical team using the same diagnostic criteria.

### *Endoscopy*

To describe the severity of *candida* esophagitis both in AIDS[7,8] and in non-AIDS populations, a grading scale was described by Kodsi *et al*[9] or a modification thereof has been

used[10]. *Candida* esophagitis was graded as the following: Grade 1 as scattered mucosal plaques involving less than 50 % of the esophageal mucosa, grade 2 as mucosal plaques involving more than 50 % esophageal mucosa, grade 3 as confluent plaque material circumferentially coating at least 50 % of the esophageal mucosa but without luminal impingement, grade 4 as circumferential plaque mat coating at least 50 % of the esophageal mucosa with luminal impingement despite air insufflations. In most cases, an ulcer could be readily distinguished endoscopically by the marked hyperemia and granularity of the ulcer base from the surrounding *candida* esophagitis.

### *Histopathology*

At the time of endoscopy, routine biopsies were performed on all endoscopic abnormalities. At least 2 biopsies were performed on each esophageal lesion with standard biopsy forceps. All tissue specimens were submitted for routine histopathology, and stained with hematoxylin-eosin (H-E) and periodic acid-Schiff stains (PAS). In the presence of ulcer tissue, immuno-histochemical staining for *CMV* and *HSV* was performed using previously described technique to confirm infection[3]. An ulcer was considered secondary to *candida* esophagitis alone when it was found endoscopically and histopathologically. Fungal organisms compatible with *candida* were seen in the superficial epithelium, there was an absence of viral cytopathic effect histopathologically, no clinical or endoscopic evidence of gastroesophageal reflex disease or drug-induced esophagitis existed.

### *Statistical analysis*

Results were expressed as mean ± standard deviation, median range for all continuous variables (e.g., age) and number (percentage) for categorical data (e.g., gender, diabetes mellitus, steroids, etc) were provided. Univariate analysis was performed using the independent sample *t*-test, Pearson Chi-square test and Fisher exact test when ever appropriate. A *P* value <0.05 was considered as statistically significant. All *P* values were two sided. Statistical interpretation of data was performed using the computerized software programme SPSS version 10.0.

## RESULTS

### *Patients*

During the study period, 15 000 upper endoscopies were performed in our endoscopy unit. Fifty-one patients were diagnosed with *candida* esophagitis on the basis of endoscopic and histopathologic criteria. The age, sex and percentage of inpatients are given in Table 1.

### *Risk factors*

The common risk factors for candida esophagitis were carcinoma (OR 8.05, 95 % CI 1.91-47.1 and *P*=0.001), uncontrolled diabetes mellitus (OR 7.34, 95 % CI 2.26-27.5 and *P*=0.001), corticosteroid therapy (OR 6.67, 95 % CI 2.20-22.3 and *P*=0.001) and antibiotics (OR 4.56, 95 % CI 1.14-21.5 and *P*=0.02) (Table 2).

### *Clinical feature*

The clinical details are given in Table 1. The clinical symptoms in group A were retrosternal discomfort in 39.2 % (20/51) patients, of these 6/20 were associated with dysphagia and 3/20 with epigastric pain. Dysphagia was present in 25.4 % (13/51) and epigastric symptoms in 35.2 % (18/51) with only 9/18 describing it as an epigastric pain (Table 1). In control group B, retrosternal discomfort was described in 30.3 % (31/102), dysphagia in 19.6 % (20/102) and epigastric symptoms in 50 % (51/102). These patients responded well to treatment with nystatin or fluconazole, 84.3 % (43/51) and 15.7 % (8/51), respectively (Table 1).

**Table 1** Details of patients presenting with *candida* esophagitis and controls

| | Cases *n*=51 | Control *n*=102 |
|---|---|---|
| Sex | | |
| Male | 27 (53) | 58 (57) |
| Female | 24 (47) | 44 (43) |
| Age in years (yrs) | | |
| Range: | 21-77 | 19-74 |
| Mean ±SD | 52.9±14.6 | 50.08±12.64 |
| No: % of In-patients | 34 (64) | 58 (54) |
| Risk factors for *candida* esophagitis | | |
| Steroid therapy | 15 (29.4 %) | 6 (5.8 %) |
| Diabetes mellitus type 1 and 2 | 14 (27.4 %) | 5 (4.9 %) |
| Carcinoma (e.g. breast, gastric, esophagus) | 10 (19.6 %) | 3 (2.9 %) |
| Broad spectrum antibiotics | 8 (15.6 %) | 4 (3.9 %) |
| Chronic liver disease | 2 (3.9 %) | 6 (5.8 %) |
| Tuberculosis | 2 (3.9 %) | 1 (0.9 %) |
| Ischemic heart disease | - | 26 (25.4 %) |
| Peptic ulcer disease | - | 26 (25.4 %) |
| Hypertension | - | 20 (19.6 %) |
| Chronic anemia | - | 2 (1.9 %) |
| Arthritis | - | 1 (0.9 %) |
| Primary Infertility | - | 1 (0.9 %) |
| Osteoporosis | - | 1 (0.9 %) |
| Clinical symptoms | | |
| Retrosternal discomfort | 20 (39.3 %) | 31 (30.3 %) |
| Dysphagia | 13 (25.4 %) | 20 (19.7 %) |
| Epigastric symptoms | 18 (35.3 %) | 51 (50 %) |
| Endoscopic grading | | |
| Grade 1 | 9 | |
| Grade 2 | 19 | |
| Grade 3 | 10 | |
| Grade 4 | 13 | |
| Treatment | | |
| Nystatin | 84.3 % (43/51) | |
| Fluconazole | 15.7 % (8/51) | |

Results were expressed as number and percentage, mean ± standard deviation (SD).

### *Polymorphonuclear leucocyte and lymphocyte counts*

All of our patients had polymorphonuclear leucocyte count within the normal range, while 33.3 % (17/51) had lymphocyte count below the normal range. These patients were mostly those who were on steroid or antibiotics therapy (Table 3).

### *Endoscopy finding*

The endoscopic appearance of plaques varied in color from yellow to white. With increasing severity, scattered mucosal plaques coalesced circumferentially coating the mucosal surface and impinged into the esophageal lumen. In group A, 9 patients had localized disease, 5 patients had disease more prominent in the distal esophagus than in proximal esophagus, while it involved middle esophagus and middle to distal esophagus equally in 4 patients (Table 1). Esophageal ulceration was noted endoscopically and histopathologically in 3.9 % (2/51). In these patients, ulcer was believed to be secondary to *candida* esophagitis alone, as other etiologies of esophageal ulceration were excluded by testing for *CMV*, *HSV* and *HIV* by serology and immuno-histochemistry. In 15.6 % (8/51) of group ‘ A’ patients, associated endoscopic findings included 7.8 % (4/51) with antral gastritis, 1.9 % (1/51) with gastric ulcer, 5.8 % (3/51) with duodenitis. In control group B

esophageal disease was found in 35.3 % (36/102) cases, gastric disease in 38.3 % (39/102) cases, and duodenal pathology was seen in 26.4 % (27/102) cases.

### *Correlation of clinical symptoms and endoscopic feature*

There was no correlation between clinical symptoms and endoscopic findings.

### *Hepatitis B, C and HIV serology*

HIV serology was negative for hepatitis B, and 9.8 % (5/51) were positive for hepatitis C virus.

### *Histopathology*

Histopathology revealed varying degrees of hyperplastic squamous mucosa with moderate-severe degree of acute-chronic inflammation in the surface epithelium. Mucosal surface was covered with desquamated epithelium and inflammatory necrotic slough. Superficial colonies of *candida* organism showed non-branching hyphae. In cases of ulcerative esophagitis, intact and focally ulcerated mucosa revealed moderate-severe inflammation, basal cell hyperplasia with non-septate fungal hyphae.

**Table 2** Results of univariate analysis of potential risk factors for acquisition of *candida* esophagitis

| Variable | No. of cases (%) | No. of control (%) | Odd ratio (95% CI) | *P* value |
|---|---|---|---|---|
| Carcinoma | 10 (19.6 %) | 3 (2.9 %) | 8.05 (1.91-47.1) | 0.001 |
| Diabetes mellitus | 14 (27.4 %) | 5 (4.9 %) | 7.34 (2.26-27.5) | 0.001 |
| Prior use of steroid | 15 (29.4 %) | 6 (5.8 %) | 6.67 (2.20-22.3) | 0.001 |
| Prior use of antibiotics | 8 (15.6 %) | 4 (3.9%) | 4.55 (1.14-21.5) | 0.02 |

**Table 3** Distribution of low lymphocyte counts in patients presenting with *candida* esophagitis

| Association | Cases (*n*=17) |
|---|---|
| Steroids | 6 |
| Antibiotics | 6 |
| Malignancy | 3 |
| Diabetes mellitus | 1 |
| Chronic liver disease | 1 |

## DISCUSSION

Our study is the first attempt to evaluate the risk factors and endoscopic manifestations of *candida* esophagitis in Pakistan, an area where AIDS is not endemic. All of our patients diagnosed with *candida* esophagitis did not have oral thrush. This was similar to a study by Bonacini *et al* in which 110 HIV positive patients with esophageal symptoms, 38 % of those without oral thrush had *candida* esophagitis[11]. In this study, the patients had oral, intravenous and nebulizer steroid treatment in varying durations and doses of therapy. Steroid levels were not measured on admission. However, steroid therapy was associated with lymphopenia and 33.3 % of our patients presented with a low lymphocyte count[12]. Diabetic patients complicated with *candida* esophagitis had uncontrolled diabetes at the time of presentation, irrespective of its type. Malignancies beside other mechanisms were also associated with esophageal stasis due to mechanical obstruction that predisposes to *candida* esophagitis[13]. However, esophageal obstruction was not a feature in our cases of *candida* esophagitis associated with malignancies. A high frequency of bacterial or mycotic infections has been reported in HCV-associated membranoproliferative glomerulonephritis and diabetes mellitus due to acquired defects of polymorphonuclear leukocyte (PMN) functions[14,15]. In HIV seropositive cases, coexistent *candida* esophagitis and tuberculosis have been frequently described [16], but in non-HIV immunosuppressed patients, there were few reports of active pulmonary or intestinal tuberculosis associated with *candida* esophagitis[17].

Epigastric pain has been known to be associated with *candida* infection[18]. In our study, no correlation was found between the symptoms and the endoscopy grade score similar to another study[19]. At endoscopy, the presence of classic whitish exudates or plaques should predict candidiasis in at least 90 % of cases, although viral infection occasionally might cause a similar appearance[11]. We found that *candida* esophagitis resulted in typical endoscopic appearances and both characteristic and uniform histopathologic findings. These *candida* esophagitis patients were treated with either nystatin 5 ml QDS/fluconazole 100 mg a day by mouth for 5 days. None of these candida esophagitis patients was found to be resistant to this treatment. As treatment with fluconazole was expensive, fewer patients were prescribed this medication. Culture for identifying *candida* species was not carried out, as it would have added cost to the management of disease. The limitation of our study included a small number of patients and a retrospective design. There were few patients with risk factors in the control group B as their stable disease did not predispose them to *candida* esophagitis (Table 1).

The implications of our study are that in Pakistan *candida* esophagitis is associated with chronic diseases and those on treatment with corticosteroids and antibiotics are predisposed to it. Patients on these medications need to be monitored and reviewed frequently. *Candida* esophagitis should be considered early in patients who have been on steroids and antibiotic treatment and presented with upper gastro-intestinal symptoms. Oral candidiasis does not accompany candida esophagitis. Our study showed that *candida* esophagitis by itself was an easily managed complication. In conclusion, *candida* esophagitis in Pakistan is more common due to chronic diseases, corticosteroid and antibiotic therapy which impaires the immune system rather than as an AIDS-defining disease. It occurs in the absence of local obstructive lesions and responds to treatment with nystatin and fluconazole.

## REFERENCES


1 **Laine L**, Boncini M. Esophageal disease in human immunodeficiency virus infection. *Arch Intern Med* 1994; **154**: 1577-1582
2 **Wilcox CM**, Karowe MW. Esophageal infections: etiology, diagnosis and management. *Gastroenterology* 1994; **2**: 188-206
3 **Schwartz DA**, Wilcox CM. Atypical cytomegalovirus inclusions in gastrointestinal biopsy specimens from patients with the acquired immunodeficiency syndrome: diagnostic role of in situ nucleic acid hybridization. *Hum Pathol* 1992; **23**: 1019-1026
4 **Connolly GM**, Hawkins D, Harcourt-Webster JN, Parsons PA, Husain OA, Gazzard G. Esophageal symptoms, their causes, treatment and prognosis in patients with the acquired immunodeficiency syndrome. *Gut* 1989; **30**: 1033-1039
5 **Hyder AA**, Khan OA. HIV / AIDS in Pakistan: the context and magnitude of an emerging threat. *J Epidemiol Community Health* 1998; **52**: 579-585
6 **Bagby GC**. Disorders of neutrophil production. In: Bennet JC, Plum F, eds. Cecil Text book of Medicine. *WB Saunders* 1996: 908-915
7 **Laine L**, Dretler RH, Conteas CN, Tuazon C, Koster FM, Sattler F, Squires K, Islam MZ. Fluconazole compared with ketoconazole for the treatment of candida esophagitis in AIDS. A randomized trial. *Ann Intern Med* 1992; **117**: 655-660
8 **Porro GB**, Parente F, Cernuschi M. The diagnosis of esophageal candidiasis in patients with acquired immune deficiency syndrome: Is endoscopy always necessary? *American J Gastroenterol* 1989; **84**: 143-146

9 **Kodsi BE**, Wickremesinghe PC, Kozinn PJ, Iswara K, Goldberg PK. Candida esophagitis: A prospective study of 27 cases. *Gastroenterology* 1976; **71**: 715-719
10 **Wilcox CM**, Schwartz DA. Endoscopic-pathologic correlates of candida esophagitis in acquired immunodeficiency syndrome. *Dig Dis Sci* 1996; **41**: 1337-1345
11 **Boncini M**, Young T, Laine L. The causes of esophageal symptoms in human immunedeficiency virus infection: a prospective study of 110 patients. *Arch Intern Med* 1991; **151**: 1567-1572
12 **Demir G**, Derman U, Berkarda B. Haematological effects of pulse steroid therapy. *Int J Clin Pharmacol Res* 1994; **14**: 101-106
13 **Rigas B**, Spiro HM. Clincal Gastroenterology. *McGraw Hill* 1995: 12-15
14 **Mazzaro C**, Panarello G, Tesio F, Santini G, Crovatto M, Mazzi G, Zorat F, Tulissi P, Pussini E, Baracetti S, Campanacci L, Pozzato G. Hepatitis C Virus risk: a HCV related syndrome. *J Intern Med* 2000; **247**: 535-545
15 **Jirillo E**, Greco B, Caradonna L, Satalino R, Pugliese V, Cozzolongo R, Cuppone R, Manghisi OG. Evaluation of cellular immune responses and soluble mediators in patients with cHCV infection. *Immunopharmacol Immunotoxicol* 1995; **17**: 347-364
16 **George J**, Hamide A, Das AK, Amarnath SK, Rao RS. Clinical and laboratory profile of sixty patients with AIDS: a South Indian study. *Southeast Asian J Trop Med Public Health* 1996; **27**: 686-691
17 **Hirasaki S**. Active intestinal tuberculosis with esophageal *candidiasis* due to idiopathic CD (+) T-lymphocytopenia in an elderly woman. *J Gastroenterol* 2000; **35**: 47-51
18 **Alexander JA**, Brouillette DE, Chien MC, YooYK, Tarter RE, Gavaler JS, Van Thiel DH. Infectious esophagitis following liver and renal transplantation. *Dig Dis Sci* 1988; **33**: 1121-1126
19 **Rodriquez Hernandez H**, Reyes Gutierrez E, Elizondo Rivera J. Esophageal candidiasis in AIDS, Clinical, endoscopic and histopathologic analysis of 19 cases. *Rev Invest Clin* 1991; **43**: 124-127

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2332-2334
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Serum malondialdehyde level in patients infected with *Ascaris lumbricoides*

Eser Kilic, Süleyman Yazar, Recep Saraymen, Hatice Ozbilge

**Eser Kilic, Recep Saraymen,** Department of Biochemistry and Clinical Biochemistry, Medical Faculty, Erciyes University, Kayseri-Turkey
**Süleyman Yazar,** Department of Parasitology, Medical Faculty, Erciyes University, Kayseri-Turkey
**Hatice Ozbilge,** Department of Microbiology, Medical Faculty, Harran University,[a] anl1urfa-Turkey
**Correspondence to:** Eser Kilic, Department of Biochemistry Medical Faculty, Erciyes, University, 38039 Kayseri-Turkeyemail. kiliceser@hotmail.com
**Received:** 2003-07-12 **Accepted:** 2003-08-20

## Abstract

**AIM:** The aim of the study was to investigate the changes of serum malondialdehyde level, i.e; the oxidative stress hypothesis in patients infected with *Ascaris lumbricoides*.

**METHODS:** Serum malondialdehyde activity was measured in 43 patients who were positive for intestinal parasite of *Ascaris lumbricoides*. Scores were obtained for the positives and their age-and sex-matched 60 *Ascaris lumbricoides* negative healthy controls.

**RESULTS:** The difference between malondialdehyde levels of patients infected with *Ascaris lumbricoides* and control group was statistically significant both for females ($P<0.05$) and for males ($P<0.05$). In the patient and control groups, no correlation was found between age and malondialdehyde levels ($P>0.05$) both in females and in males. In addition, no significant correlation could be found between malondialdehyde levels of both females and males for patients and control groups ($P>0.05$).

**CONCLUSION:** Malondialdehyde levels clearly increase in patients infected with *Ascaris lumbricoides*.

Kilic E,Yazar S, Saraymen R, Ozbilge H. Serum malondialdehyde level in patients infected with *Ascaris lumbricoides*. *World J Gastroenterol* 2003; 9(10): 2332-2334
http://www.wjgnet.com/1007-9327/9/2332.asp

## INTRODUCTION

*Ascaris lumbricoides (A. lumbricoides)* is one of the largest nematode (roundworm) parasitizing the human intestine[1-5]. It is estimated that 25 % of the world' s population was infected with this nematode[4]. The adult worms live in the small intestine and eggs are passed in the feces. A single female can produce up to 200 000 eggs each day. About two weeks after passage in the feces the eggs contain an infective larval or juvenile stage, and humans are infected when they ingest such infective eggs. The eggs hatch in the small intestine, the juvenile penetrates the small intestine and enters the circulatory system, and eventually the juvenile worm enters the lungs. In the lungs the juvenile worm leaves the circulatory system and enters the air passages of the lungs. The juvenile worm then migrates up the air passages into the pharynx where it is swallowed, and once in the small intestine the juvenile grows into an adult worm.Why *Ascaris lumbricoides* undergoes such a migration through the body to only end up where it started is unknown.Such a migration is not unique to *Ascaris lumbricoides*, as its close relatives undergo a similar migration in the bodies of their hosts[1-3,5].

*Ascaris lumbricoides* infections in humans can cause significant pathology.Infection with ascarids is called ascariasis. The migration of the larvae through the lungs causes the blood vessels of the lungs to hemorrhage, and there is an inflammatory response accompanied by edema. The resulting accumulation of fluids in the lungs results in "ascaris pneumonia", and this can be fatal[1,3,5,6]. The large size of the adult worms also presents problems, especially if the worms physically block the gastrointestinal tract. *Ascaris lumbricoides* is notorious for its reputation to migrate within the small intestine, and when a large worm begins to migrate there is not much that can stop it. Instances have been reported in which *Ascaris lumbricoides* has migrated into and blocked the bile or pancreatic duct or in which the worms have penetrated the small intestine resulting in acute (and fatal) peritonitis. *Ascaris lumbricoides* seems to be especially sensitive to anesthetics, and numerous cases have been documented where patients in surgical recovery rooms have had worms migrating from the small intestine, through the stomach, and out the patient' s nose or mouth[1-3,5].

Infections are diagnosed by finding the typical eggs in the patient' s feces, on occasion the larval or adult worms are found in the feces, or especially for *Ascaris lumbricoides*, in the throat, mouth, or nose[1]. This infection may self-cure after the larvae have matured into adults or may require anthelmintic treatment. In severe cases, surgical removal may be necessary. Allergic symptoms (especially but not exclusively of the asthmatic sort) are common in long-lasting infections or upon reinfection in ascariasis[1,3,7]. Eggs of *Ascaris lumbricoides*. have been detected on fresh vegetables[2,3,5]. This infection is cosmopolitan, but ascariasis is more common in North America and in Europe. Relative infection rates on other continents are not available[3,4].

Lipid peroxidation is a well-established mechanism of cellular injury in human, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides derived from polyunsaturated fatty acids, are unstable and can decompose to form a complex series of compounds. These include reactive carbonyl compound, which is the most abundant malondialdehyde (MDA). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans and model systems[8-10]. The aim of this study was to test the hypothesis of decreased activity of defense system protecting tissues from free radical damage in patients with *A. lumbricoides* by measuring the level of MDA (an end-product of lipid peroxidation) in serum samples.

## MATERIALS AND METHODS

### *Patients*

We assayed MDA activities of 103 subjects aged between 12-44 years (48 males and 55 females). None of them was smoker,

and had any known pathologies and taking steroids or medications such as iron for anemia at the time of sampling. Serum samples for control group were obtained from healthy people who came to the different departments of Medical Faculty Erciyes University, for regular check-up and students or employees of the University. All subjects weer fasted after midnight before blood collection the next morning. 43 patients and 60 controls were examined in this study. The mean age of the patient group, which consisted of 21 men and 22 women were 25±13 years and 27±13 years, respectively. The mean age of the control group, which included 27 men and 33 women were 30±14 years and 29±12 years respectively. Wet mount preparations in 0.9 % NaCl, diluted Lugol's iodine and flotation technique in saturated saline solution were used for the detection of intestinal parasites.

### *Assay*

All venous blood samples taken between 8 and 9 a.m. after 8 h of fasting were collected in polystyrene tubes and vacutainers containing heparin. The tubes were centrifuged at 500×g for 15 min. Sera were then removed and stored at -20 ℃ until analysis. Serum MDA levels were measured by the double heating method[11,12]. The principle of the method was based on the spectrophotometric measurement of the color occurred during the reaction to thiobarbituric acid with MDA. Concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of malondialdehyde-thiobarbituric acid complex and expressed in nmol/ml.

### *Statistical analysis*

Statistical analysis was performed with SPSS software package (Version 11.0 for Windows). The data were expressed as mean ± standard deviation (SD). For comparison of two groups of continuous variables, independent sample *t*-test was used. A probability value of $P<0.05$ indicated a statistically significant difference.

## RESULTS

Malondialdehyde scores are given in Table 1.

**Table 1** MDA levels of patients infected with *A. lumbricoides* and control group

| **Patients** | Age(year) | MDA levels (nmol/ml) |
|---|---|---|
| Female (22) | 27±13 | 0.67±0.16 |
| Male (21) | 25±13 | 0.62±0.11 |
| **Controls** | Age(year) | MDA levels (nmol/ml) |
| Female (33) | 29±12 | 0.21±0.15 |
| Male (27) | 30±14 | 0.22±0.14 |

The difference between MDA levels of patients and control group was statistically significant both for females ($P<0.05$) and males ($P<0.05$), (Table 1). In the patient and control groups, no correlation was found between age and MDA levels ($P>0.05$) both in females and in males. In addition, no significant correlation could be found between MDA levels of both females and males for patients and control group ($P>0.05$).

## DISCUSSION

This present study was aimed to evaluate and characterize the relationship between intestinal parasite infection of *ascariasis*, which can cause pathology and oxidative stress mechanism as a mediator of tissue damage concurrent with ascariasis infection.

Ascariasis is the most common human worm infection. Human can become infected after touching mouth with hands contaminated with eggs from soil or other contaminated surfaces. Infection has occurred worldwide and has been most common in tropical and subtropical areas where sanitation and hygiene were poor[1-3,5]. Children are infected more often than adults. Estimates suggest that 1 in 4 of the world's population, or more than 1 billion people, are infected with the intestinal roundworm *A. lumbricoides*. In Europe, infection was common, but the most common in rural areas of the southeast[3-5].

Although *Ascaris lumbricoides* has only a single host and it is found in the small intestine, its life cycle is far from simple. It has been suggested that from an evolutionary perspective that *Ascaris lumbricoides* originally had two hosts and has secondarily lost its intermediate host[1-3,5-7]. Although most people have no symptoms, symptoms can be broken down into 2 categories: early (larval migration, 4-16 days after egg ingestion) fever, cough and wheezing and late (mechanical effects, 6-8 weeks after ingestion). All symptoms resulted from mechanical irritation include; vague abdominal complaints (i.e, cramping, nausea, vomiting), small bowel obstruction (mostly in children), pancreatitis (secondary to worm migration), cholecystitis (secondary to worm migration), appendicitis (less common, secondary to worm migration). Secondary complications could arise with *Ascaris lumbricoides* infections because sometimes when the worms were undergoing this migration they appeared to get lost and started wandering through other organs such as the brain, bile duct, pancreas or appendix[1-3,5,7].

*Ascaris lumbricoides* proteins are very immunogenic and people can become very sensitive to the worm and have strong allergic reactions. The parasite could be treated very easily with drugs such as mebendazole or pyrantel pamoate[1,3,5,13,14]. However, reinfections frequently occur if other control measures are not taken. This is a particular problem where night soil is used as a fertilizer.

Oxidative stress as a mediator of tissue damage concurrent with *A. lumbricoides* infection was investigated. This was the first study to characterize the relationship between *A. lumbricoides*, (may cause no symptoms however, some complains of cramping, nausea, vomiting, small bowel obstruction, pancreatitis, cholecystitis, appendicitis can be seen) and MDA (lipid peroxidation), which is a well-established mechanism of cellular injury in human, and is used as an indicator of oxidative stress in cells and tissues.

Levels of MDA were significantly increased in patients infected with *A. lumbricoides*. The results of our study strongly suggested that one of the main reasons for high MDA levels in patients infected with *A.lumbricoides* could be decreased activity of defense system protecting tissues from free radical damage. However, in the patients and control groups, no correlation was found between age and MDA levels both in females and in males. In addition, no significant correlation could be found between MDA levels of both females and males for *A. lumbricoides* infected and control groups. These results for patients infected with *A. lumbricoides* could possibly be explained as that with high MDA activity in all ages.

As it is known that lipid peroxidation is a free radical-related process that in biologic systems may occur under enzymatic control, e.g., for the generation of lipid-derived inflammatory mediators, or nonenzymatically. This latter form was associated mostly with cellular damage as a result of oxidative stress, which also involved cellular antioxidants in this process[10]. The high infection/control ratio of MDA concentration and the significant correlation strongly indicate the occurrence of oxidative stress and lipid peroxidation as a mechanism of tissue damage in cases of *A.lumbricoides* infection.

## REFERENCES

1 **Crompton DW**. Ascaris and ascariasis. *Adv Parasitol* 2001; **48**:

285-375
2 **Goncalves ML**, Araujo A, Ferreira LF. Human intestinal parasites in the past: new findings and a review. *Mem Inst Oswaldo Cruz* 2003; **98**: 103-118
3 **O'Lorcain P**, Holland CV. The public health importance of Ascaris lumbricoides. *Parasitology* 2000; **121**: 51-71
4 **Hall A**, Holland C. Geographical variation in Ascaris lumbricoides fecundity and its implications for helminth control. *Parasitol Today* 2000; **16**: 540-544
5 **Sarinas PS**, Chitkara RK. Ascariasis and hookworm. *Semin Respir Infect* 1997; **12**: 130-137
6 **Geiger SM**, Massara CL, Bethony J, Soboslay PT, Carvalho OS, Correa-Oliveira R. Cellular responses and cytokine profiles in Ascaris lumbricoides and Trichuris trichiura infected patients. *Parasite Immunol* 2002; **24**: 499-509
7 **Palmer LJ**, Celedon JC, Weiss ST, Wang B, Fang Z, Xu X. Ascaris lumbricoides infection is associated with increased risk of childhood asthma and atopy in rural China. *Am J Respir Crit Care Med* 2002; **165**: 1489-1493
8 **Draper HH**, Hadley M. A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. *Xenobiotica* 1990; **20**: 901-907
9 **Nayak DU**, Karmen C, Frishman WH, Vakili BA. Antioxidant vitamins and enzymatic and synthetic oxygen-derived free radical scavengers in the prevention and treatment of cardiovascular disease. *Heart Dis* 2001; **3**: 28-45
10 **Romero FJ**, Bosch-Morell F, Romero MJ, Jareno EJ, Romero B, Marin N, Roma J. Lipid peroxidation products and antioxidants in human disease. *Environ Health Perspect* 1998; **106**: 1229-1234
11 **Stocks J**, Dormandy TL. The autoxidation of human red cell. Lipids induced by hydrogen peroxide. *Br J Haematol* 1971; **20**: 95-111
12 **Jain SK**. Evidence for membrane lipid peroxidation during the invivo aging of human erythrocytes. *Biochem Biophys Acta* 1998; **937**: 205-210
13 **Montresor A**, Awasthi S, Crompton DW. Use of benzimidazoles in children younger than 24 months for the treatment of soil-transmitted helminthiasis. *Acta Trop* 2003; **86**: 223-32S
14 **Georgiev V**. Pharmacotherapy of ascariasis. *Expert Opin Pharmacother* 2001; **2**: 223-239

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2335-2337
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Problems in screening colorectal cancer in the elderly

Davidoviæ M. Mladen, Milosevic P. Dragoslav, Zdravkovic Sanja, Bojic Bozidar, Djurica Snezana

**Davidovic M. Mladen, Milosevic P. Dragoslav, Zdravkovic Sanja, Bojic Bozidar, Djurica Snezana**, Center of Geriatric Medicine, 11050 Beograd, Rifata Burdzevica 31, Yugoslavia
**Correspondence to:** Davidoviæ M. Mladen, at Center, 11050 Beograd, Rifata, Burdževiæa 31, Yugoslavia. davidovi@EUnet.yu
**Telephone:** +381-11-417094 **Fax:** +381-11-765731
**Received:** 2003-05-11 **Accepted:** 2003-06-20

## Abstract

**AIM:** To explore the problems in the screening of colorectal carcinoma in the elderly.

**METHODS:** Three models of colorectal cancer prevention were examined: standard screening, active check-up of suspected cases and summons to have endoscopic check-up for previously diagnosed colorectal polyps. The study was performed among three groups of elderly individuals: Group 1 (167 cases), hospitalized asymptomatic individuals without symptoms in large intestines. Group 2 (612 cases): old individuals at home for the aged, out of which 32 showed symptoms of colon disorders; Group 3 (44 cases): elderly people with diagnosed polyps. As a result of 1788 rectosigmoidoscopies, we identified 61 individuals with polyps, out of which 44 patients were over 65 years old. However, only 9 of these 44 individuals agreed to have the endoscopy performed again.

**RESULTS:** One cancer and 13 polyps were detected in Group 1, and two polyps in Group 2. However, it should be noted that only eleven individuals from Group 2 agreed to have the endoscopy. In Group 3, there were no relapses of the polyps among the nine individuals who came back for the endoscopy.

**CONCLUSION:** Poor understanding of the screening procedures is one of the greatest problems in early detection of the cancer in the aged. Paradoxically, the cooperation is better with hospitalized patients, than with "successfully old" persons.

Mladen DM, Dragoslav MP, Sanja Z, Bozidar B, Snezana D. Problems in screening colorectal cancer in the elderly. *World J Gastroenterol* 2003; 9(10): 2335-2337
http://www.wjgnet.com/1007-9327/9/2335.asp

## INTRODUCTION

Incidence of colorectal cancer in the world has increased over the past few decades, which is in turn, the cause for the increased importance of the screening for colorectal *carcinoma* (CRC). The incidence of colorectal cancer increases with age and doubles every ten years after the age of 40. In USA, the incidence of CRC is 35/100 000 population, thus one from 20 people will suffer from CRC during their life. High incidence of CRC has been recorded in Western European countries, which stands opposed to the numbers from the Far-East countries and Africa where the incidence is low, i.e. Nigeria has the incidence of 3.4/100 000. The average colorectal cancer age-adjusted death rates in Serbia from 1971 to1996 were 11.2/100 000 for men, and 8.3/100 000 for women[1].

Colorectal cancer screening includes fecal occult blood tests (FOBT), sigmoidoscopy, air-contrast *barium enema* examination and colonoscopy. These methods are recommended for persons in moderate risk, but its merits and limitations are still under investigation. Persons at high risk, i.e. family history of hereditary non-polyposis CRC, patients with ulcerous colitis, should be screened by colonoscopy. Screening method can surely have a positive effect on the survival rate of CRC. Since survival rate of CRC correlates to the anatomical spread of tumor, as well as to the surgical treatment at the right time, rectosigmoidoscopy can prevent and disclose the earliest stages of distal colon *carcinoma*.

Colonoscopy is certainly the "gold standard" for colorectal examination, but it is not most commonly accepted due to costs, bowel preparation, sedation and perforation risks (1/2 000 examinations). It is the most efficient method for detecting adenomas and probably has greater significance in the detection of the advanced tumor than other screening methods.

In carrying out our project on screening rectosigmoidoscopy, we found two kinds of problems. Like in all screening projects, one was the motivation of the participants (both the researchers and especially the subjects). The other problem was related to, what was in the developed countries called the cost/benefit issue, and in the under-developed countries was simply the issue of cost, which was extra high.

In the recent "American Cancer Society Guidelines for the Early Detection of Cancer"[2], the prevalence of screening procedure in American adult population was 19.0 % for FOBT, and 32.3 % for colonoscopy or sigmoidoscopy. Chinese author Wan J[3] claimed that multimorbidity was noted in 87 % of the investigated elder cases and the incidence of complication was only 0.05 %.

## MATERIALS AND METHODS

### *Patients*

The study was performed among three groups of elderly (from 65 to 80 yr, mean age 74.37) individuals: Group 1 had 167 hospitalized asymptomatic individuals without any symptoms in the large intestine, Group 2 consisted of 612 old individuals at home for the aged, out of which 32 had symptoms of colon disorders and Group 3 consisted of 44 individuals with polyps. Group 3 resulted from 1788 rectosigmoidoscopies, among which 61 individuals with polyps were found. 44 of these individuals were over the age of 65, and only 9 agreed to come back for a check-up of endoscopy.

### *Endoscopy*

In our study, screening rectosigmoidoscopy was used with the purpose of assessing the importance of the rectoscopic examination for the early diagnosis and prevention of colorectal cancer. Rectosigmoidoscopy was performed with OLYMPUS endoscope after colon cleaning which was usually with SENNA.

### *Grouping*

We selected three groups of elderly persons. The first one was the group of asymptomatic individuals (regarding digestive

tract) hospitalized at our institute. The answer to the screening was optimal, however it was difficult to obtain such results in the majority of the old population. The second trial was more selective. We included in our endoscopy proposition only the persons with digestive disorders. This selection was based on and followed the assessment procedure in home for the aged. There was a very low percentage of agreement to the endoscopy. Finally, Group 3 was reselected from the elderly persons with previously diagnosed colon polyps, and invited for a control endoscopy. Only 25 % of the group was available for the control rectosigmoidoscopy.

## RESULTS

Three models of colorectal cancer prevention were examined: standard screening, active check-up of suspected cases and summons to endoscopic check-up for previously diagnosed colorectal polyps.

Group 1 consisted of hospitalized asymptomatic persons (without symptoms in large intestines).

The second trial (Group 2)was more selective. We included only the persons with digestive disorders in our endoscopy proposition, after the assessment procedure in home for the aged.

Group 3 consisted of the patients who already had polyps of the *rectum* removed and had never reported for endoscopic follow up although advised to do so. All the subjects were summoned by phone and letter.

**Table 1** Data of three groups

| Number of investigated cases | Selection criteria | Results |
|---|---|---|
| 167 | Hospitalized asymptomatic | 1 cancer and 13 polyps |
| 612 (32 with symptoms of colon disorders), 11 agreed to have the endoscopy | Home for the aged | 2 polyps |
| 1788 rectosigmoidoscopies (61 polyps) 44 older than 65 y 9 agreed on the endoscopy | With previous diagnosis of colon polyp | 0 relapses of polyps |

## DISCUSSION

As colorectal tumor represents a considerable medical problem in the elderly, early detection of adenomatous polyps, as the precursors of intestinal *carcinomas*, is increasingly important and relevant to geriatricians.

The incidence of colorectal cancer increases with age, and approximately half of patients who develop it die from it. The possibility of developing cancer during the lifetime is approximately 5-6 %. Those that belong to the risk groups are more in a position to get this disease. In all cases, however, early diagnosis is decisive.

Sigmoidoscopy studies showed that regarding the possibility of CRC development, people older than 65 years were equaled in the incidence with patients having an *adenoma* of distal colon for more than 90 % CRC cases were diagnosed after the age of 55 and the peak of incidence of sigmoidoscopically discovered *adenomas* was before 70 years old.

The reason for lack of improvement in survival is that in most cases of CRC the disease has already been at an advanced stage by the time of diagnosis. Therefore, the main goal of screening is to discover CRC at the early stage when successful radical treatment is possible. Screening is an examination in asymptomatic individuals for CRC.

The American Cancer Society' s (ACS) Colorectal Cancer Advisory Group recommends FOBT once a year for people older than 50, rectosigmoidoscopy every 5 years, *barium enema* every 5 years and colonoscopy every 10 year[4].

According to WHO, screening tests must be sensitive, specific, applicable in the asymptomatic population and it must aim at lowering morbidity and mortality rate. Although rectosigmoidoscopy has certain disadvantages (such as being invasive, unpleasant and expensive compared to FOBT), it is more reliable than other screening tests in that it is highly sensitive (no false negative results even for lesions less than 5 mm in diameter) and the lesion can be removed during the screening examination.

In almost all patients with CRC, preceding lesions were asymptomatic *adenoma*. Therefore, it is of great importance to locate and remove *adenoma* before the development of *dysplasia* and the following malignant alterations, which, at the same time, represents secondary prevention of colorectal cancer. This is also obvious in British National Polyp Study from 1993, which showed 90 % decline of CC incidence in a group of 1500 patients who underwent polypectomy and were followed up for 6-8 years[5].

There are different problems regarding colorectal screening in old age: (1) The problem of performing screening or not seems that consensus was reached that different panel group and association have agreed that the answer was YES[7-15,17]; (2) Recently there were more references that supported colonoscopy as a choice[11,12,16,17]; (3) Are there any age limits for the colorectal screening procedure? There were two opposed opinions: No limits, or regarding biological age, and individual limit as consequence of the personal expected survival, on average, 75 to 80 y[19]; (4) What about national programs, accepted by the national authority supported by health funds? There were no such data[11]; (5) Regarding cost/ benefits ratio, it is rational to invest in screening procedure. Developing countries are between high standards and low economies. With minute financial support, the choice is to modify screening method, to reduce the number of investigated persons, or to quit the program of prevention; (6) It seemed very important to have influence on public opinions regarding screening procedure[15,16 ,19].

The authors do not have any doubts about the justification of the screening procedure, so an attempt should be made to find a solution for active prevention. However, in developing countries which have fewer technological and financial options, better choice, and the only choice for prevention must be a less costly one. The relation of cost and benefit is principally dependent on the economic possibilities of a country' s health service, within which the priorities of a health system play an especially meaningful role.

Further, the selection of hospitalized patients with other diagnosis is not statistically proper for screening of rectosigmoidoscopy. Hypothetically, optimal way to find patients for screening is to start from the homes for the aged or with the patients with colon symptoms that are treated at their homes. This is the best way to find patients when it is not possible to perform massive screening. Periodical checkup of the patients with polyipectomy is necessary.

Judging by the commentary[6], the idea on a prior questionnaire in active search for persons with high risk of contracting (developing) colon malignome is not recommended. In the same article, the recommendation of USA on compulsory screening of persons over 50 is adjusted to the needs of UK service. If the choice is either screening an insignificantly small population or absolute impossibility of screening or just screening of the high-risk group, including those actively detected after the questionnaire, it is highly probable that the latter solution is the one that should be chosen.

Results of our investigation showed that the main problem in the prevention of cancer was the extent of acceptance of

this method by the elderly patients. Screening methods for hospitalized patients (Group 1) were necessary, since they were already motivated for medical treatment. However, the screening method had the lowest acceptance among the old persons from the homes for the aged. A very small number of patients were going for endoscopic checkups. Those patients usually conducted controls on repeated doctor' s invitations.

## REFERENCES


1 **Gajic-Veljanoski O**, Jarebinski M, Jovicevic-Bekic A, Pekmezovic T. Analysis of mortality in cancer of the large intestine in a cohort group in Serbia from 1971 to 1996. *Srp Arh Celok Lek* 2002; **130**: 173-177

2 **Smith RA,** Cokkinides V, von Eschenbach AC, Levin B, Cohen C, Runowicz CD, Sener S**.** American cancer society guidelines for the early detection of cancer. *CA Cancer J Clin* 2002; **52**: 8-22

3 **Wan J,** Zhang ZQ, Zhu C, Wang MW, Zhao DH, Fu YH, Zhang JP, Wang YH, Wu BY. Colonoscopic screening and follow-up for colorectal cancer in the elderly. *World J Gastroenterol* 2002; **8**: 267-269

4 **Levin B,** Brooks D, Smith RA, Stone A. Emerging technologies in screening for colorectal cancer: CT colonography, immunochemical fecal occult blood tests, and stool screening using molecular markers. *CA Cancer J Clin* 2003; **53**: 44-55

5 **Atkin W**. Implementing screening for colorectal cancer. *BMJ* 1999; **319**: 1212-1213

6 **Scholefield JH**. ABC of colorectal cancer Screening. *BMJ* 2000; **321**: 1004-1006

7 **Steele RJC,** Parker R, Patnick J, Warner J, Fraser C, Mowat NAG, Wilson J, Alexander FE, Paterson JG. A demonstration pilot trial for colorectal cancer screening in the United Kingdom: a new concept in the introduction of healthcare strategies. *J Med Screen* 2001; **8:** 197-203

8 **Martyres R**, St John DJ, Irving FH, Wyman K. Colorectal cancer screening in general practice. A survey of current practice and attitudes in Victoria. *Aust Fam Physician* 1999; **28**: 755-758

9 **Lieberman DA**, Weiss DG, Bond JH, Ahnen DJ, Garewal H, Chejfec G. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. Veterans Affairs Cooperative Study Group 380. *N Engl J Med* 2000; **343**: 162-168

10 **Rossos PG**, Yeung E. Screening for colorectal cancer in older adults. *Geriatrics aging* 2002;**5/ 3**:16-18 http: / / www.geriatricsandaging. com

11 **Anderson J**. Clinical practice guidelines: Review of the recommendations for colorectal screening. *Geriatrics* 2000; **55**: 67-73

12 **Sonnenberg A**, Delc F, Inadomi JM. Cost-effectiveness of colonoscopy in screening for colorectal cancer. *Ann Intern Med* 2000; **133**: 573-584

13 **O'Connor AM,** Stacey D, Rovner D, Holmes-Rovner M, Tetroe J, Llewellyn-Thomas H, Entwistle V, Rostom A, Fiset V, Barry M, Jones J. Decision aids for people facing health treatment or screening decisions (Cochrane Review)**.** *The Cochrane Libary, Issue* 2001. http: / / www.medlib.com

14 **Lieberman DA,** Weiss DG, Bond JH. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. *N Engl J Med* 2000; **343**: 162-168

15 **Lieberman DA**, Harford WV, Ahnen D J, Provenzale D, Sontag SJ, Schnell TG, Chejfec G, Campbell DR, Durbin TE. One-time screening for colorectal cancer with combined fecal occult-blood testing and examination of the distal colon. *N Engl J Med* 2001; **345**: 555-560

16 **Wilson LS**, Lightwood J. Model of estimated rates of colorectal cancer from polyp growth by year of surveillance. *J Med Scr* 2001; **8**: 187-196

17 **Mayor S**. Single flexible sigmoidoscopy screening could help prevent colorectal cancer. *BMJ* 2002; **324**: 934

18 **Wardle J**, Taylor T, Sutton S, Atkin W. Does publicity about cancer screening raise fear of cancer?Randomised trial of the psychological effect of information about cancer screening. *BMJ* 1999; **319:** 1037-1038

19 **Smith RA**, Cokkinides V, Eyre HJ. American Cancer Society. American Cancer Society guidelines for the early detection of cancer, 2003. *CA Cancer J Clin* 2003; **53**: 27-43

20 **Early DS**. Colorectal Cancer Screening: An overview of available methods and current recommendations. *South Med J* 1999; **92**: 258-265

21 **Frame PS.** Implementing clinical preventive medicine: time to fish or cut bait. *J Am Board Fam Pract* 2000; **13**: 84-85

22 **Spencer MP**. Screening colonoscopy. *Medscape Gastroenterol* 2000; **2** http: **//**www.medscape.com

23 **Slattery ML**, Edwards SL, Ma KN, Friedman GD. Colon cancer screening, lifestyle, and risk of colon cancer. *Cancer Causes Control* 2000; **11**: 555-563

24 **Saltzstein SL**, Behling CA. 5- and 10-year survival in cancer patients aged 90 and older: A study of 37,318 patients from SEER. *J Surg Oncol* 2002; **81**: 113-116

25 **Huang X**, Zhu HM, Deng CZ, Porro GB, Sangaletti O, Pace F. Gastroesophageal reflux: the features in elderly patients. *World J Gastroenterol* 1999; **5**: 421-423


**Edited by** Xu XQ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2338-2340
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Significant factors associated with fatal outcome in emergency open surgery for perforated peptic ulcer

Mario Testini, Piero Portincasa, Giuseppe Piccinni, Germana Lissidini, Fabio Pellegrini, Luigi Greco

**Mario Testini, Giuseppe Piccinni, Germana Lissidini,** Section of General Surgery and Vascular Surgery and Clinical Oncology, Department of Applications in Surgery of Innovative Technologies (DACTI), University Medical School, Bari, Italy
**Piero Portincasa,** Section of Internal Medicine, Department of Internal Medicine and Public Medicine (DIMIMP), University Medical School, Bari, Italy
**Fabio Pellegrini,** Department of Clinical Pharmacology and Epidemiology, Pharmacological Research Institute, Consortium "Mario Negri" South, Maria Imbaro (L' Aquila), Italy
**Luigi Greco,** Section of General Surgery, Department of Emergency and Organ Transplantations (DETO), University Medical School, Bari, Italy
This paper is dedicated to the memory of Prof. Francesco Paccione, Head of the Department of Surgery who died prematurely in 1996.
**Correspondence to:** Mario Testini, MD, Sezione Chirurgia Generale, Vascolare ed Oncologia Clinica, Dipartimento per le Applicazioni in Chirurgia delle Tecnologie Innovative (D.A.C.T.I.). Università degli Studi di Bari. mario.testini@tin.it
**Telephone:** +39-80-5592882 **Fax:** +39-80-5478759
**Received:** 2003-05-13 **Accepted:** 2003-08-02

## Abstract

**AIM:** To evaluate the main factors associated with mortality in patients undergoing surgery for perforated peptic ulcer referred to an academic department of general surgery in a large southern Italian city.

**METHODS:** One hundred and forty-nine consecutive patients (M:F ratio=110:39, mean age 52 yrs, range 16-95) with peptic ulcer disease were investigated for clinical history (including age, sex, previous history of peptic ulcer, associated diseases, delayed abdominal surgery, ulcer site, operation type, shock on admission, postoperative general complications, and intra-abdominal and/or wound infections), serum analyses and radiological findings.

**RESULTS:** The overall mortality rate was 4.0 %. Among all factors, an age above 65 years, one or more associated diseases, delayed abdominal surgery, shock on admission, postoperative abdominal complications and/or wound infections, were significantly associated ($\chi^2$) with increased mortality in patients undergoing surgery ($0.0001<P<0.03$).

**CONCLUSION:** Factors such as concomitant diseases, shock on admission, delayed surgery, and postoperative abdominal and wound infections are significantly associated with fatal outcomes and need careful evaluation within the general workup of patients admitted for perforated peptic ulcer.

Testini M, Portincasa P, Piccinni G, Lissidini G, Pellegrini F, Greco L. Significant factors associated with fatal outcome in emergency open surgery for perforated peptic ulcer. *World J Gastroenterol* 2003; 9(10): 2338-2340
http://www.wjgnet.com/1007-9327/9/2338.asp

## INTRODUCTION

There has been a marked decrease in elective surgery for peptic ulcer disease (PUD) following introduction of medical therapies including $H_2$-receptor antagonists, and more recently proton pump inhibitors with or without antibiotics for *H pylori* eradication. By contrast, the number of acute complications *e.g.* ulcer perforation and bleeding requiring emergency surgery, have remained quantitatively constant[1,2]. Peptic ulcer perforation is a serious complication which affects almost 10 % of PUD patients. Overall, PUD accounts for more than 70 % of mortality associated with the disease[3,4]. Several potential predicting factors for perforation have been evaluated in the literature, including use of ulcerogenic drugs (*e.g.* steroids, NSAIDs, immunosuppressive agents, *etc*.), and the development of an acute, rather than chronic peptic ulcer[5-8].

In this paper, we studied the main factors associated with mortality in a large number of patients undergoing surgery for perforated peptic ulcers (PPU) in a large referral academic hospital in southern Italy.

## MATERIALS AND METHODS

### Patients

The study population comprised 149 consecutive patients with an established intra-operative final diagnosis of PUD referred for emergency surgery to the 1st Department of General Surgery of the University of Bari. Bari is the main city of a province of about 1 500 000 inhabitants in the south-eastern coast of Italy. During a time spanning from 1988 to 1997 all patients were treated exclusively by open surgical approach, as agreed by all staff members. Since then, additional patients have been treated also by laparoscopy for PPU, but due to the scant number of cases, they were not included in the present analysis. Overall, there were 39 females and 110 males (mean age 52 years, range 16-95). The diagnosis of gastrointestinal ulcers was based on clinical features, blood tests, routine laboratory tests, and radiological findings (*i.e.* plain abdominal X-ray in all cases and abdominal CT scan in 87 % of patients). Invariably, the definitive diagnosis of PPU was obtained at surgery. The time between presumed perforation and surgery was considered delayed if longer than 12 h. The following factors were analysed: age >65 years, sex, previous ulcer history, associated medical diseases, delayed operation, site of ulcer, type of operation, shock on admission, postoperative general complications, postoperative intra-abdominal and/or wound infections.

### Surgical procedure

An open surgical approach was performed leading to a non definitive operation (*i.e.* ulcer excision and suture with or without pyloroplasty) in 120 patients (80.5 %) and to definitive operations (*i.e.* Billroth II resection) in 29 patients (19.5 %). The decision to perform one or the other type of surgery depended on several known factors including location and extent of lesions, feasibility of a safe non-definitive surgery, presence or absence of anaesthesiological risk factors, and surgeon' s attitude. No truncal or selective vagotomies were performed. All operations were performed by the same surgical staff whose colleagues were well trained in gastrointestinal surgery.

### *Statistical analysis*

All calculations were performed with the *NCSS 2001* statistical software (Kaysville, UT, USA). The chi-square test was used to compare proportions. A two-tailed probability (*P*) value of less than 0.05 was considered statistically significant[9,10].

## RESULTS

The time between perforation and surgery was delayed in 51 patients (34.2 %), 79 patients (53.0 %) had associated diseases which are listed in Table 1. Cardiovascular, chronic obstructive pulmonary diseases and diabetes mellitus were the most frequently (over 65 %) associated conditions. A previous history of PUD was found in 53 (35.6 %) patients and 9 (6.0 %) were shocked on admission. Gastric and duodenal ulcers were perforated in 23 (15.4 %) and 126 (84.6 %) patients, respectively.

**Table 1** Associated diseases in study group

| | |
|---|---|
| Cardiovascular disease | 27 |
| Diabetes mellitus | 20 |
| Chronic obstructive pulmonary disease | 19 |
| Impaired liver function | 8 |
| Renal failure | 7 |
| Coagulation disorders | 6 |
| Cerebrovascular disease | 4 |
| Neurological disease (others) | 3 |
| Malignancy | 2 |
| Thyroid disease | 1 |
| Gallstones | 1 |
| Acute pancreatitis | 1 |
| Total | 99 |

Types of postoperative complications are reported in Table 2. The most frequent events were due to general, rather than abdominal complications or wound infections.

**Table 2** Postoperative complications

| | |
|---|---|
| General | |
| Cardiac | 7 |
| Respiratory | 7 |
| Sepsis | 7 |
| Renal | 5 |
| Mental disorders | 2 |
| Ictus | 1 |
| Deep venous thrombosis | 1 |
| Total | 30 |
| Abdominal | |
| Abscess | 6 |
| Bleeding | 2 |
| Stenosis | 2 |
| Total | 10 |
| Wound infections | 8 |
| Total | 48 |

The analysis of factors associated with mortality is depicted in Table 3. Of the 149 patients, 6 died yielding an overall mortality rate of 4.0 %. The presence of one or more associated diseases, delay in surgical approach, shock on admission, postoperative abdominal complications (6 dehiscence/abscess, 2 bleedings, 2 stenosis) and the postoperative wound infections were all significantly ($0.0001<P<0.04$) associated with increased mortality in patients undergoing surgery for PPU. By contrast, age, sex, previous history and site of peptic ulcer, type of surgical treatment and the development of postoperative general complications were not associated with increased mortality.

**Table 3** Analysis of factors associated with mortality in 149 patients undergoing surgery for perforated peptic ulcer

| | *n* | Mortality (%) | *P* value |
|---|---|---|---|
| Male:Female | 110:39 | 3.6 *vs.* 5.1 | NS |
| Age (<65: >65 years) | 63:86 | 1.6 *vs.* 5.8 | NS |
| Previous ulcer history (yes:no) | 53:96 | 5.7 *vs.* 3.1 | NS |
| Associated disease (yes:no) | 79:70 | 7.6 *vs.* 0.0 | 0.02 |
| Delayed operations (yes:no) | 41:108 | 9.8 *vs.* 1.9 | 0.04 |
| Site (duodenal:gastric) | 126:23 | 3.1 *vs.* 8.7 | NS |
| Operation type (non definitive:definitive) | 120:29 | 2.5 *vs.* 10.3 | NS |
| Shock on admission (yes : no)* | 9:140 | 55.6 *vs.* 0.7 | 0.0001 |
| Postop. general complications (yes:no) | 30:119 | 6.7 *vs.* 3.4 | NS |
| Postop. abdominal complications (yes:no)* | 10:139 | 50.0 *vs.* 0.7 | 0.0001 |
| Postoperative wound infections (yes:no)* | 8:141 | 37.5 *vs.* 2.1 | 0.0001 |

Data analyzed by $\chi^2$ test and *Fisher's exact test.

## DISCUSSION

Several factors might contribute to increased postoperative mortality in patients with PPU. Perforation has been found to be a major complication of PUD with a mortality rate ranging from 6 % to 31 %[6-8, 11-20].

Age of patients with PPU has been gradually increasing over the last years[21-23]. In this series, an age >65 years tended to be associated with increased mortality. This finding is in line with other studies in which older patients frequently had associated diseases, or they were more on NSAIDs treatment[8,16,22]. It should be also noted that the mean age of patients from this series was considerably lower than that from patients included in different studies. Thus, such differences might account for the markedly lower overall mortality rate (4.0 %), as compared to other series[6-8, 11-20].

In accord with others[19, 23], we could not find that male sex was associated with a greater mortality rate. Also, there was no significant difference in mortality rate between gastric or duodenal ulcer and in patients with or without previous ulcer history. Apparently, these findings were at variance with those from two other studies[13, 24] reporting a higher mortality rate in gastric peptic ulcer than in duodenal peptic ulcer and in acute peptic ulcer than in chronic peptic ulcer. Such apparent discrepancies might be explained by the characteristics of patients included in the study, and/or by different age or different surgical procedures[16,18].

This study confirmed the previous observations[5,8,15,25-28] that shock on admission and delayed operation were both associated with a greater mortality rate.

Despite the fact that surgery remains the choice of treatment for PPU, the type of procedure in emergency is still debated. In some series definitive surgery had lower rates of recurrence and mortality than non definitive surgery[16,18,19,29,30]. Otherwise, non-definitive surgery was more frequently performed in patients admitted with more risk factors than definitive surgery, and this might explain the higher mortality rate of such studies. Moreover, diffusion of the laparoscopic approach to PPU with less surgical trauma and less metabolic and physiological disturbances, has determined an increase of non definitive surgical procedures performed by simple closures[3,20,22,31]. In the present study, there was no difference in mortality rate between definitive (*i.e.* Billroth II resection) or non-definitive (*i.e.* ulcer excision and suture with or without pyloroplasty)

surgical procedure.

It has been reported that mortality rate increased progressively with increasing numbers of risk factors[6,8]. Indeed, the mortality rate was 0 % and 7.6 % in the group of patients without and with associated diseases, respectively. In the present study cardiovascular, chronic obstructive pulmonary diseases and diabetes mellitus were the most frequent concomitant diseases. Besides, 6 patients developing a postoperative abscess had a previous history of chronic obstructive pulmonary disease. A possible explanation for such an outcome could be the reduced tissue oxygenation resulting in damage of post-surgical wound healing process. This possibility was supported by recent studies from our group at the intestinal level in both experimental and clinical conditions[32-36].

We also observed that in patients developing postoperative abdominal complications (*i.e.* 6 abscesses, 2 bleedings, and 2 stenosis) and wound infections, the mortality rate was significantly higher ($P$=0.0001) than those without abdominal complications. We would like to explain that such a striking difference was due to the development of a generalized sepsis in the group of patients with intra-abdominal abscess. Indeed, 83.3 % (*i.e.* 5/6) of patients with dehiscence and abdominal abscess, died in the postoperative period, otherwise, in this group with postoperative complications, the appearance of stenosis or bleeding was not associated with a higher mortality rate. In our experience the presence of wound infection appeared to be a predictive factor for mortality. A careful analysis of the 3 patients who died of wound infection, however, revealed that the cause of exitus was septicaemia complicating an abdominal abscess. By contrast, postoperative general complications did not influence the prognosis of patients with PPU.

In conclusion, concomitant diseases, shock on admission, delayed surgery, and postoperative abdominal and wound infections are factors significantly associated with fatal outcomes in patients undergoing emergency surgery for perforated peptic ulcer. Older age tends to fulfill a similar trend. Thus, such factors need to be carefully taken into account during the general workup of patients admitted for PPU.

## REFERENCES


1 **Christensen A**, Bousfield R, Christiansen J. Incidence of perforated and bleeding peptic ulcers before and after the introduction of H2-receptor antagonist. *Ann Surg* 1988; **207**: 4-6

2 **Bliss DW**, Stabile BE. The impact of ulcerogenic drugs on surgery for the treatment of peptic ulcer disease. *Arch Surg* 1991; **126**: 609-612

3 **Lau WL**, Leung KL, Kwong KH, Davey IC, Robertson C, Dawson JJ, Chung SC, Li AK. A randomised study comparing laparoscopic versus open repair of perforated peptic ulcer using suture or sutureless technique. *Ann Surg* 1996; **224**: 131-138

4 **Svanes C**, Salvesen H, Stangeland L, Svanes K, Soreide O. Perforated peptic ulcer over 56 year. Time trends in patients and disease characteristics. *Gut* 1993; **34**: 1666-1671

5 **Boey J**, Wong J, Ong GB. A prospective study of operative risk factors in perforated duodenal ulcers. *Ann Surg* 1982; **195**: 265-269

6 **Boey J**, Choi SKY, Alagaratnam TT, Poon A. Risk stratification in perforated duodenal ulcers. *Ann Surg* 1987; **205**: 22-26

7 **Boey J**, Wong J. Perforated duodenal ulcers. *World J Surg* 1987; **11**: 319-324

8 **Evans JP**, Smith R. Predicting poor outcome in perforated peptic ulcer disease. *Aust N Z J Surg* 1997; **67**: 792-795

9 **Armitage P**, Berry G. Statistical methods in medical research. *Blackwell Scientific Publ* 1994

10 **Dawson B**, Trapp RG. Basic & Clinical Biostatistics. New York: *McGraw-Hill* 2001

11 **Gunshefsky L**, Flancbaum L, Brolin R, Frankel A. Changing pattern in perforated peptic ulcer disease. *Am Surg* 1990; **56**: 270-274

12 **Greiser WB**, Bruner BW, Shamoun JM, Jurkovich GJ, Ferrara JJ. Factors affecting mortality in patients operated upon for complications of peptic ulcer disease. *Am Surg* 1989; **55**: 7-11

13 **Hodnett RM**, Gonzalez F, Lee WC, Nance FC, Deboisblanc R. The need for definitive therapy in the management of the perforated gastric ulcers. Review of 202 cases. *Ann Surg* 1989; **209**: 36-39

14 **Horowitz J**, Kukora JS, Ritchie WP Jr. All perforated ulcers are not alike. *Ann Surg* 1989; **209**: 693-697

15 **Irvin TT**. Mortalità and perforated peptic ulcer: a case for risk stratification in elederly patients. *Br J Surg* 1989; **76**: 215-218

16 **Suter M**. Surgical treatment of perforated peptic ulcer. Is there a need for a change? *Acta Chir Belg* 1993; **93**: 83-87

17 **Lee FY**, Leung KL, Lai BS, Ng SS, Dexter S, Lau WY. Predicting mortality and morbidity of patients operated on for perforated peptic ulcers. *Arch Surg* 2001; **139**: 90-94

18 **Blomgren LGM**. Perforated peptic ulcer: long-term results after simple closure in the elderly. *World J Surg* 1997; **21**: 412-415

19 **Sillakivi T**, Lang A, Tein A, Peetsalu A. Evaluation of risk factors for mortality in surgically treated perforated peptic ulcer. *Hepatogastroenterology* 2000; **47**: 1765-1768

20 **Michelet I**, Agresta F. Perforated peptic ulcer: laparoscopic approach. *Eur J Surg* 2000; **166**: 405-408

21 **Svanes C**, Salvesen H, Stangeland L, Svanes K, Soreide O. Perforated peptic ulcer over 56 years. Time trend in patients and disease characteristics. *Gut* 1993; **34**: 1666-1671

22 **Cocks JR**. Perforated peptic ulcer - the changing scene. *Dig Dis* 1992; **10**: 10-16

23 **Walt R**, Katschinski B, Logan R, Ashley J, Langman M. Rising frequency of ulcer perforation in elderly people in the United Kingdom. *Lancet* 1986; **3**: 489

24 **McGee GS**, Sawyers JL. Perforated gastric ulcers. *Arch Surg* 1987; **122**: 555-561

25 **Hamby LS**, Zweng TN, Strodel WE. Perforated gastric and duodenal ulcer: an analysis of prognostic factors. *Am Surg* 1993; **59**: 319-324

26 **Mattingly SS**, Ram MD, Griffin WO Jr. Factors influencing morbidity and mortality in perforated duodenal ulcer. *Am Surg* 1980; **46**: 61-66

27 **McIntosh JH**, Berman K, Holliday FM, Byth K, Chapman R, Piper DW. Some factors associated with mortality in perforated peptic ulcer: a case control study. *J Gastroenterol Hepatol* 1996; **11**: 82-87

28 **Wakayama T**, Ishizaki Y, Mitsusada M, Takahashi S, Wada T, Fukushima Y, Hattori H, Okuyama T, Funatsu H. Risk factors influencing the short-term results of gastroduodenal perforation. *Surg Today* 1994; **24**: 681-687

29 **Jordan PH**, Thornby J. Perforated pyloroduodenal ulcers. Long-term results with omental patch closure and parietal cell vagotomy. *Ann Surg* 1995; **221**: 479-488

30 **Robles R**, Parrilla P, Lujan JA, Torralba JA, Cifuentes J, Liron R, Pinero A. Short note: long-term follow-up of bilateral truncal vagotomy and pyloroplasty for perforated duodenal ulcer. *Br J Surg* 1995; **82**: 665

31 **Matsuda M**, Nishiyama M, Hanai T, Saeki K, Watanabe T. Laparoscopic omental patch repair for perforated peptic ulcer. *Ann Surg* 1995; **221**: 336-240

32 **Testini M**, Scacco S, Loiotila L, Regina G, Vergari R, Papa F, Paccione F. Comparison of oxidative phosphorilation in the small vs. large bowel anastomosis. *Eur Surg Res* 1998; **30**: 1-7

33 **Testini M**, Piccinni G. Wound healing of intestinal anastomosis after digestive surgery under septic condition. *World J Surg* 1999; **23**: 1315-1316

34 **Testini M**, Margari A, Amoruso M, Lissidini G, Bonomo GM. Le deiscenze nelle anastomosi colo-rettali: fattori di rischio. *Ann Ital Chir* 2000; **71**: 433-440

35 **Testini M**, Piccinni G, Amoruso M, Di Venere B, Nicolardi V, Bonomo GM. Chronic obtructive pulmonary disease and failure of large bowel anastomosis. *It J Coloproctol* 2000; **3**: 91-94

36 **Testini M**, Portincasa P, Scacco S, Piccinni G, Minerva F, Lissidini G, Papa F, Loiotila L, Bonomo GM, Palasciano G. Contractility *in vitro* and mitochondrial response in small and large anastomosed rabbit bowel. *World J Surg* 2002; **26**: 493-498


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2341-2343
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Effect of resveratrol on cell cycle proteins in murine transplantable liver cancer

Liang Yu, Zhong-Jie Sun, Sheng-Li Wu, Cheng-En Pan

**Liang Yu, Zhong-Jie Sun, Sheng-Li Wu, Cheng-En Pan,** Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China
**Supported by** Traditional Chinese Medicine Bureau Foundation of Shaanxi Province, No. 2001-035
**Correspondence to:** Dr. Liang Yu, Department of Hepatobiliary Surgery, First Hospital of Xi' an Jiaotong University, Xi' an 710061, Shaanxi Province, China. yuliang@163.com
**Telephone:** +86-29-5324009
**Received:** 2002-11-11 **Accepted:** 2002-12-25

## Abstract

**AIM:** To study the antitumour activity of resveratrol and its effect on the expression of cell cycle proteins including cyclin D1, cyclin B1 and p34cdc2 in transplanted liver cancer of murine.

**METHODS:** Murine transplanted hepatoma H22 model was used to evaluate the *in vivo* antitumor activity of resveratrol. Following abdominal administration of resveratrol, the change in tumour size was recorded and the protein expression of cyclin D1, cyclin B1 and p34cdc2 in the tumor and adjacent noncancerous liver tissues were measured by immunohistochemistry.

**RESULTS:** Following treatment of H22 tumour bearing mice with resveratrol at 10 or 15 mg/kg bodyweight for 10 days, the growth of murine transplantable liver cancer was inhibited by 36.3 % or 49.3 %, respectively. The inhibitory effect was significant compared to that in control group ($P<0.05$). The level of expression of cyclin B1 and p34cdc2 protein was decreased in the transplantable murine hepatoma 22 treated with resveratrol whereas the expression of cyclin D1 protein did not change.

**CONCLUSION:** Resveratrol exhibits anti-tumour activities on murine hepatoma H22. The underlying anti-tumour mechanism of resveratrol might involve the inhibition of the cell cycle progression by decreasing the expression of cyclinB1 and p34cdc2 protein.

Yu L, Sun ZJ, Wu SL, Pan CE. Effect of resveratrol on cell cycle proteins in murine transplantable liver cancer. *World J Gastroenterol* 2003; 9(10): 2341-2343
http://www.wjgnet.com/1007-9327/9/2341.asp

## INTRODUCTION

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a kind of phytoalexin found in root extract of the weed polygonum cuaspidatum and in grape skins as well as red wine. Studies have demonstrated that resveratrol can alter the synthesis and secretion of lipids and lipoproteins by liver cells, block human platelet aggregation and inhibit the synthesis of pro-aggregatory and proinflammtory eicosanoids by platelets and neutrophils[1-5]. Moreover, some reports have shown that resveratrol is a potential chemopreventive agent of cancer and it can prevent tumor growth and metastasis in human lung carcinoma, pancreatic cancer, prostate cancer, bronchial epithelioma cancer and breast cancer models[6-10]. The present investigation evaluated the potency of resveratrol on tumour cell growth and proliferation and on expression of cell cycle proteins in a transplantable murine hepatoma 22 model.

## MATERIALS AND METHODS

### *Materials*

Resveratrol was purchased from Sigma Co (USA). The resveratrol was dissolved and sterilized in dimethyl sulfoxide (DMSO) first and then diluted to the required working concentration in RPMI-1640 (Gibco, USA) containing 10 % calf serum (SiJiQing Co, Hangzhou, China). MS-110cdk1/p34cdc2 Ab-1 0.1 ml, MS-210 cyclin D1/bcl-1 Ab-1 0.1 ml, MS-338 cyclin B1 Ab-1 0.1 ml were purchased from Santa Cruz Co (USA). S-P ultra sensitive immunostaining kit was purchased from Maixin Biotechnology Developing Co (Fuzhou, China). Mouse hepatocellular carcinoma cell line H22 was kindly supplied by Cheng Wei (Center of Molecular Biology, First Affiliated Hospital, Xi' an Jiaotong University). Balb/c mice were purchased from the Animal Center of Xi' an Jiaotong University.

### *Methods*

**Suppressive effect of resveratrol on transplanted liver cancer of mouse** H22 cells were first subcultured in RPMI 1640 containing 10 % fetal bovine serum. Then the cells were washed twice and resuspended in RPMI 1640 culture medium ($1\times10^8$/ml). About 0.2 ml cell solution (including $2\times10^7$ cells) was taken and injected into the right groin of 5 Balb/c mice. After 14 days, when tumors of 3-5 mm in diameter formed in the right groin of these mice, they were taken out and sheared into small pieces of 1 $mm^3$ under sterile condition. Forty Balb/c mice were anesthetized by using coelio-injection of pentobarbitone (70 mg/Kg) and laparotomy was performed. Under sterile condition their middle lobes of liver were punctured to form a 3 mm-long sinus tract and a small piece of tumor tissue was put into each sinus tract. Then these mice were divided into 4 groups randomly: one control group and 3 experimental groups. The experimental groups were designed to be injected with resveratrol (dissolved in DMSO and diluted to the working concentration of 25 mM in RPMI-1640 containing 10 % calf serum) at 5, 10 or 15 mg/kg body weight while the control group was designed to be given the same volume of the solution as for experimental group but absent of resveratrol. Twenty-four hours following liver tumour transplantation, each mouse was injected corresponding dosage of resveratrol into its abdominal cavity once a day for 10 days. These mice were then sacrificed on the following day after the last injection. After the maximum diameter and transverse length of tumour were measured, the hepatocellular carcinoma tissues and adjacent noncancerous liver tissues were sampled and fixed in 10 % formalin.

**Measurement of tumour volume** The tumour volume was

calculated using formula V=1/2 (maximum diameter x transverse length$^2$). The suppressive rate of tumour growth was calculated as [(mean V of tumour in control group - mean V of tumour in experimental group)/mean V of tumour in control group]×100 %.

**Determination of protein expression of cyclin D1, cyclin B1 and p34cdc2 in murine transplantable liver cancer tissue**

The tumour tissues and adjacent noncancerous liver tissues taken from therapeutic group (resveratrol in 10 mg/kg) and control group were 10 % formalin-fixed, paraffin-embedded and cut into 4 μm thick sections for staining. PBS of 0.01 mol· L$^{-1}$ was used to substitute for primary antibody for negative control while a breast cancerous tissue expressing cyclin D1 and a tonsil tissue expressing cyclin B1 and cdk1/p34cdc2 were used for positive controls. The working concentration of each antibody was cyclin B1 (1:100), cyclin D1 (1:40) and cdk1/p34cdc2 (1:70), respectively. The staining procedures used were as described in S-P immunostaining kit. The sections were examined twice on different days by the same pathologist and the distribution of positively stained cells for each protein was evaluated semi-quantitatively by calculating the percentage of positive cells in nonoverlaping microscopic fields. The protein expression was then scored arbitrarily as "-" <5 % positive cells, "+" 5-50 % positive cells, "++" >50 % positive cells. $\chi^2$ test was used to evaluate the statistical significance of the difference between experimental and control groups.

## RESULTS

### *Suppressive effect of resveratrol on murine transplantable liver cancer*

Except 2 Balb/c mice (one in control group and one in 15 mg/kg resveratrol therapeutic group), all the mice inoculated with hepatocarcinoma cell line H22 were successively transplanted with liver cancer. After treatment of the tumour bearing mice with 5, 10 or 15 mg/kg resveratrol for 10 days, the tumour size was reduced from 134 mm$^3$±40 mm$^3$ in control group to 105 mm$^3$±14mm$^3$, 85 mm$^3$±22mm$^3$ and 68 mm$^3$±17mm$^3$ in experimental groups, resulting in an inhibition rate of tumour growth of 21.6 %, 36.3 % and 49.3 %, respectively. The inhibition effect in the latter 2 therapeutic groups was significant compared with that in control group (all $P$<0.05) (Table 1).

**Table 1** The suppressing effect of resveratrol on murine transplanted liver tissue

| | Dose (mg/kg) | No. of mice (begin/end) | Tumour size (mm$^3$, $\bar{x}$±s) | GIR (%) |
|---|---|---|---|---|
| Control group | 0 | 10/9 | 134±40 | |
| Resveratrol | 5.0 | 10/10 | 105±14[a] | 21.6 |
| | 10.0 | 10/10 | 85±22[b] | 36.3 |
| | 15.0 | 10/9 | 68±17[b] | 49.3 |

[a]$P$>0.05, [b]$P$<0.01, *vs* control.

### *Effect of resveratrol on expression of cyclin D1, cyclin B1 and p34cdc2 in murine liver cancer tissue*

Cyclin D1 immunoreactivity was observed in the nucleolus of tumour cell, cyclin B1 immunoreactivity and p34cdc2 immunoreactivity were observed in the cytoplasm of tumour cell. The expression level of cyclin D1, cyclin B1 and p34cdc2 in transplanted liver cancer tissue was significantly higher than that of adjacent noncancerous liver tissues in control group (All $P$<0.05) (Tables 2-4). Following treatment with resveratrol, a depressed expression of cyclin B (77.8 % *vs* 30.0 %, $P$<0.05) and p34cdc2 protein (88.9 % *vs* 40.0 %, $P$<0.05) was observed, but the expression of cyclin D1 protein (66.7 % *vs* 60.0 %, $P$>0.05) did not change.

**Table 2** Effect of resveratrol on the expression of cyclin D1 in murine liver tumor and adjacent noncancerous liver tissues

| | No. of mice | Cyclin D1 | | | Positive rate (%) (+, ++) |
|---|---|---|---|---|---|
| | | - | + | ++ | |
| Noncancerous tissues | 9 | 9 | 0 | 0 | 0 |
| Control group | 9 | 3 | 2 | 4 | 66.7[a] |
| Therapeutic group | 10 | 4 | 3 | 3 | 60.0[b] |

[a]$P$<0.01, *vs* noncancerous tissues; [b]$P$>0.05, *vs* control.

**Table 3** Effect of resveratrol on the expression of cyclin B1 in murine liver tumor and adjacent noncancerous liver tissues

| | No. of mice | CyclinB1 | | | Positive rate (%) (+,++) |
|---|---|---|---|---|---|
| | | - | + | ++ | |
| Noncancerous tissues | 9 | 7 | 1 | 1 | 22.2 |
| Control group | 9 | 2 | 2 | 5 | 77.8[a] |
| Therapeutic group | 10 | 7 | 2 | 1 | 30.0[b] |

[a]$P$<0.05, *vs* noncancerous tissues; [b]$P$<0.05, *vs* control.

**Table 4** Effect of resveratrol on the expression of p34cdc2 in murine liver tumor and adjacent noncancerous liver tissues

| | No. of mice | p34cdc2 | | | Positive rate (%) (+,++) |
|---|---|---|---|---|---|
| | | - | + | ++ | |
| Noncancerous tissues | 9 | 7 | 2 | 0 | 22.2 |
| Control group | 9 | 1 | 3 | 5 | 88.9[a] |
| Therapeutic group | 10 | 6 | 3 | 1 | 40.0[b] |

[a]$P$<0.01, *vs* noncancerous tissues; [b]$P$<0.05, *vs* control.

## DISCUSSION

In 1997 Park *et al*, showed that resveratrol inhibited tumorigenesis in a mouse skin cancer model[11]. Since then, many studies have shown that resveratrol has suppressive effects on tumour cells in human lung carcinoma, prostate cancer, bronchial epithelioma and breast cancer models[12-14]. The ability of resveratrol to inhibit cellular events associated with tumour initiation, promotion, and progression might be attributed to its anticyclooxygenase activity (COX-1), inducing apoptosis of tumour cells, antagonizing mutation, antioxidation and anti-free radicals activity and its effect on cell cycle[15,16].

Recent studies have shown that many drugs exhibited their anti-tumour activity by interfering cell cycles. The cell cycle regulators have thus become a new target in drug discovery research. Of the 3 cell cycle regulators of cyclins, CDKs, and CKIs which have been found, CDKs is the core of the modulation network, which is positively regulated by cyclins and negatively by CKIs. Cyclin D1, a member of cyclins family, modulates mainly G1/S stage transition. Under normal conditions, the amount of cyclin D1 is invariable in G1 stage and its overexpression will lead to cells passing through G1/S without control, which is considered to be associated with carcinogenesis. Cyclin B1 is related mainly to the completion of M stage. CDC2 (CDK1) gene, a modulating core of G2/M inspect point, codes for p34cdc2 protein. The p34cdc2 combines with cyclin B1 to form MPF which plays an important role in the process from G2 stage to M stage. Studies have shown that cyclin B1-CDC2 was in activated state in many tumour cell lines no matter what the state of DNA was. Even

though cells had DNA damage, they could still enter the cleavage stage. Therefore, p34cdc2 and cyclinB1 were also associated with carcinogenesis[17].

In the present investigation, resveratrol was administered into murine abdomen and its potency on growth and proliferation of H22-innoculated tumour was evaluated by measuring the size of hepatoma and examining the expression of cell cycle proteins. The tumour size was found to be reduced by each dosage of 5, 10 or 15 mg/kg of resveratrol for 10 days. When the higher dosage of resveratrol was applied, the tumour size was significantly reduced, the inhibition rate of tumour growth by 10 or 15 mg/kg reached to 36.3 % and 49.3 %, respectively ($P$<0.01). We also found the protein expression of cyclin D1, cyclin B1 and p34cdc2 in murine transplanted liver cancer tissue was significantly higher than that in adjacent noncancerous liver tissues, suggesting that the overexpression of these cell cycle proteins may play a role in the onset and development of carcinoma. Compared with control group, a depressed expression of cyclin B1 and p34cdc2 protein was observed in the transplantable murine hepatoma following treatment with resveratrol, suggesting that the anti-tumor mechanism of resveratrol may be to prevent mitosis of tumor cells by suppressing the protein expression of cyclin B1and p34cdc2, and thus, interfering with the process of tumor cells from S stage to G2/M stage.

In short, the data presented in this paper suggest that resveratrol has an apparent antitumour activity, and blocking of S stage of tumour cells may be the underlying mechanism. The demonstration of modulating effect of resveratrol on cell cycle should provide certain theoretical basis for further study of resveratrol and other cell cycle interfering agents.

## REFERENCES


1 **Afaq F**, Adhami VM, Ahmad N. Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice small star, filled. *Toxicol Appl Pharmacol* 2003; **186**: 28-37

2 **Fremont L**. Biological effects of resveratrol. *Life Sci* 2000; **66**: 663-673

3 **Sato M**, Ray PS, Maulik G, Maulik N, Engelman RM, Bertelli AA, Bertelli A, Das DK. Myocardial protection with red wine extract. *J Cardiovasc Pharmacol* 2000; **35**: 263-268

4 **Roemer K**, Mahyar-Roemer M. The basis for the chemopreventive action of resveratrol. *Drugs Today* 2002; **38**: 571-580

5 **Zou JG**, Wang ZR, Huang YZ, Cao KJ, Wu JM. Effect of red wine and wine polyphenol resveratrol on endothelial function in hypercholesterolemic rabbits. *Int J Mol Med* 2003; **11**: 317-320

6 **Kimura Y**, Okuda H. Resveratrol isolated from Polygonum cuspidatum root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *J Nutr* 2001; **131**: 1844-1849

7 **Narayanan BA**, Narayanan NK, Re GG, Nixon DW. Differential expression of genes induced by resveratrol in LNCaP cells: P53-mediated molecular targets. *Int J Cancer* 2003; **104**: 204-212

8 **Ding XZ**, Adrian TE. Resveratrol inhibits proliferation and induces apoptosis in human pancreatic cancer cells. *Pancreas* 2002; **25**: e71-76

9 **Kuo PL**, Chiang LC, Lin CC. Resveratrol- induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. *Life Sci* 2002; **72**: 23-34

10 **Banerjee S**, Bueso-Ramos C, Aggarwal BB. Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9. *Cancer Res* 2002; **62**: 4945-4954

11 **Park JW**, Choi YJ, Jang MA, Lee YS, Jun DY, Suh SI, Baek WK, Suh MH, Jin IN, Kwon TK. Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. *Cancer Lett* 2001; **163**: 43-49

12 **Mollerup S**, Ovrebo S, Haugen A. Lung carcinogenesis: resveratrol modulates the expression of genes involved in the metabolism of PAH in human bronchial epithelial cells. *Int J Cancer* 2001; **92**: 18-25

13 **Kozuki Y**, Miura Y, Yagasaki K. Resveratrol suppresses hepatoma cell invasion independently of its anti-proliferative action. *Cancer Lett* 2001; **167**: 151-156

14 **Rodrigue CM**, Arous N, Bachir D, Smith-Ravin J, Romeo PH, Galacteros F, Garel MC. Resveratrol, a natural dietary phytoalexin, possesses similar properties to hydroxyurea towards erythroid differentiation. *Br J Haematol* 2001; **113**: 500-507

15 **Young J**, Barker M, Fraser L, Walsh MD, Spring K, Biden KG, Hopper JL, Leggett BA, Jass JR. Mutation searching in colorectal cancer studies: experience with a denaturing high-pressure liquid chromatography system for exon-by-exon scanning of tumour suppressor genes. *Pathology* 2002; **34**: 529-533

16 **Takahashi M**, Shimomoto T, Miyajima K, Iizuka S, Watanabe T, Yoshida M, Kurokawa Y, Maekawa A. Promotion, but not progression, effects of tamoxifen on uterine carcinogenesis in mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine. *Carcinogenesis* 2002; **23**: 1549-1555

17 **Pommier Y**, Kohn KW. Cell cycle and checkpoints in oncology: new therapeutic targets. *Med Sci* 2003; **19**: 173-186


**Edited by** Liu HX and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2344-2347
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Rapid and high throughput detection of HBV YMDD mutants with fluorescence polarization

Yui-Jie Bai, Jin-Rong Zhao, Guan-Ting Lv, Wen-Hong Zhang, Yan Wang, Xiao-Jun Yan

**Yui-Jie Bai, Jin-Rong Zhao, Guan-Ting Lv, Wen-Hong Zhang, Yan Wang, Xiao-Jun Yan,** Institute of Genetic Diagnosis, Fourth Military Medical University, Xi' an 710032, Shannxi Province, China
**Supported by** Shen Zhen Syno Gene Digital Co. Ltd
**Correspondence to:** Dr. Yu-Jie Bai, Institute of Genetic Diagnosis, Fourth Military Medical University, Xi' an 710032, Shannxi province, China. yujiebai@163.com
**Telephone:** +86-29-3216587 **Fax:** +86-29-3285729
**Received:** 2003-04-09 **Accepted:** 2003-05-19

## Abstract

**AIM:** To develop a simple and rapid detection of HBV gene variants and prediction of lamivudine-resistance in patients.

**METHODS:** Initially, plasmids harboring the wild-type or mutant HBV DNA fragments were used in a model system. The technique was then applied to clinical samples for an analysis of YMDD mutations. The sera were extracted from chronic hepatitis patients who had received lamivudine treatment for more than one year. P region gene of HBV was amplified by polymerase chain reaction. The excess primers and dNTPs in PCR products were removed by cleaning-up reagents. Template-directed dye-terminator incorporation reaction was performed and R110 or TAMRA labeled acyclo-terminator was added on the 3' end of TDI-primer specifically. Fluorescence polarization value was measured with Victor 2 multilabel counter and the genotypes of HBV were analyzed.

**RESULTS:** The YMDD genotypes in recombined positive plasmid and 56 serum samples of HBV infected patients were analyzed by using our TDI-FP method and the specificity and sensitivity were confirmed by DNA sequencing. Five of 56 serum samples showed YVDD phenotype (9 %), including 1 YMDD and YVDD mixed infection. Four of 56 showed YIDD phenotype (7.1 %).

**CONCLUSION:** This is a simple, rapid, low cost and high throughput assay to detect HBV polymerase gene variants and suitable for large-scale screening and prediction of the lamivudine-resistance in clinical samples.

Bai YJ, Zhao JR, Lv GT, Zhang WH, Wang Y, Yan XJ. Rapid and high throughput detection of HBV YMDD mutants with fluorescence polarization. *World J Gastroenterol* 2003; 9(10): 2344-2347
http://www.wjgnet.com/1007-9327/9/2344.asp

## INTRODUCTION

Hepatitis virus B (HBV) is the causative agent of acute and chronic hepatitis. Approximately 400 million people worldwide are chronically infected with HBV. There are 170 million people infected in China[1,2]. HBV infection can lead to cirrhosis and primary hepatocellular carcinoma[3-5]. To date, interferon alfa and lamivudine are the only two agents approved for chronic hepatitis[6-10]. Because interferon alfa shows significant dose-dependent side effects, lamivudine has emerged as the first therapeutic agent[11-13]. Chinese patients are immunotolerant to interferon alfa because of acquisition of the disease during early childhood. The efficacy of interferon alfa in Chinese is lower than in white patients[14]. However, the efficacy of lamivudine is equal in Chinese and white patients.

Lamivudine is an antiviral nucleoside analogue. It can inhibit HBV reverse transcriptase activity and also act as a viral DNA chain terminator[15]. It is an inactive form before activated in hepatocytes by addition of phosphate group[2]. Lamivudine has two pathways of viral suppression. First, the active triphosphate metabolite is incorporated into newly synthesized HBV DNA to stop the chain extension. Second, it inhibits HBV reverse transcriptase. Its clinical use has resulted in HBeAg seroconversion and undetectable HBV DNA[16]. Unlike some nucleoside analogues, lamivudine is well tolerated and has an excellent safety profile. It has little or no effect on bone marrow, hepatocytes, kidney, or muscle tissues. However, long-term lamivudine treatment has led to emergence of HBV variants resistant to lamivudine therapy in some patients. The major sites of mutation are situated in the highly conserved motif, tyrosine (Y), methionine (M), aspartate (D), aspartate (D) (YMDD), of the catalytic (C) domain of the reverse transcriptase. Mutations consist of an amino acid substitution from M to either valine (A739G, $Met_{552} \rightarrow Val_{552}$) or isoleucine (G741T, $Met_{552} \rightarrow Ile_{552}$). Those mutations at the YMDD motif could render HBV resistant to lamivudine[17].

Fluorescence polarization assay (FPA) is a well established method used to analyze associations and dissociations between molecules in solution. This technique relates the change in the molecular size of a fluorophore to a change in the fluorescence polarization value (mp unit). The value changes indicate molecular associations or dissociations, so that FPA have been used to examine the interactions between molecules, such as protein-protein interactions, DNA-protein interaction and DNA-DNA hybridization.

Based on the FP detection method and the combined template-directed dye-terminator incorporation technique, a novel SNP (single nucleotide polymorphism) detection system (TDI-FP) has been developed[18]. It relies on the ability of AcycloPol™, a novel mutant thermostable polymerase from the Archeon family, to extend accurately an annealed probe by a single dye-terminator that is complementary to the opposite strand. It performs an assay to determine the base at a SNP site with many advantages over the other methods, such as homogeneity, low cost, high accuracy, high throughput and ready adaptation to automation. This method has been used to genotyping and SNP detections but has not, to our knowledge, been used to microbial mutant analysis yet.

Routine detection of HBV YMDD mutation after lamivudine treatment will be required to increase the efficacy. In this study, we undertook such a study using TDI-FP to detect the YMDD variants using mutagenesis plasmids and serum samples of 56 patients. This new method is a rapid, accurate and high throughout method to analyses the YMDD mutations**.** It will be a very useful tool for the clinical diagnosis and monitor of the lamivudine resistance.

## MATERIALS AND METHODS

### *Instrumentation*

Fluorescence polarization values were determined with the VICTOR2 multilabel counter (Perkin-Elmer, USA). The excitation and emission wavelengths were 544 nm and 595 nm for R110 and 485 nm and 535 nm for TAMRA, respectively. The samples were measured in 384 well PCR plates (MSP-3862 MJ Research, USA).

### *Reagents*

Taq DNA polymerase, DNA extractor kit were obtained from Hua-mei Bio-tech Co., LTD. AcycloPrime™-FP detection kit (including AcycloPol™, AcycloTeminators™ labeled with R110 and TAMRA, shrimp alkaline phosphatase, exonuclease I) was product of Perkin-Elmer Co., Ltd.

### *Oligonucleotides*

A set of PCR primers was designed to amplify the P region of HBV gene including the YMDD motif encoding sequences. 2 TDI-primers were also designed to encompass 17-21 bp 5' (A739Gtdi, sense) or 3' (G741T, antisense) to the nucleotide adjacent to the mutation sites using DNAStar software. 3 sets of primers for the site-directed mutagenesis were also designed using DNAStar software. All the primers, whose sequences are shown in Table 1, were synthesized using 391 DNA synthesizer (Perkin-Elmer, USA) by Bao Tai Ke Biotech Co.

### *Site-directed mutagenesis*

The site-directed HBV YMDD mutants were made using Takara MutanBEST Kit by TaKaRa Biotechnology Co., Ltd. The A719G mutation was created using primers A739G*for* and A739G*rev*. The G741T mutation was created using primers G741T*for* and G741T*rev*. The A739G/G741T double mutants were made using primers diMut*for* and diMut*rev* with the plasmid pMD/HBV as a template respectively. (All the sequences of primers are shown in Table 1). The mutagenesis was detected according to the manufacture' s manual and verified by DNA sequencing.

**Table 1** Primer sequences

| Primer | Sequence |
|---|---|
| PCR*for* | 5'-GCACTTGTATTCCCATCCCATCAT-3' |
| PCR*rev* | 5'-GTATACCCAAAGACAAAAGAA-3' |
| 739*tdi* | 5'-CACTGTTTAGCTTTCAGTTAT-3' |
| 741*tdi* | 5'-CCCAATACCACATCATC-3' |
| A719G*for* | 5'-CTTTCAGTTATGTGGATGATGTGGTA 3' |
| A719G*rev* | 5' -CCAGACAGTGGGGGAAAGC 3' |
| G741T*for* | 5'-CTTTCAGTTATATTGATGATGTGGTA 3' |
| G741T*rev* | 5'- CCAGACAGTGGGGGAAAGC 3' |
| diMut*for* | 5'-CTTTCAGTTATGTTGATGATGTGGTA 3' |
| diMut*rev* | 5' -CCAGACAGTGGGGGAAAGC 3' |

### *DNA samples*

Venous blood (1 ml) was obtained from 56 patients with HBV chronic hepatitis B in Tangdu Hospital and Second Hospital of Jiaotong University, Xi' an.

### *Amplification of P region of HBV gene*

PCR amplification was performed in a total volume of 50 μL, containing 1.5U Taq polymerase, PCR buffer, 0.1 mmol/L dNTPs, 1 μL of DNA sample (10 ng plasmid DNA or 100 ng genome DNA), 100 nmol/L PCR*for* and PCR*rev* primer each. The amplification procedure consisted of an initial denaturation and enzyme activation step at 94 ℃ for 5 min, followed by 40 cycles containing denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s and extension at 72 ℃ for 1 min. After a final extension step at 72 ℃ for 10 minutes, the reactions were cooled to 4 ℃ until further use. The PCR reaction was carried out in a Touchgene gradient termal cycler (TECHNE Co. USA). In order to evaluate the amplification, each 5 μL of PCR products was separated on a 20 g/L agarose gel in 0.5× TBE buffer. The products were visualized and photographed under ultraviolet (UV) light.

### *Fluorescence polarization detection*

Following PCR amplification, 2 μL clean-up reagent (including shrimp alkaline phosphatase and exonuclease I and reaction buffer) was added into 5 μL of PCR product and incubated at 37 ℃ for 60 min to remove unincorporated dNTPs and free primers. The enzymes were then heat-inactivated at 80 ℃ for 30 min.

TDI-FP was performed according to the protocol supplied by the manufacturer (Perkin-Elmer Life Sciences, Inc, USA). The reaction system consisted of 13 μL of AcycloPrime-FP mixture (containing 0.05 μL of AcycloPol™ polymerase, 2 μL of 10× reaction buffer, 1 μL of Acyclo-Terminator Mix, 0.5 μL mutant detection primer and 9.45 μL of water) and 7 μL of amplified and processed target DNA. After an initial denaturation at 95 ℃ for 2 min, 25 cycles at 95 ℃ for 15 sec and at 55 ℃ for 30 s were performed on a black 384-well PCR plate (MSP-3862, MJ Research). The fluorescence polarization values were measured on the 384-well plate using Victor2 multilabel counter directly.

## RESULTS

### *PCR amplification*

The PCR products were analyzed by agarose gel electrophoresis (Figure 1). A single band corresponding to a molecular size of 200 bp was detected, which was in concordance with expectation.

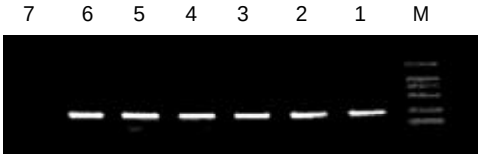



**Figure 1** Electrophoretic analysis of PCR products. 1: plasmid pMD / HBV-A739G as PCR template, 2-6: serum samples as PCR template, 7: water as template (negative control), M: DNA molecular weight marker.

### *TDI-FP assay*

Following PCR amplification, excess primers and dNTPs were removed. In the TDI extension reaction, 729*tdi* primer and dye-terminators mix (R110-acyGTP/ TAMRA-acyATP) were used to detect the A739G mutation (Figure 2). 741*tdi* primer and R110-acyCTP/TAMRA- acyATP) were used to detect the G741T mutation. The dye-terminator was incorporated and the TDI-primer was extended by one base complementary to the specific mutation site. The fluorescence intensity was measured using Victor 2 multilabel counter and the FP value was calculated by the instrument software automatically. The graph was created from an Excel workbook and the cluster of AcycloPrime-FP data was obtained by plotting the TAMRA polarization *vs* the R110 polarization. The FP reading of the samples was clustered into four distinct groups (Figure 3). As expected, for HBV YVDD samples, the values for R110-acyGTP were high and the values for TAMRA-acyATP were low, reflecting incorporation of the R110-acyGTP but not

TAMRA-acyATP. The genotypes were represented by the cluster in the lower left. Conversely, the YMDD genotypes appeared in the upper right. YMDD and YVDD double mutations or mixed infection samples appeared in the upper right. Negative controls were in the lower left cluster (Figure 3). For the G741T detection, the YIDD samples appeared in the upper left, YMDD samples in the lower right and the negative control in the lower left.

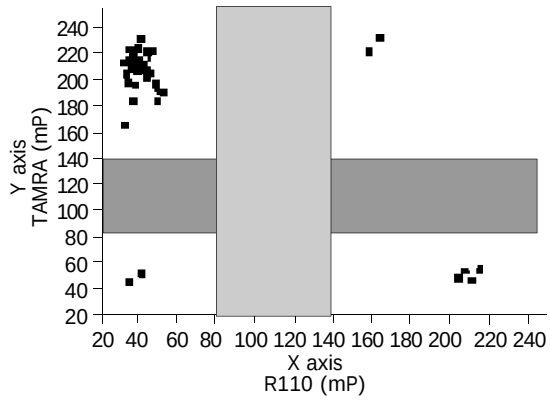



**Figure 3** TDI-FP analysis data of YMDD mutation.

### *Detection of YMDD mutation in clinical samples*

To evaluate the utility and accuracy of the TDI-FP in clinical samples, 56 serum samples from patients with chronic hepatitis B were analyzed. Among the sera analyzed, the wild-type YMDD variant was detected in 46 (82 %), the YVDD variant in 5 (8.9 %) and the YIDD in 4 (7.1 %). In one patient (1.8 %), both YMDD and YVDD variants were detected, which might reflect a mixed infection.

## DISCUSSION

As reported before, the major drawback to the therapy of lamivudine is the emergence of drug-resistance due to the HBV DNA mutations. The patients who have lamivudine resistant HBV variants infection are more likely to develop more severe liver damage than those who do not have drug-resistant mutants. The mutation at YMDD (YMDD→YVDD or YMDD →YIDD) is considered to be the major lamivudine-resistance related variants. DNA sequencing is considered as the ideal method for characterizing DNA mutants, but it can not be afforded by most laboratories, especially clinical laboratories due to the cost and equipment requirement. Type-specific PCR, PCR-RLFP and PCR-SSCP have been used to detect HBV YMDD mutants. However, all the methods have drawbacks, e.g. lack of accuracy, difficulty in determining the mutation sites exactly or detecting mixed infection[19].

We reported here, for the first time, the successful application of the TDI-FP to detect the HBV YMDD mutation in hepatitis B patients. As a homogenous assay, the FP value reflected the total sum of free and incorporated dye-terminators. Two major factors affecting the result were faced: (1) The concentration of the first PCR product in TDI extension was too high or too low, which would create misincorporation or lower incorporation of dye-terminators, (2) the excess primers

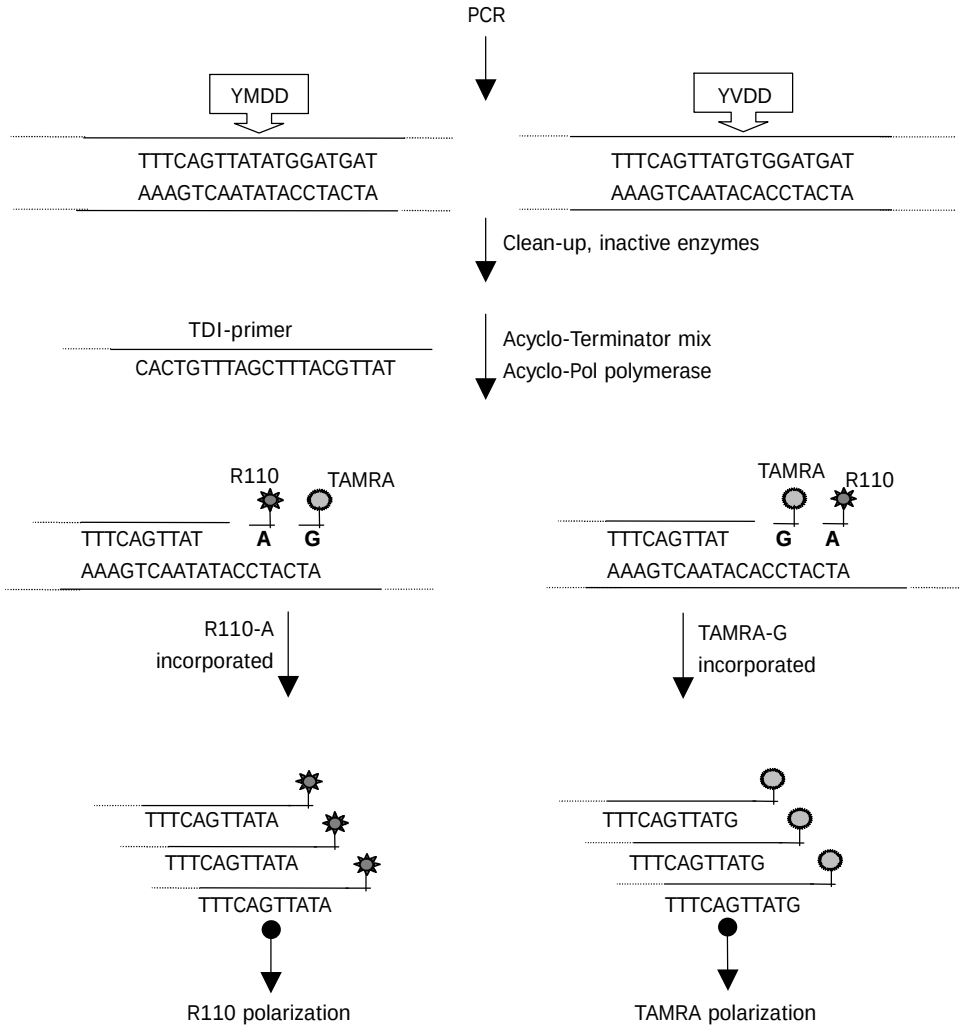



**Figure 2** The scheme of TDI assay of YMDD.

in the first PCR product might lead to misincorporation, while excess dNTPs would interfere the dye-terminator incorporation competitively. We increased the number of thermal cycles to 40-45 to ensure that all PCR components were exhausted and adjusted to the concentration of dNTPs and primer in PCR reaction, which were lower than standard PCR protocol. We also prolonged the digestion time from 30 min to 60 min to completely remove the dNTPs and primers. At the same time, to get best TDI-FP performance, forward and reverse TDI primers were used and compared in our study (data not shown). The predicted reaction conditions (annealing temperature and $MgCl_2$) were tested on several nonessential DNA samples to optimize the system.

DNA diagnostic tests will no doubt be applied more and more in clinical rather than in research laboratories. The main advantages of our technique over the other methods for scoring DNA variation, are high accuracy, high throughput and easy to adaptation automatic. It will improve the analysis and prediction of drug-resistance in clinic.

## REFERENCES


1 **Yan JC**, Ma JY, Pan BR, Ma LS. The study of chronic hepatitis B in China. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 611-616

2 S**u Q**, Fu Y, Liu YF, Zhang W, Liu J, Wang CM. Laminin induces the expression of cytokeratin 19 in hepatocellular carcinoma cells growing in culture. *World J Gastroenterol* 2003; **9**: 921-929

3 **Cao XY**, Liu J, Lian ZR, Clayton M, Hu JL, Zhu MH, Fan DM, Feitelson M. Differentially expressed genes in hepatocellular carcinoma induced by woodchuck hepatitis B virus in mice. *World J Gastroenterol* 2001; **7**: 575-578

4 **Huang YX**, Wu GH. The relationship between hepatitis B and hepatocellular carcinoma. *Shijie Huaren Xiaohua Zazhi* 2001; **9**: 682-685

5 **Wang FS**, Xing LH, Liu MX, Zhu CL, Liu HG, Wang HF, Lei ZY. Dysfunction of peripheral blood dendritic cells from patients with chronic hepatitis B virus infection. *World J Gastroenterol* 2001; **7**: 537-541

6 **Han HL**, Lang ZW. Changes in serum and histology of patients with chronic hepatitis B after interferon alpha-2b treatment. *World J Gastroenterol* 2003; **9**: 117-121

7 **Lai YC**, Hu RT, Yang SS, Wu CH. Coinfection of TT virus and response to interferon therapy in patitents with chronic hepatitis B or C. *World J Gastroenterol* 2002; **8**: 567-570

8 **Zhuang L**, You J, Tang BZ, Ding SY, Yan KH, Peng D, Zhang YM, Zhang L. Preliminary results of Thymosin-α1 versus interferon-a-treatment in patients with HBeAg negative and serum HBV DNA positive chronic hepatitis B. *World J Gastroenterol* 2001; **7**: 407-410

9 **You J**, Zhuang L, Tang BZ, Yang WB, Ding SY, Li W, Wu RX, Zhang HL, Zhang YM, Yan SM, Zhang L. A randomized controlled clinical trial on the treatment of Thymosin α1 versus interferon-α in patients with hepatitis B. *World J Gastroenterol* 2001; **7**: 411-414

10 **Cheng ML**, Wu YY, Huang KF, Luo TY, Ding YS, Lu YY, Liu RC, Wu J. Clinical study on the treatment of liver fibrosis due to hepatitis B by IFN-alpha(1) and traditional medicine preparation. *World J Gastroenterol* 1999; **5**: 267-269

11 **Zoulim F**. A preliminary benefit-risk assessment of lamivudine for the treatment of chronic hepatitis B virus infection. *Drug Saf* 2002; **25**: 497-510

12 **Yang SS**, Hsu CT, Hu JT, Lai YC, Wu CH. Lamivudine does not increase the efficacy of interferon in the treatment of mutant type chronic viral hepatitis B. *World J Gastroenterol* 2002; **8**: 868-871

13 **Han T**, Qian SC, Chen Y, Xiao SX, Wang FM, Han ZC, Lu HM. Treatment of 198 patients with chronic hepatitis B with lamivudine and Yi –Gan-Jian. *Shijie Huaren Xiaohua Zazhi* 2002; **10**: 1236-1237

14 **Lai CL**, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687-696

15 **Cao H**, Tao P. Anti-hepatitis B virus effects of lamivudine and other five drugs *in vitro*. *Zhonghua Yixue Zazhi* 2001; **81**: 1004-1007

16 **Dienstag JL**, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256-1263

17 **Li G**, Shu X, Ma HH, Chen W, Chen WS, Chen Q Jiang YS, Yao JL. Detection of HBV, HCV and HBV YMDD mutants by DNA microarray. *Shijie Huaren Xiaohua Zazhi* 2003; **11**: 178-181

18 **Hsu TM**, Chen X, Duan S, Miller RD, Kwok PY. Universal SNP genotyping assay with fluorescence polarization detection. *Biotechniques* 2001; **31**: 560-568

19 **Jardi R,** Buti M, Rodriguez-Frias F, Cotrina M, Costa X, Pascual C, Esteban R, Guardia J. Rapid detection of lamivudine-resistant hepatitis B virus polymerase gene variants. *J Virol Methods* 1999; **83**: 181–187


**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2348-2349
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Ultrasonography in predicting and screening liver cirrhosis in children: A preliminary study

Jia-An Zhu, Bing Hu

**Jia-An Zhu, Bing Hu,** Department of Ultrasound in Medicine, Affiliated Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Correspondence to:** Dr. Zhu Jia-An, Department of Ultrasound in Medicine, Shanghai Sixth People' s Hospital, 600 Yishan Road, Shanghai 200233, China. zhujiaan@tom.com
**Telephone:** +86-21-64369181-8751
**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To evaluate the value of ultrasonography in predicting and screening liver cirrhosis in children.

**METHODS:** Twenty-eight children with liver cirrhosis of various etiologies were examined by routine ultrasonography. A percutaneous liver biopsy guided by ultrasound was also performed on each patient, and the results of liver biopsy and ultrasonography were compared.

**RESULTS:** When compared with the biopsy results, ultrasonography in combination of clinical and laboratory findings gave accurate diagnoses of children liver cirrhosis. Although ultrasound imaging of children with liver cirrhosis revealed abnormal characteristics, these images were not specific to this disease, thus reinforcing the necessity of ultrasound-guided liver biopsy in the diagnosis of children liver cirrhosis.

**CONCLUSION:** Ultrasonography is reliable in the diagnosis of children liver cirrhosis, and its usefulness should be stressed in the screening and follow-up of high-risk pediatric patients.

Zhu JA, Hu B. Ultrasonography in predicting and screening liver cirrhosis in children: A preliminary study. *World J Gastroenterol* 2003; 9(10): 2348-2349
http://www.wjgnet.com/1007-9327/9/2348.asp

## INTRODUCTION

Cirrhosis of the liver is a morphological entity, and it has been assumed to be an end-stage condition of all chronic active liver diseases. There are many types of liver cirrhosis, including posthepatitic, alcoholic and mixed types of cirrhosis, and congestive, biliary and parasitic cirrhosis, etc. The etiological factors underlying this disease, as well as its morbidity, have been shown to be different in different geographical regions[1-8]. It should be mentioned that morphological changes are different in liver cirrhosis, which include the structural changes not only in the parenchymal disorganizations, but also in the stromal vascular changes in the cirrhotic process[9,10]. It has been widely accepted that ultrasonography is a useful diagnostic procedure for advanced liver cirrhosis. However, liver cirrhosis of children and its applicable techniques are usually different from those of adults. Several types of the cirrhosis resulted from metabolic diseases including glycogen storage disease and hepatolenticular degeneration are rare in adults. In children, the onset of cirrhosis is often occult. Children cirrhosis lacks the early typical clinical symptoms as seen in adult patients, thus contributing to its frequent misdiagnosis[11]. Unfortunately, reports on children cirrhosis are few. To determine if ultrasonography could be of clinical value in predicting the etiology of children cirrhosis, we performed this test in 28 children whose cirrhosis was confirmed by histopathology.

## MATERIALS AND METHODS

### *Patients*

We studied 45 children with clinically and ultrasonically suspected cirrhosis of the liver. Only 28 children (23 males and 5 females) aged from 11 months to 12 years (mean 7.9 years) who had a definite biopsy were included in this study. The other 17 children who had no biopsy were excluded.

All the patients were regularly evaluated by clinical assessment, biochemical tests, ultrasound study, and liver biopsy when clinically indicated. Among them, six had chronic hepatitis, and two lived in areas with a high prevalence of *Schistosoma mansoni* while the other 20 had no known etiology. In eight cases, their mothers were positive for HbsAg, among these cases three had hepatitisB. In three cases, their fathers were positive for HbsAg, among these cases one had hepatitisB. In two cases, both parents were positive for HbsAg. Seventeen patients came to hospital because of indigestion or abdominal bloating, four because of diarrhoea, and one each because of dyspneic respiration, bellyache, or jaundice. One patient was identified during routine physical examination.

### *Sonography*

Ultrasonography was performed by conventional techniques with a high-resolution, real-time scanner (Sonoline AC) equipped with 5 MHz and 7 MHz rectilinear array or convex scan probes combined with a puncture guider. Liver size was assessed according to routine methods (transverse and longitudinal sections, relationship between the hepatic border and the right kidney). The contour of the liver (smooth and nodular) was observed. The ultrasound patterns of the liver (nonspecific hepatomegaly, homogeneously increased echogenicity, heterogeneously increased echogenicity, and nodular liver) were observed. The presence of ascites, signs of portal hypertension, and splenomegaly were also recorded.

Ultrasound guided biopsy was performed on all the patients, each biopsy was subsequently examined immunohistochemically.

## RESULTS

The diagnosis of children cirrhosis was confirmed in the 28 cases by histopathology, and liver cirrhosis was classified into six groups: post-hepatitis B, biliary disease, congestive disease, schistosomiasis, glycogen storage disease and hepatolenticular degeneration cirrhosis according to the results from biopsy (Table 1).

Among the patients, most were post hepatitis B, accounting for 67.9 % (Table 1). The ultrasound patterns of post-hepatitis B cirrhosis were similar in appearance to the adult disease, with increased parenchymal echo, coarsened echo texture,

irregularities on the liver surface, abnormalities of the intrahepatic biliary tree and splenomegaly. Because the etiology of liver cirrhosis was multifactorial, each particular patient might have a different pathogenesis and different hepatic ultrasound changes. Sonography revealed intrahepatic cholangiectasis in three children with cholestasic cirrhosis. One case of congestive cirrhosis showed an enlargement of the venae hepaticae and inferior vena cava, and almost no change in the inner diameter of the vessels during respiratory movement. In two cases of schistosomiasis cirrhosis, who lived in an epidemic region for *Schistosoma mansoni*, a grid pattern was observed. By sonography, two patients with glycogen storage disease and one hepatolenticular degeneration showed relatively characteristic ultrasound patterns. The echo patterns of liver parenchyma of glycogen storage disease were found to have coarsened echo texture, but the surface of their livers appeared smooth. The pattern of hepatolenticular degeneration showed a coarsened parenchymal echo, with a normal distribution, and the route of the intrahepatic biliary duct was not altered.

**Table 1** Diagnosis of children with cirrhosis

| Classification | <2 years | 2-7 years | 8-12 years | Total | % |
|---|---|---|---|---|---|
| Post hepatitis B cirrhosis | 1 | 2 | 16 | 19 | 67.9 |
| Biliary cirrhosis | | 1 | 2 | 3 | 10.7 |
| Congestive cirrhosis | | | 1 | 1 | 3.6 |
| Schistosomiasis cirrhosis | | | 2 | 2 | 7.1 |
| Glycogen storage disease | | 1 | 1 | 2 | 7.1 |
| Hepatolenticular degeneration | | | 1 | 1 | 3.6 |
| Total | 1 | 4 | 23 | 28 | 100 |

## DISCUSSION

The value of ultrasonography in diagnosing cirrhosis of the liver has been recognized clinically[12-14]. By observing the press extents of the surface of the left hepatic septum in front of the inferior vena cava, real time ultrasound could directly determine the degree of cirrhosis. Estimating the condition of esophageal varices by detecting portal hypertension was important in differentiating the source of gastrointestinal hemorrhage[14]. Because the child liver was smaller in size, we obtained high-resolution ultrasound images by using high frequency probes (5 MHz and 7 MHz).

Our results showed that the echo pattern revealed by ultrasound in combination of the patient' s medical history, could be useful in the diagnosis of children liver cirrhosis and prediction of its the etiology. Then, physicians could select appropriate subsequent diagnostic methods to confirm the diagnosis. Since ultrasound does not allow for the specificity of imaging, we suggest that percutaneous liver biopsy guided by ultrasound should be performed when possible, in order to provide the most accurate method for clinical diagnosis. Ultrasonic scans could localize areas for biopsy away from major vessels and the gall bladder.

Most children with chronic hepatitis have no history of acute hepatitis. Chronic hepatitis has few clinical symptoms and most children patients cannot be diagnosed early in the course of their disease. Thus, by the time they do present the symptoms, the disease is well advanced, and their prognosis is poor. Of the 28 children described here, only six had a previous history of hepatitis. More than half of them came to hospital because of indigestion and abdominal bloating. One patient was misdiagnosed as having biliary ascarid, and was not diagnosed as cirrhosis until bleeding of the digestive tract was observed following ineffective antihelminthic treatment. One child with chronic diarrhoea was found to have complications of ascites. Children cirrhosis was thus easily misdiagnosed. For this reason, if a child manifests symptoms associated with cirrhosis, such as hypodynamia, poor appetite, nausea, vomiting, diarrhea, and intense stomach pains, an ultrasound examination and correlated clinical examination should be performed.

Of the 28 children in this study, 19 (67.9 %) had post-hepatitis B cirrhosis. China is a country with a high prevalence of hepatitis B infection. Perinatal infection is recognized as the predominant mode of transmission of the virus, resulting in many carriers of HbsAg. Infection occurred predominantly at or after birth by maternal-infant transmission, and might even occur as early as the oosperm stage[15]. Embryos infected in the uterus have been reported to develop cirrhosis. Thus the children' s parents who are positive for HbsAg or have a history of hepatitis B infection should be examined by ultrasound and correlated clinical tests at regular intervals. This may lead to an early detection of children liver cirrhosis.

In conclusion, ultrasonography can accurately reveal morphological characteristics and predict the etiologic factors underlying children liver cirrhosis. This technique can be used to screen high-risk groups and allows for early treatment.

## REFERENCES


1 **Imperial JC**. Natural history of chronic hepatitis B and C. *J Gastroenterol Hepatol* 1999; **14**(Suppl): 1-5
2 **Noble JA**, Caces MF, Steffens RA, Stinson FS. Cirrhosis hospitalization and mortality trends, 1970-87. *Public Health Rep* 1993; **108**: 192-197
3 **Jorke D**, Reinhardt M. Contributions to the epidemiology of liver cirrhosis and chronic hepatitis. *Dtsch Z Verdau Stoffwechselkr* 1982; **42**: 129-137
4 **Haraszti A**, Vadnay I, Toth K, Baranyai T. Importance of environmental factors for the development of liver cirrhosis. *Zentralbl Allg Pathol* 1983; **128**: 411-423
5 **Li H**, Li RC, Liao SS, Gong J, Zeng XJ, Li YP. Long-term effectiveness of infant low-dose hepatitis B vaccine immunization in Zhuang Minority Area in China. *World J Gastroenterol* 1999; **5**: 122-124
6 **Wu J**, Cheng ML, Zhang GH, Zhai RW, Huang NH, Li CX, Luo TY, Lu S, Yu ZQ, Yao YM, Zhang YY, Ren LZ, Ye L, Li L, Zhang HN. Epidemiological and histopathological study of relevance of Guizhou Maotai liquor and liver diseases. *World J Gastroenterol* 2002; **8**: 571-574
7 **Roussos A**, Goritsas C, Pappas T, Spanaki M, Papadaki P, Ferti A. Prevalence of hepatitis B and C markers among refugees in Athens. *World J Gastroenterol* 2003; **9**: 993-995
8 **Harbin WP**, Robert NJ, Ferrucci JT Jr. Diagnosis of cirrhosis based on regional changes in hepatic morphology: a radiological and pathological analysis. *Radiology* 1980; **135**: 273-283
9 **Okudaira M**, Atari E, Oubu M. Liver cirrhosis, its definition and classification-from a morbid anatomical point of view. *Nippon Rinsho* 1994; **52**: 5-10
10 **Kamegaya K.** Definition and classification of liver cirrhosis. *Nippon Rinsho* 1994; **52**: 11-18
11 **Peter L**, Dadhich SK, Yachha SK. Clinical and laboratory differentiation of cirrhosis and extrahepatic portal venous obstruction in children. *J Gastroenterol Hepatol* 2003; **18**: 185-189
12 **Ohta M**, Hashizume M, Kawanaka H, Akazawa K, Tomikawa M, Higashi H, Kishihara F, Tanoue K, Sugimachi K. Prognostic significance of hepatic vein waveform by Doppler ultrasonography in cirrhotic patients with portal hypertension. *Am J Gastroenterol* 1995; **90**: 1853-1857
13 **Chou YH**, Chiou HJ, Tiu CM, Chiou SY, Lee SD, Hung GS, Wu SC, Kuo BI, Lee RC, Chiang JH, Chang T, Yu C. Duplex Doppler ultrasound of hepatic Schistosomiasis japonica: a study of 47 patients. *Am J Trop Med Hyg* 2003; **68**: 18-23
14 **Gorg C**, Riera-Knorrenschild J, Dietrich J. Pictorial review: Colour Doppler ultrasound flow patterns in the portal venous system. *Br J Radiol* 2002; **75**: 919-929
15 **Gu CH**. New development of Lemology(No 2). 1st ed. *Chongqing*: *Science Technol Press* 1990: 105


**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2350-2352
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Experimental study of diode-laser induced thermocoagulation on hepatic tissue with scanner fiber tip

De-Fei Hong, Shu-You Peng, Song-Ying Li, Li-Min Tong

**De-Fei Hong, Shu-You Peng,** Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
**Song-Ying Li,** Department of Pathology, Medical College of Zhejiang University, Hangzhou 310006, Zhejiang Province, China
**Li-Min Tong,** Department of Physics, State Key Laboratory of Silicon Marterial, Zhejiang University, Hangzhou 310027, Zhejiang Province, China
**Supported by** the Youth Science Foundation of National 863 Laser Technique
**Correspondence to:** De-Fei Hong, Department of General Surgery, Sir Run Run Shaw Hospital, Zhejiang University, Hangzhou 310016, Zhejiang Province, China. hongdefi@mail.hz.zj.cn
**Telephone:** 13605700656
**Received:** 2002-12-22 **Accepted:** 2003-02-13

## Abstract

**AIM:** To explore a safe, efficient, and cost-effective technique for local thermo-ablation of hepatic tumors.

**METHODS:** The livers of 16 healthy rabbits were thermocoagulated by diode-laser with a hand-made scanner fiber tip, 6 w for 10 min. At the same time, the temperature was measured at 5 and 10 mm from the laser tip. Liver function 7 days post-thermocoagulation was compared to pre-thermocoagulation. Pathological changes were also studied 1 month after laser thermocoagulation.

**RESULTS:** All the rabbits lived and the temperature of hepatic tissues at 0 mm, 5 mm, 10 mm from laser tip reached 96.39±3.97 ℃, 60.79±6.21 ℃, 46.10±4.58 ℃, respectively after 10 min thermocoagulation. There was no significant change in liver function. The hepatic thermocoagulated necrosis and the surrounding fibrosis was 26.0 mm in diameter. Light microscopy observation revealed no surviving cells in the coagulated area.

**CONCLUSION:** Hepatic tissue can be locally ablated safely and effectively by diode-laser with scanner fiber tip, and this technique may be a new method to treat hepatic tumors.

Hong DF, Peng SY, Li SY, Tong LM. Experimental study of diode-laser induced thermocoagulation on hepatic tissue with scanner fiber tip. *World J Gastroenterol* 2003; 9(10): 2350-2352
http://www.wjgnet.com/1007-9327/9/2350.asp

## INTRODUCTION

In recent years, thermo-ablation of liver cancer has received increasing attention. Generally this can be achieved by radio-frequency (RF) or Nd: YAG laser. Several factors have prevented these techniques from gaining widespread use including availability and cost, in addition to some uncertainty about their therapeutic effectiveness. With these concerns in mind, it is appropriate to consider a new technique for thermo-ablation that we believe is safe, stable, convenient and cost effective. Semiconductor taper-scattered light for thermo-ablation of liver tissue in an experimental model is advanced as such a technique with details presented as follows.

## MATERIALS AND METHODS

### *Material and apparatus*

Hand-made semiconductor taper-scattered laser therapeutic system (HTLTS) has a wavelength of 810±10 nm. The working tip is 10 mm long and 1 mm in diameter. Power utilization was 6 w and after dispersion output power was at 2.1 w with an exposure time of 10 min. Sixteen adult rabbits were obtained from the Animal Laboratory of Zhejiang University, weighing 2 to 3 kg.

### *Methods*

Intravenous anesthesia was administered via ear vein using 2 % sodium pentothal. The abdomen was entered with a midline incision and right or left lobes of the liver were exposed. A 18G PTC needle was introduced into the liver and through it, the laser tip was advanced into the liver tissue before turning on the power for the laser. Care was taken to advance the laser tip fully into the liver tissue. Power input was 6 w and 2.1w after dispersion.

For temperature measurement during laser application, a nickel-chrome-nickel thermocouple 300 μm in diameter was placed intrahepatically at 5 and 10 mm from the laser tip. Temperature was continuously measured during application. After thermocoagulation the abdomen was closed with silk suture. Liver function was measured before surgery and 7 days afterwards. One month after irradiation the rabbits were sacrificed and the liver specimen was fixed in formaldehyde. Multiple sections were taken from the ablative and surrounding area in the verticle plane of the optical fiber. After H&E staining the sections were examined grossly and microscopically with particular attention to the diameter of the coagulated area. Liver function data were analyzed using the *t*- test with the "SAS" software.

## RESULTS

The temperature variation is shown in Table 1. Note that two of the rabbits were irradiated for only six minutes because of carbonization of tissue at the center of the optical fiber.

**Table 1** The temperature variation

| Time (Seconds) | Temperature (℃) | | |
|---|---|---|---|
| | 0 mm | 5 mm | 10 mm |
| 0 | 34.00±0.00 | 34.00±0.00 | 34.00±0.00 |
| 120 | | 55.70±4.81 | 40.50±1.47 |
| 240 | | 58.75±5.65 | 43.30±2.54 |
| 360 | | 60.26±5.52 | 45.00±2.67 |
| 480 | | 59.89±6.65 | 46.10±3.87 |
| 600 | 96.39±3.97 | 60.79±6.21 | 46.10±4.58 |

Liver function changes are shown in Table 2.

**Table 2** Liver function changes

| | Pre-coagulation | 7 days after coagulation | *P* |
|---|---|---|---|
| ALT(IU / L) | 67.78±24.98 | 77.11±49.79 | >0.05 |
| AST(IU / L) | 87.22±58.82 | 66.55±44.75 | >0.05 |
| TBIL(mg / dl) | 1.84±1.79 | 1.21±0.51 | >0.05 |
| ALB(g / L) | 17.90±3.98 | 14.77±2.79 | >0.05 |
| TP (g / L) | 66.54±13.95 | 59.40±9.55 | >0.05 |

All rabbits were alive after the procedure. When the animals were sacrificed after one month the livers were cut in the vertical plane of the optical fiber and the gray coagulated necrotic tissue was measured. Gross evaluation demonstrated a diameter of 23 mm with a perimeter zone of 3 mm (Figure 1). Under light microscopy the central zone of coagulation necrosis was followed by a layer showing inflammatory cell infiltration and finally the layer of fibrosis, the last two were 0.5 mm and 2.4 mm, respectively.

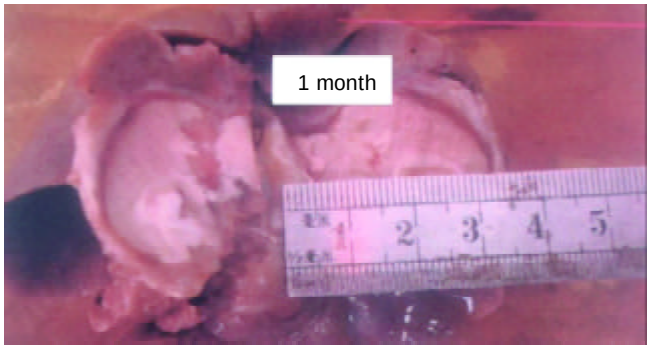



**Figure 1** The necrotic center with surrounding fibrosis (HE).

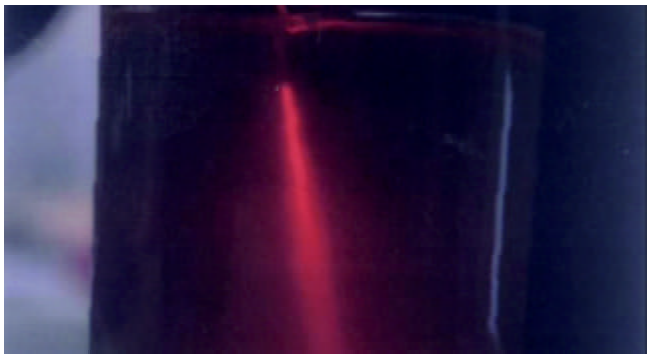


**Figure 2** High density linear laser energy from fiber.

**Figure 3** Low density up-tip taper scattered laser energy after conversion.

## DISCUSSION

Currently the optimal treatment for liver cancer is surgical excision with curative intent. However, because of the frequency of coexistent cirrhosis and the difficulty of early detection, only 25 to 30 percent of newly diagnosed patients with primary or metastatic liver cancer undergo potential curative resection and the recurrence rate is at least 50 %[1-2]. This means many patients are not even candidates for a surgical procedure and have little chance even for palliation, which has led to increasing interest in alternatives such as thermo-ablative procedures including radio-frequency, Nd:YAG laser and microwave. However, limited benefit, high cost and difficulties in technical application have limited the usefulness of these modalities as well[3-6].

The basis for laser thermo-ablation is that light energy can be absorbed by tissues after its conversion into heat, resulting in deep penetration with therapeutic destruction of tumor tissues. The temperature and therefore the extent of destruction are controlled by the intensity and duration of the energy transmitted. This has already been shown effective using Nd: YAG laser as the energy source. However high cost and difficulties in use have restricted its application in this setting[6-9]. More recently, semi-conductor focused laser energy has come to the forefront due to decreased cost, stable-performance, durability and ease of use. In addition it does not require water cooling equipment[7,8]. In this report, we have examined a similar process using semiconductor taper-scattered light. We have shown 1064 nm Nd:YAG and 800-900 nm semiconductor laser light both exhibit deep penetration in tissue. Dong[10] has shown normal liver cells necrotize in one minute with a temperature of 54 ℃ and immediately with a temperature of 60 ℃. In addition he has shown liver carcinoma cells are more sensitive than normal cells if the temperature is less than 130 ℃. We also know penetration of laser energy is significantly reduced by vaporization and carbonization of tissues. Obviously, to enlarge the treatment area and avoid injury to adjacent areas it is necessary to elevate the temperature of the treatment area but not so high as to cause vaporization and carbonization. It is our opinion that semiconductor taper-scattered laser light may be superior to other systems in this regard. HTLTS can convert high density linear laser energy into up-tip taper scattered laser light which is lower in energy and density as shown in Figure 2 and Figure 3. In effect it can increase the power of laser light equipment, enlarge the treatment area, shorten the duration of application and avoid injury to important areas adjacent to the treatment area such as major hepatic vessels. Specifically, the power is only 6 watts and after scatter only 2.1 W. Ten minutes after thermocoagulation the temperature at the center of the optical fiber is 96.39±3.97 ℃ and 10 mm from the center the temperature is 46.1±4.58 ℃. One month after the procedure the diameter of necrosis was 26 mm and there was no viable tissue in this area. HTLTS is more efficient than Nd:YAG or semiconductor focused laser light. In the study by Ping Liang[5] Nd:YAG provided only 15 mm necrosis using 2 to 6 W energy after 15 to 40 min application. Fan[7] demonstrated only 7-13 mm necrosis with semiconductor focused laser energy from a British laser using 2 W and 2800-8400 J respectively. Injury beyond the intended area is less likely since it is not necessary to place the tip of the instrument exactly in the center of the lesion and this makes the procedure also applicable for more superficial lesions. The optical fiber can be separated from the tissue with the catheter thus avoiding contamination from the blood and the length of the fiber tip can be altered to fit the diameter of the tumor. Furthermore, the optical fiber can be sterilized and reused, the fiber is flexible, allowing access to areas one side or the other of the primary objective. Overall,

HTLTS is more convenient, economical and effective than the currently available ablative procedures.

In regards to the effect of HTLTS on hepatic carcinoma or metastatic lesions, we have shown greater penetration than in normal tissue. For example, the penetration of YAG (wavelength 670 nm) in normal liver tissue, liver sclerosis, HCC, and metastatic lesions is 0.75 mm, 0.97 mm, 1.43 mm and 2.78 mm, respectively[11]. HCC is also more sensitive to thermal ablation than normal tissue. Again HTLTS is more effective and efficient than the other methods. HTLTS can also be applied to the clinical area easily. The fiber is passed into the intended tissue through a 18G paracentesis needle guided by B-ultrasound. The length of the fiber tip can be adjusted according to the size of the target tissue. Special techniques are also available to utilize more than one fiber at a time to improve the resulting effect according to the exact size and location of the target tissue.

In conclusion, we believe that the foregoing report indicates HTLTS may be the best technique yet devised for thermo-ablation of hepatic lesions.

## REFERENCES


1 **Cance WG,** Stwart AK, Menck HR. The national cancer data base report on treatment patterns for hepatocellular carcinoma: improved of surgically resected patiens, 1985-1986. *Cancer* 2000; **88**: 912-920

2 **Wu MC,** Chen H, Sheng F. Surgical treatment of primary liver cancer: report of 5524 cases. *Zhonghua Waike Zazhi* 2001; **39**: 25-28

3 **Curley SA,** Lzzo F, Delrio P, Ellis LM, Granchi J, Vall Fiore F, Pignata S, Daniele B, Cremona F. Radiofrequency ablation of unresectable primary and metastatic hepatic malignancies: results in 123 patients. *Ann Surg* 1999; **230**: 1-8

4 **Ohmoto K,** Miyake I, Tsuduki M, Shibata N, Takesue M, Kunieda T, Ohno S, Kuboki M, Yamamoto S. Percutaneous Microwave coagulation therapy for unresectable hepatocellular carcinoma. *Hepatogastroenterology* 1999; **46**: 2894-2900

5 **Liang P,** Dong BW, Gu Y, Li JH, Yu XN, Su L, Zhang Y. Effect of Nd:YAG laser coagulation on hepatic tissue and its application to hepatic cancer. *Zhonghua Liliao Zazhi* 1999; **22**: 158-160

6 **Heisterkamp J,** Van Hillegersberg R, Ijzermans JN. Interstitial laser coagulation for hepatic tumor. *Br J Surg* 1999; **86**: 293-304

7 **Fan XH,** Shen TZ, Lu W, Gong DX. Laser ablation of liver malignant tumor. *Zhongguo Yixue Jishuanji Chengxiang Zazhi* 2000; **6**: 404-407

8 **Devaux BC,** Roux FX, Nataf F, Turak B. Cioloca C. High-power diode laser in neurosurgery: clinical experience in 30 cases. *Surg Neurol* 1998; **50**: 33-40

9 **Zhang ZX,** Jiang DZ. Laser-Tissue Interactions: Foundaments and Application. 1st ed. *Xi'an*: *Xian Jiaotong University Publishing House* 1999: 64-65

10 **Dong BW,** Liang P, Yu XL, Zeng XQ, Wang PJ, Su L, Wang H, Li S. Sonographically guided microwave coagulation treatment of liver cancer: an experimental and clinical study. *Am J Roentgenol* 1998; **171**: 449-454

11 **Li DJ,** Hu ZS. Tumor thermotherapy. 1st ed. *Henan: Henan Medical University Publishing House* 1995: 186-187


**Edited by** Pang LH

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2353-2355
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Is a low dose of hepatitis B vaccine enough for a rapid vaccination scheme?

Ru-Xiang Wang, Greet Boland, Ying Guo, Shao-Ping Lei, Chang-Hong Yang, Juan Chen, Jie Tian, Jin-Ying Wen, Ke-Hong Du, Jan van Hattum, Gijsbert C. de Gast

**Ru-Xiang Wang, Ying Guo, Juan Chen,** Shenyang Center for Disease Control and Prevention, Shenyang 110031, Liaoning Province, China
**Greet Boland, Jan van Hattum,** Department of Gastroenterology, University Hospital Utrecht, Netherlands
**Shao-Ping Lei,** Hospital of Institute of Shenyang Electricity Power, Shenyang 110031, Liaoning Province, China
**Chang-Hong Yang,** Dongling District Anti-epidemic Station, Dongling District, Shenyang 110031, Liaoning Province, China
**Jie Tian,** Shenyang No.4 Hospital, Shenyang 110031, Liaoning Province, China
**Jin-Ying Wen,** Shenyang 606 Hospital, Shenyang 110031, Liaoning Province, China
**Ke-Hong Du,** Shenzhen Kangtai Biological Products Company, Shenzhen 518000, Guangdong Province, China
**Gijsbert C. de Gast,** Netherlands Cancer Institute, Amsterdam, Netherlands
**Correspondence to:** Dr. Ru-Xiang Wang, Shenyang Center for Disease Control and Prevention, 37 Qishanzhong Road, Huanggu District, Shenyang 110031, China. rxwtxh@pub.sy.ln.cn
**Telephone:** +86-24-86853243 **Fax:** +86-24-86863778
**Received:** 2003-04-12 **Accepted:** 2003-06-11

## Abstract

**AIM:** To determine whether or not a low dose of HB vaccine can be effectively used in the rapid vaccination.

**METHODS:** Rapid vaccination (0, 1, 2 months) with low dose (5 μg) or routine dose (10 μg) HB vaccine was studied in 250 subjects (130 school children and 120 university students). Serum from all the participants was tested for HBsAg, anti-HBs and anti-HBc at 1, 3 and 7 months after the first dose of vaccination and all subjects were serum HBV marks negative before the vaccination. Non-responders to a complete initial vaccination from university students were given an additional vaccination with 10 μg of HB vaccine and their serum anti-HBs was tested again one month later.

**RESULTS:** One month after the third dose of vaccination (third month) sero-conversion rates and geometric mean titer (GMTs) were significantly ($P<0.01$) higher in the routine dose (resp. 89 % and 106.8) than in the low dose group (resp. 72 % and 59.5). Sero-conversion rates and GMTs were maintained stable for another 4 months in both groups. After an additional vaccination to non-responders with 10 μg HB vaccine, 17/23 subjects (13/15 from those vaccinated with 5 μg vaccine and 4/8 from those vaccinated with 10 μg vaccine) became anti-HBs positive, yielding similar sero-conversion rates for both dose groups.

**CONCLUSION:** Higher sero-conversion rates and GMTs were reached in those vaccinated with 10 μg HB vaccine than in those vaccinated with 5 μg HB vaccine after a complete vaccination with a 0, 1, 2 month scheme. But the subjects vaccinated with 5 μg vaccine can also reach the similar sero-conversion rate after an additional vaccination.

Wang RX, Boland G, Guo Y, Lei SP, Yang CH, Chen J, Tian J, Wen JY, Du KH, van Hattum J, de Gast GC. Is a low dose of hepatitis B vaccine enough for a rapid vaccination scheme? *World J Gastroenterol* 2003; 9(10): 2353-2355
http://www.wjgnet.com/1007-9327/9/2353.asp

## INTRODUCTION

Although hepatitis B vaccine has been available for 20 years, hepatitis B nowadays remains prevalent in the world, especially in a majority of the developing countries. In China, 5 μg of vaccine (Merck) has been used in the HB vaccination for several years and acquired acceptable results. The long-term effectiveness of low-dose HB vaccine immunization in the infancy has been observed, but that in school children or adults remains to be determined. It is known that protective anti- HBs antibody titers can generally be reached in 80-90 % of individuals vaccinated with 10 μg vaccine according to a 0,1, 6 month vaccination scheme. Considering of the relative length of the current HB vaccination scheme and the availability of low dose HB vaccine in the market, it seemed, therefore, worthwhile to test a routine dose (10 μg) vaccination in a rapid scheme (0, 1, 2 months) compared to a low dose (5 μg) vaccination. We here report that significantly higher sero-conversion rates and GMTs are reached in those vaccinated with a 10 μg dose vaccination than those vaccinated with 5 μg dose vaccination after 3 months from the initial dose. School children and university students showed no significant differences apart from a more rapid response in school children.

## MATERIALS AND METHODS

Two hundred and fifty subjects (130 school children aged 8-10 years old and 120 university students aged 18-20 years old) with negative HB marks (HBsAg, anti-HBs, anti- HBc) were included in the study. One hundred and thirty subjects (72 school children and 58 university students) nominated as low dose group and were vaccinated with 5 μg HB vaccine, 120 subjects (55 school children and 65 university students) named as routine dose group received 10 μg HB vaccine (5 μg dose x 2) respectively according to a 0, 1, 2 month vaccination scheme. All non-responding university students after a complete initial vaccination series were given a fourth dose with 10 μg HB vaccine at the 8$^{th}$ month. All injections were given intramuscularly at the site of deltoid muscle. The HB vaccine used in this study is 5 μg yeast recombinant HB vaccine (Lot no: 2990104-1, from Merck), provided by Kangtai Biological Pharmaceutical Company, China.

Blood samples that were collected from all vaccinees for three times performed were used to detect serum anti-HBs (Ausab EIA, No 642841 M401) at month 1, 3 and 7 after the first dose of vaccination respectively. The third month serum specimen from all non responders after the initial vaccination was tested for HBsAg and anti-HBc by ELISA (Lizon Kit, China) in order to make sure that they are real no-responders to HB vaccine and not HBV carriers. Those with negative tests

received a fourth dose of vaccine at 8th month after vaccination and were again tested for serum anti- HBs one month later.

## RESULTS

128/130 subjects in low dose group were tested for serum anti-HBs levels one month after the first dose of vaccination and only 114 subjects were available for the test one month after the third vaccination. The anti-HBs sero-conversion rate and GMT in this group was 71.9 % and 9.5 respectively (Table 1). 116/120 subjects in the routine group were tested for anti-HBs levels one month after the first dose of vaccination and only 110 subjects were available for the test one month after the third dose of vaccination. A significantly higher anti-HBs sero-conversion rate (89.1 %, $P<0.01$) and GMT (106.8, $P<0.01$) in routine dose group were observed after the third dose of vaccination compared with that in low dose group. At the seventh month the sero-conversion rate and GMT persisted at similar level to that after the second dose of vaccination.

**Table 1** Serum conversion rate and GMT induced by low and routine dose of vaccines

| Dose of vaccine | Time after first injection | *n* | Sero- conversion rate (%) | GMT |
|---|---|---|---|---|
| 5 μg | 1st time | 128 | 12.5 | |
| | 2nd time | 114 | 71.9 | 59.5 |
| | 3rd time | 113 | 69.9 | 58.5 |
| 10 μg | 1st time | 116 | 19 .0 | |
| | 2nd time | 110 | 89.1[b] | 106.8 |
| | 3rd time | 98 | 91.8[b] | 94.6 |

[b]$P<0.01$ *vs.* 5 μg group.

Twenty three non-responding university students (15 vaccinated with 5 μg vaccine and 8 with 10 μg vaccine) were given a fourth dose of 10 μg HB vaccine after completion of the initial vaccination. Of these individuals, 13/15 non-responders in the low dose group and 4/8 non-responders in the routine dose group responded with anti-HBs one month after the additional dose. As a result of this additional vaccination, serum conversion rate in the low dose group was nearly similar to that in the routine dose group (95.2 % *vs* 92.9 %, Figure 1).

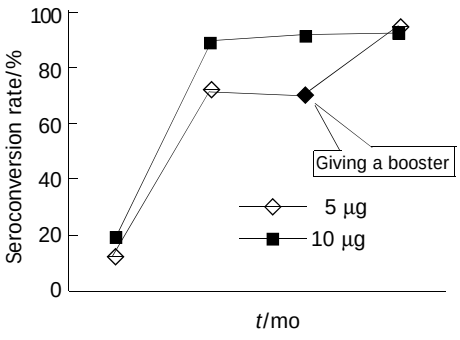



**Figure 1** Seroconversion rate before and after a booster.

## DISCUSSION

Since the introduction of hepatitis B vaccination in the early 1980s, the safety and immunogenicity of the plasma-derived and yeast-derived hepatitis B vaccines have been well demonstrated[1-12]. However, the disease remains a global problem. The failure of obtaining a complete 3 doses HB vaccination due to the length of the scheme probably contributes to the current prevalence of hepatitis B in the developing countries. Clearly, the protection against hepatitis B could be improved if a shorter vaccination regimen of achieving protective anti-HBs levels in >90 % of vaccinees was available. Our results clearly showed that with a rapid vaccination scheme (0, 1, 2 months) using 10 μg HB vaccine, serum anti-HBs levels can be induced, while it can not be reached by using 5 μg of HB vaccine. The similar results were also reported previously by Carlsson *et al*[13] who vaccinated some medical staff and compared the effects of intramuscular (I m, 20 μg.) vaccination with the low-dose intradermal (i d, 2 μg) vaccination on their early sero-conversion rates according to a 0, 4, 8 week scheme or to an accelerated 0, 2, 6 week scheme. He concluded that when a rapid protection against hepatitis B virus (HBV) infection was needed, such as the post-exposure prophylaxis, low-dose i.d. vaccination schedule could not be used.

A study on evaluating the potential for developing rapid seroprotection was performed by Young *et al*[14], beneficial results of a one month/two dose regimen with a novel triple antigen vaccine (Hepacare) have been achieved[14]. These results confirmed that an accelerated vaccination could be achieved with appropriate vaccinating protocols.

Low-dose vaccination has been proposed as a cost-saving strategy to implement mass vaccination of neonates with HB vaccine world-wide, particularly in developing countries. In a previous study, Shokri F compared the effectiveness of three doses of recombinant HB vaccine (Heberbiovac) in three different doses, among three groups of healthy Iranian neonates. His results showed that there were no significant differences in sero-protection rate and GMTs between the 10 and 5 μg dose recipients. Both parameters, however, were significantly lower in neonates vaccinated with a 2.5 μg vaccine dose[12]. Leroux-Roels G observed the immunogenicity and reactogenicity profiles of different doses of Engerix-B(R) (10 microg hepatitis B surface antigen) and Recombivax (R) (5 microg hepatitis B surface antigen), on a 0, 1, 6 month schedule in healthy adolescents. One month following the third dose of vaccination, seroprotection rates of Engerix-B and Recombivax vaccination were similar but the geometric mean titer was significantly higher in those vaccinated with Engerix-B than that with Recombivax[15]. Similar results were also reported that higher immunogenicity is usually related with higher vaccine dosages, especially in older adult population[13-18]. Our results showed that a higher dose (10 μg) of vaccine can induce much higher sero- conversion rate and higher GMTs compared with the reduced dose (5 μg) vaccination whereas the GMTs can be sustained for at least 5 months in both groups after the primary vaccination.

In most studies on immunocompetent subjects, 5 % to 10 % vaccinees failed to respond to HB vaccination. The possible reasons for lack of adequate antibody response have been well reported[16-22]. In order to confirm if the reduced dose of HB vaccination could cause a higher rate of non- or hypo-response to HB vaccine, we gave all non-responders an additional dose after the initial HB vaccination scheme and one month later, the majority of them converted into anti-HBs positive. The final sero-conversion rates of non-responders in low dose group and routine dose group were 95.2 % and 92.9 % respectively, showing that it is rather a hyporesponse to a lower dose of HB vaccine than a non-response as found earlier [23-31]. Based on the results, we conclude that it is better for adults to choose 10 μg HB vaccine, especially when a rapid vaccination program is needed.

## REFERENCES

1 **Kojouharova M**, Teoharov P, Bahtchevanova T, Maeva I, Eginlian

A, Deneva M. Safety and immunogenicity of a yeast-derived recombinant hepatitis B vaccine in Bulgarian newborns. *Infection* 2001; **29**: 342-344

2 **Liao SS**, Li RC, Li H, Yang JY, Zeng XJ, Gong J, Wang SS, Li YP, Zhang KL. Long-term efficacy of plasma-derived hepatitis B vaccine among Chinese children: a 12-year follow-up study. *World J Gastroenterol* 1999; **5**: 165-166

3 **Li H**, Li RC, Liao SS, Yang JY, Zeng XJ, Wang SS. Persistence of hepatitis B vaccine immune protection and response to hepatitis B booster immunization. *World J Gastroenterol* 1998; **4**: 493-496

4 **Rendi-Wagner P**, Kundi M, Stemberger H, Wiedermann G, Holzmann H, Hofer M, Wiesinger K, Kollaritsch H. Antibody-response to three recombinant hepatitis B vaccines: comparative evaluation of multicenter travel-clinic based experience. *Vaccine* 2001; **19**: 2055-2060

5 **Ozaki T**, Mochizuki H, Ichikawa Y, Fukuzawa Y, Yoshida S, Morimoto M. Persistence of hepatitis B surface antibody levels after vaccination with a recombinant hepatitis B vaccine: a 3-year follow-up study. *J Oral Sci* 2000; **42**: 147-150

6 **Jain A**, Mathur US, Jandwani P, Gupta RK, Kumar V, Kar P. A multicentric evaluation of recombinant DNA hepatitis B vaccine of Cuban origin. *Trop Gastroenterol* 2000; **21**: 14-17

7 **Al-Faleh FZ**, Al-Jeffri M, Ramia S, Al-Rashed R, Arif M, Rezeig M, Al-Toraif I, Bakhsh M, Mishkkhas A, Makki O, Al-Freihi H, Mirdad S, AlJuma A, Yasin T, Al-Swailem A, Ayoola A. Seroepidemiology of hepatitis B virus infection in Saudi children 8 years after a mass hepatitis B vaccination programme. *J Infect* 1999; **38**: 167-170

8 **Li H**, Li RC, Liao SS, Gong J, Zeng XJ, Li YP. Long-term effectiveness of infant low dose hepatitis B vaccine immunization in Zhuang Minority Area in China. *World J Gastroenterol* 1999; **5:** 122-124

9 **Liu HB**, Meng ZD, Ma JC, Han CQ, Zhang YL, Xing ZC, Zhang YW, Liu YZ, Cao HL. A 12 year cohort study on the efficacy of plasma-derived hepatitis B vaccine in rural newborns. *World J Gastroenterol* 2000; **6**: 381-383

10 **Li H**, Wang L, Wang SS, Gong J, Zeng XJ, Li RC, Nong Y, Huang YK, Chen XR, Huang ZN. Research on optimal immunization strategies for hepatitis B in different endemic areas in China. *World J Gastroenterol* 2000; **6**: 392-394

11 **Zeng XJ**, Yang GH, Liao SS, Chen AP, Tan J, Huang ZJ, Li H. Survey of coverage, strategy and cost of hepatitis B vaccination in rural and urban areas of China. *World J Gastroenterol* 1999; **5**: 320-323

12 **Shokri F**, Jafarzadeh A. High seroprotection rate induced by low doses of a recombinant hepatitis B vaccine in healthy Iranian neonates. *Vaccine* 2001; **19**: 4544-4548

13 **Carlsson T**, Struve J, Sonnerborg A, Weiland O. The anti-HBs response after 2 different accelerated intradermal and intramuscular schemes for hepatitis B vaccination. *Scand J Infect Dis* 1999; **31**: 93-95

14 **Young MD**, Rosenthal MH, Dickson B, Du W, Maddrey WC. A multi-center controlled study of rapid hepatitis B vaccination using a novel triple antigen recombinant vaccine. *Vaccine* 2001; **19**: 3437-3443

15 **Leroux-Roels G**, Abraham B, Fourneau M, De Clercq N, Safary A. A comparison of two commercial recombinant vaccines for hepatitis B in adolescents. *Vaccine* 2000; **19**: 937-942

16 **Treadwell TL**, Keeffe EB, Lake J, Read A, Friedman LS, Goldman IS, Howell CD, DeMedina M, Schiff ER, Jensen DM. Immunogenicity of two recombinant hepatitis B vaccines in older individuals. *Am J Med* 1993; **95**: 584-588

17 **de Rave S**, Heijtink RA, Bakker-Bendik M, Boot J, Schalm SW. Immunogenicity of standard and low dose vaccination using yeast-derived recombinant hepatitis B surface antigen in elderly volunteers. *Vaccine* 1994; **12**: 532-534

18 **Bennett RG**, Powers DC, Remsburg RE, Scheve A, Clements ML. Hepatitis B virus vaccination for older adults. *J Am Geriatr Soc* 1996; **44**: 699-703

19 **Yucesoy B**, Sleijffers A, Kashon M, Garssen J, de Gruijl FR, Boland GJ, van Hattum J, Simeonova PP, Luster MI, van Loveren H. IL-1beta gene polymorphisms influence hepatitis B vaccination. *Vaccine* 2002; **20**: 3193-3196

20 **Jabaaij L**, van Hattum J, Vingerhoets JJ, Oostveen FG, Duivenvoorden HJ, Ballieux RE. Modulation of immune response to rDNA hepatitis B vaccination by psychological stress. *J Psychosom Res* 1996; **41**: 129-137

21 **Sleijffers A**, Yucesoy B, Kashon M, Garssen J, De Gruijl FR, Boland GJ, Van Hattum J, Luster MI, Van Loveren H. Cytokine polymorphisms play a role in susceptibility to ultraviolet B-induced modulation of immune responses after hepatitis B vaccination. *J Immunol* 2003; **170**: 3423-3428

22 **Goldwater PN**. Randomized, comparative trial of 20 micrograms vs 40 micrograms Engerix B vaccine in hepatitis B vaccine non-responders. *Vaccine* 1997; **15**: 353-356

23 **Wismans P**, van Hattum J, Stelling T, Poel J, de Gast GC. Effect of supplementary vaccination in healthy non-responders to Hepatitis B vaccination. *Hepatogastroenterol* 1988; **35**: 78-79

24 **Wismans P**, van Hattum J, Mudde GC, Endeman HJ, Poel J, de Gast GC. Is booster injection with hepatitis B vaccine necessary in healthy responders? *J Hepatol* 1988; **8**: 236-240

25 **Zaruba K**, Grob PJ, Bolla K. Thymopentin as adjuvant therapy to hepatitis B vaccination in formerly non-or hyporesponding hemodialysis patients. *Surv Immunol Res* 1985; **4**: S102-S106

26 **Maruyama N**, Sata M, Ishii K, Atono Y, Ono K, Matuda T, Suzuki H, Muraoka H, Nakano H, Hino K. Hepatitis B vaccination in alcoholics. *Kansenshogaku Zasshi* 1989; **63**: 27-34

27 **Fabrizi F**, Andrulli S, Bacchini G, Corti M, Locatelli F. Intradermal versus intramuscular hepatitis b re-vaccination in non-responsive chronic dialysis patients: a prospective randomized study with cost-effectiveness evaluation. *Nephrol Dial Transplant* 1997; **12**: 1204-1211

28 **Hollinger FB**. Factors Influencing the Immune Response to Hepatitis B Vaccine, booster dose guidelines and vaccine protocol recommendations. *Am J Med* 1989; **87**: 3A-36S

29 **Erensoy S,** Bilgic A, Arda B, Ozer O. Low-dose intramuscular hepatitis B vaccination in medical students: 4-year follow-up. *Infection* 2002; **30**: 303-305

30 **Huang LM**, Chiang BL, Lee CY, Lee PI, Chi WK, Chang MH. Long-term response to hepatitis B vaccination and response to booster in children born to mothers with hepatitis B e antigen. *Hepatology* 1999; **29**: 954-959

31 **Clemens R**, Sanger R, Kruppenbacher J, Hobel W, Stanbury W, Bock HL, Jilg W. Booster immunization of low- and non-responders after a standard three dose hepatitis B vaccine schedule-results of a post-marketing surveillance. *Vaccine* 1997; **15**: 349-352

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2356-2358
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Role of activation-induced cell death in pathogenesis of patients with chronic hepatitis B

Chun-Sheng Hou, Gui-Qiang Wang, Shu-Lan Lu, Bei Yue, Ming-Rong Li, Xiao-Yan Wang, Jian-Wu Yu

**Shu-Lan Lu, Bei Yue, Ming-Rong Li, Xiao-Yan Wang, Jian-Wu Yu,** Department of Infectious Diseases, The 2nd Affiliated Hospital, Harbin Medical University, Harbin 150086, Heilongjiang Province, China
**Chun-Sheng Hou,** Jining Infectious Diseases Hospital, Jining 272031, Shandong Province, China
**Gui-Qiang Wang,** Department of Infectious Diseases, The First Affiliated Hospital, Beijing Medical University, Beijing 100034, China
**Supported by** the National Natural Science Foundation of China, No. 39570655
**Correspondence to:** Dr. Chun-Sheng Hou, Jining Infectious Diseases Hospital, Jining 272031, Shandong Province, China. cshou2000@sina.com
**Telephone:** +86-537-2032536
**Received:** 2003-03-05 **Accepted:** 2003-06-19

## Abstract

**AIM:** To study and compare the difference of activation-induced cell death (AICD) in peripheral blood T-lymphocytes (PBL-Ts) from patients with chronic hepatitis B (CHB) and the normal people *in vitro*, and to explore the role of AICD in chronic hepatitis B virus (HBV) infection and the pathogenesis of CHB.

**METHODS:** Twenty-five patients and fourteen healthy people were selected for isolation of PBL-Ts. During cultivation, anti-CD3 mAb, PMA and ionomycin were used for AICD of PBL-Ts. AICD ratio of PBL-Ts was detected with TdT-mediated dUTP nick end labeling and assessed by flow cytometry.

**RESULTS:** When induced with anti-CD3, PMA and ionomycin *in vitro*, AICD ratio of PBL-Ts from CHB patients was significantly higher than that from healthy control (17.24±1.21 *vs*. 6.63±1.00, $P<0.01$) and that from CHB patients without induction (17.24±1.21 *vs*. 9.88±1.36, $P<0.01$). There was a similar AICD ratio of PBL-Ts between induction group and without induction group, but no difference was found before and after induction in healthy control. The density of INF-γ in culture media of induction groups of CHB was lower than that of other groups ($P<0.01$). There was no difference between these groups in density of IL-10 ($P>0.05$).

**CONCLUSION:** When induced during cultivation *in vitro*, PBL-Ts from CHB have AICD very commonly. This phenomenon has a potentially important relation with pathogenesis of CHB and chronicity of HBV infection.

Hou CS, Wang GQ, Lu SL, Yue B, Li MR, Wang XY, Yu JW. Role of activation-induced cell death in pathogenesis of patients with chronic hepatitis B. *World J Gastroenterol* 2003; 9(10): 2356-2358
http://www.wjgnet.com/1007-9327/9/2356.asp

## INTRODUCTION

Currently, the exact pathogenesis of chronic hepatitis B (CHB) and the reason of chronic hepatitis B virus (HBV) infection are still not completely understood. Activation-induced cell death (AICD) is related with lymphocytes decrease and functional defect. This phenomenon can cause decrease of immune clearance. Alloreactive T cells can effectively be depleted from allogeneic T cells by induction of AICD to prevent graft-versus-host disease[1]. AICD is essential for the function, growth and differentiation of T-lymphocytes[2]. This may be an important reason of persistent infection of HBV. AICD in peripheral blood T-lymphocytes (PBL-Ts) of CHB *in vivo* has been approved by some reports, but does AICD occur more easily in PBL-Ts of CHB than in those of healthy control? In order to explore the role of AICD in chronic HBV infection, we studied and contrasted the difference of AICD in PBL-Ts from patients with CHB and from normal people *in vitro*.

## MATERIALS AND METHODS

### *Patients*

Twenty-five patients (17 men, 8 women, aged 19-49, mean age 35.6 years) with CHB between March 2000 and April 2001 were selected from the Second Affiliated Hospital of Harbin Medical University. The diagnoses of all the patients were in accord with the Fifth National Conference on the Diagnostic Criteria of Virus Hepatitis (Beijing, 1995). And 14 healthy persons were selected as control.

### *PBL-Ts isolation*

10 mL peripheral blood was taken and heparin was added for anticoagulation. After equivalent Ficoll-Paque (from Amersham-Pharmacia, USA) was gently added, peripheral blood monocytes (PBMCs) were isolated by density gradient centrifugation (600 g, 20 min). Then PBL-Ts were purified with negative selection technique using immune-magnetic beads as follow. After PBMC was washed, mouse-anti-human anti-$CD_{14}$, anti-$CD_{16}$, anti-$CD_{19}$ (2 μg· $mL^{-1}$, DAKO Company, Denmark) were added and incubated for 30 min at 0 ℃, centrifuged for removing the uncombined antibody. Then goat-anti-mouse CD3 mAb coating with magnetic beads (1 cell *vs.* 30 beads, Promega Company, USA) was added and incubated for 30 min at 0 ℃. B cells, NK cells and monocytes were all linked with immune-magnetic beads and absorbed by magnetic stock (DAKO Company, Denmark). After the liquor was gently extracted by centrifugation, PBL-Ts were purified. The viability (95 %) of the cells was confirmed by trypan blue staining. When detected by flow cytometry, the purity of PBL-Ts was over 97 %.

### *Cultivation and AICD induction of PBL-Ts in vitro*

After washed 3 times with PBS, $2\times10^6$· $mL^{-1}$ PBL-Ts were added to a 24 well plate (NENC Company, USA) for cultivation. The samples were divided into treatment group and control group. The culture medium was RPMI1640 (GIBCO Company, USA) containing 10 % calf blood serum (BANDIN TECH Company, China), penicillin (100 U· $mL^{-1}$, BANDIN TECH Company, China) and streptomycin (100 U· $mL^{-1}$, BANDIN TECH Company, China). The wells of treatment group were pre-coated with anti-$CD_3$ mAb (5 μg· $mL^{-1}$, DAKO

Company, Denmark). Phorbol 12-myrisate 13-acetate (PMA) (50 ng· $mL^{-1}$, Sigma Company, USA) and ionomycin (50 ng· $mL^{-1}$, Sigma Company, USA) were added to the culture medium[3]. The culture medium of control group did not contain $CD_3$, PMA and ionomycin. The liquid of culture medium was adjusted to 1 mL. After cultured for 14 h (37 ℃, 5 % $CO_2$), PBL-Ts were harvested for AICD detection.

### *Observation by fluorescence microscope*

Some PBL-Ts were put on the carry sheet glass, dried naturally and fixed by 4 % formaldehydum polymerisatum. Then, all the cells were stained with TdT-mediated dUTP nick end labeling (TUNEL, procedure according to clarification of the kit) (Promega Company, USA). The positive cells of TUNEL staining were detected by fluorescence microscope (BG-12, Olympass, Japan).

### *Flow cytometry detection*

$1\times10^6$ PBL-Ts were washed, fixed by1 % formaldehydum polymerisatum, stayed overnight in 70 % ethanol (-20 ℃) and stained with TUNEL for apoptosis detection (procedure according to clarification of the kit). The apoptotic ratio of PBL-Ts was detected by a flow cytometer (Fort, B-D Company, USA).

### *Cytokine detection*

100 μl supernatant of medium was collected respectively from each group after cultured for 14 h and frozen in -20 ℃ refrigerator for detection. The contents of IFN-γ and IL-10 were detected by using an ELISA kit. The parallel sample was set up for each sample. The OD450 value of each sample was measured with an enzyme label meter (550 model, Bio-RAD Program, USA), and then the content of each sample was converted according to the standard curve.

### *Statistical analysis*

The data were presented as $\bar{x}\pm s$. ANOVA was used to compare the means.

## RESULTS

### *Observation by fluorescence microscope*

The apoptotic PBL-Ts presented DNA breakage. The breakage DNA could be linked by fluorescence labeling dUTP when TUNEL staining was adopted. The apoptotic cells took on kelly fluorescence under fluorescence microscope (Figure 1). This was named positive TUNEL staining. The plasm of PBL-Ts with AICD took on red fluorescence and the nuclei took on kelly fluorescence when TUNEL and PI double staining were adopted. But the cells without AICD only took on red fluorescence (Figure 2). The positive cells of TUNEL staining in PBL-Ts of CHB (with and without anti-CD3mAb, PMA and ionomycin) were more excessive than that of healthy control.

### *Results of flow cytometry detection*

After cultivated for 14 h with induction, the PBL-Ts of CHB patients displayed distinct apoptosis. Apoptosis was also found in groups without anti-CD3 and other inductions, but their apoptotic ratio was lower. There was a similar AICD ratio of PBL-Ts between induction group and healthy control without induction. AICD ratio of PBL-Ts from CHB patients (with or without induction) was significantly higher than that from healthy control (Table 1).

### *Results of cytokine detection*

Activated T lymphocytes may produce plentiful endogenous cytokine. $Th_1$ mainly produces IFN-γ, IL-2 and TNF-α. But $Th_2$ mainly produces IL-4, IL-5, IL-6, IL-10, etc. Cytokine IFN-γ, IL-10 were detected in this test. In all groups, the density of INF-γ in culture media of healthy control with induction group was the highest, and the patient in groups with induction was the lowest. But there was no difference among these groups in density of IL-10 (Table 2).

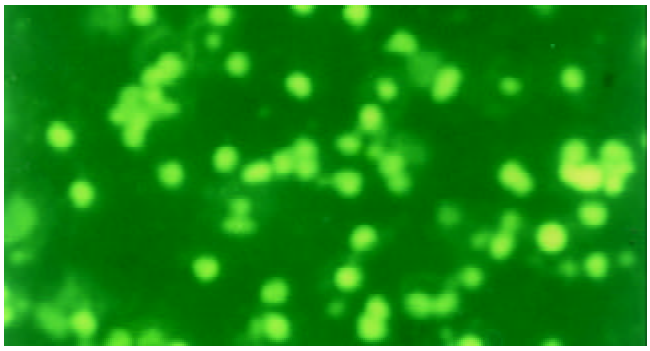


**Figure 1** PBL-T with AICD took on kelly fluorescence and the cell without AICD did not take on any fluorescence (TUNEL staining, 200×, fluorescence microscope).

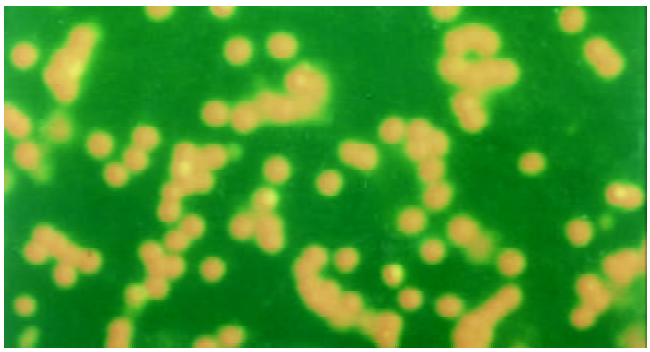


**Figure 2** The plasm of PBL-T with AICD took on red fluorescence and the nucleus took on kelly fluorescence. But the cell without AICD only took on red fluorescence (TUNEL and PI double staining, fluorescence microscope, 200×).

**Table 1** AICD ratio of each group ($\bar{x}\pm s$)

| Group | *n* | AICD Ratio (%) |
|---|---|---|
| Patient with induction | 26 | $17.24\pm1.21^{a,b,c}$ |
| Patient without induction | 26 | $9.88\pm1.36^{b}$ |
| Healthy control with induction | 15 | $6.63\pm1.00^{c}$ |
| Healthy control without induction | 15 | 6.44±1.01 |

$^aP<0.01$ ($F$=164.34) *vs*. other groups; $^bP<0.01$ ($F$=660.45) *vs*. healthy control groups; $^cP<0.01$ ($F$=326.37) *vs*. without induction groups.

**Table 2** The density of IFN-γ and IL-10 from medium of each group ($x\pm s$, pg·$mL^{-1}$)

| Group | *n* | IFN-γ | IL-10 |
|---|---|---|---|
| Patient with induction | 26 | $728.32\pm149.59^{a,b,c}$ | $175.75\pm34.65^{d}$ |
| Patient without induction | 26 | $1\,313.35\pm403.98^{b}$ | 74.48±37.21 |
| Healthy control with induction | 15 | $2\,255.18\pm465.56^{c}$ | 188.86±66.26 |
| Healthy control without induction | 15 | 2 379.22±465.33 | 190.58±46.65 |

$^aP<0.01$ ($F$=7.37) *vs*. other three groups; $^bP<0.01$ ($F$=232.94) *vs*. healthy control groups; $^cP<0.01$ ($F$=25.90) *vs*. groups without induction; $^dP>0.05$ ($F$=0.02) *vs*. other three groups.

## DISCUSSION

Chronic HBV infection is mainly related to the immune function of patients. In a large degree, immune tolerance, especially neonatal immune tolerance, results in persistence of chronic HBV infection. Because naive T cells are sensitive to Fas-mediated AICD and more easily deleted by Ag restimulation than primed T cells[4]. AICD of PBL-Ts plays a key role in central and peripheral immune tolerance[5,6]. AICD is one kind of apoptosis of reactivated lymphocytes when these lymphocytes are induced by activation signals (especially by complex of TCR/$CD_3$). Ashwell and his colleagues first detected the AICD phenomenon in 1987 when they studied T lymphocyte hybrid tumors. AICD plays an important role in the negative selection of T lymphocytes in thymus, peripheral elimination and clearance of T lymphocytes that have already cleaned the foreign antigens. Therefore, AICD is an important mechanism in maintaining immunoregulation and achieving immune system homeostasis[6,7]. If one' s AICD mechanism is disordered (up-regulation or down-regulation), immune tolerance or autoimmune disease would occur.

In this experiment, AICD of PBL-Ts was successfully induced using anti-CD3 mAb, PMA and ionomycin. The responses of PBL-Ts from CHB and healthy control were different. The results indicated that when induced with anti-CD3, PMA and ionomycin *in vitro*, AICD ratio of PBL-Ts from CHB patients was significantly higher than that from healthy control and that from CHB patients without induction. But there was a similar AICD ratio of PBL-Ts between induction group and healthy control without induction. The results imply that AICD exists in PBL-Ts of CHB and causes decrease of T lymphocytes especially $Th_1$ cells and functional defect. Specific immune response aiming directly at HBV should not occur. Finally, immunology tolerance to HBV would occur. Ji *et al* using staphylococcus aureus enterotoxin B and rHBcAg proved that AICD of PBMCs in patients would lead to persistent infection of HBV[8].

Reduction of deferent cytokines in culture medium implies apoptosis of deferent subtype T lymphocytes, because the types of cytokine secreted by Th1 and Th2 are different. The detection results revealed that the density of INF-γ in culture media of induction groups from CHB was lower than that of other groups ($P<0.01$). There was no difference between these groups in density of IL-10 ($P>0.05$). These results imply AICD cells are mainly Th1 cells.

After infection of HBV, the virus elimination depends on specific cell immunity of the body. Mostly, specific cell immunity responses are induced by Th1 lymphocytes, but humoral immunity responses are induced by Th2 type lymphocytes. The sensitivity of the two types of T lymphocytes is not equal. The occurrence of AICD is easily induced by Th1 but not Th2 when induced by Anti-CD3 and corresponding antigen[9-11]. Fan *et al* have proved that enhanced Th2 responses are present in chronic HCV infection, and this should be responsible for the persistent HCV infection[12-14]. So, if specific PBL-Ts of CHB are reactivated by HBV antigens, AICD would occur mostly in Th1 type lymphocytes. Thus, specific cell immunity response aiming directly at HBV would be defective, and HBV permanent infection would occur. However, it would be a possible method to surmount immune tolerance and to clean HBV of CHB patients that we have managed to block the apoptosis of activated T lymphocytes[6,15] and raise the amount of specific T lymphocytes.

## ACKNOWLEDGEMENT

We are grateful to professor Hu-Lun Li (Harbin Medical University), Fang Liu, Wei Liu, Jin-Bai Guo, Shu-Yun Zhang, the staff members of Department of Infectious Diseases (the 2nd Affiliated Hospital, Harbin Medical University), Qin-Huan Wang (the First Affiliated Hospital, Beijing Medical University), Xue-Hai Zhang, Jin-Jian Bi (Jining Infectious Diseases Hospital) for their excellent technical assistant.

## REFERENCES

1 **Hartwig UF**, Robbers M, Wickenhauser C, Huber C. Murine acute graft-versus-host disease can be prevented by depletion of alloreactive T lymphocytes using activation-induced cell death. *Blood* 2002; **99:** 3041-3049
2 **Baumann S**, Krueger A, Kirchhoff S, Krammer PH. Regulation of T cell apoptosis during the immune response. *Curr Mol Med* 2002; **2:** 257-272
3 **Kottilil S**, Bowmer MI, Trahey J, Howley C, Gamberg J, Grant MD. Fas / FasL-independent activation-induced cell death of T lymphocytes from HIV-infected individuals occurs without DNA fragmentation. *Cell Immunol* 2001; **214:** 1-11
4 **Inaba M**, Kurasawa K, Mamura M, Kumano K, Saito Y, Iwamoto I. Primed T cells are more resistant to Fas-mediated activation-induced cell death than naive T cells. *J Immunol* 1999; **163:** 1315-1320
5 **Hamad AR**, Schneck JP. Antigen-induced T cell death is regulated by CD4 expression. *Int Rev Immunol* 2001; **20:** 535-546
6 **Tanimoto Y**, Kizaki H. Proteasome inhibitors block Ras / ERK signaling pathway resulting in the down regulation of Fas ligand expression during activation-induced cell death in T cells. *J Biochem* 2002; **131:** 319-326
7 **Maher S**, Toomey D, Condron C, Bouchier-Hayes D. Activation-induced cell death: the controversial role of Fas and Fas ligand in immune privilege and tumour counterattack. *Immunol Cell Biol* 2002; **80:** 131-137
8 **Ji W**, Wang HF, Feng CQ. Activation-induced cell death in peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis B may be related to abnormal production of interleukin 12 and 10. *J Viral Hepat* 2001; **8:** 30-33
9 **Varadhachary AS**, Peter ME, Perdow SN, Krammer PH, Salgame P. Selective up-regulation of phosphatidylinositol 3'-kinase activity in Th2 cells inhibits caspase-8 cleavage at the death-inducing complex: a mechanism for Th2 resistance from Fas-mediated apoptosis. *J Immunol* 1999; **163:** 4772-4779
10 **Gorak-Stolinska P**, Truman JP, Kemeny DM, Noble A. Activation-induced cell death of human T-cell subsets is mediated by Fas and granzyme B but is independent of TNF-alpha. *J Leukoc Biol* 2001; **70:** 756-766
11 **Roozendaal R**, Vellenga E, de Jong MA, Traanberg KF, Postma DS, de Monchy JG, Kauffman HF. Resistance of activated human Th2 cells to NO-induced apoptosis is mediated by gamma-glutamyltranspeptidase. *Int Immunol* 2001; **13:** 519-528
12 **Fan XG**, Liu WE, Li CZ, Wang ZC, Lou LX, Tan DM, Hu GM. Determination of serum cytokines in individuals with HCV infection. *Zhonghua Shiyan He Linchuangbing Duxue Zazhi* 2000; **14:** 145-147
13 **Fan XG**, Tang FQ, Yi H, Liu WE, Houghton M, Hu GL. Effect of IL-12 on T-cell immune responses in patients with chronic HCV infection. *APMIS* 2000; **108:** 531-538
14 **Fan XG**, Liu WE, Li CZ, Wang ZC, Luo LX, Tan DM, Hu GL, Zhang Z. Circulating Th1 and Th2 cytokines in patients with hepatitis C virus infection. *Mediators Inflamm* 1998; **7:** 295-297
15 **Da Rocha Dias S**, Rudd CE. CTLA-4 blockade of antigen-induced cell death. *Blood* 2001; **97:** 1134-1137

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2359-2361
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Temporary partially-covered metal stent insertion in benign esophageal stricture

Ying-Sheng Cheng, Ming-Hua Li, Wei-Xiong Chen, Ni-Wei Chen, Qi-Xin Zhuang, Ke-Zhong Shang

**Ying-Sheng Cheng, Ming-Hua Li, Qi-Xin Zhuang, Ke-Zhong Shang,** Department of Radiology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Wei-Xiong Chen, Ni-Wei Chen,** Department of Gastroenterology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Supported by** the National Key Medical Research and Development Program of China during the 9th Five-year Plan Period (No.96-907-03-04), Shanghai Nature Science Funds (No.02Z1314073), Shanghai Medical Development Funds (No.00419)
**Correspondence to:** Dr. Ying-Sheng Cheng, Department of Radiology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China. chengys@sh163.net
**Telephone:** +86-21-64368920 **Fax:** +86-21-64701361
**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To study the therapeutic efficacy of temporary partially-covered metal stent insertion on benign esophageal stricture.

**METHODS:** Temporary partially-covered metal stent was inserted in 83 patients with benign esophageal stricture. All the patients had various dysphagia scores.

**RESULTS:** Insertion of 85 temporary partially-covered metal stents was performed successfully in 83 patients with benign esophageal stricture and dysphagia was effectively remitted in all the 83 cases. The dysphagia score was 3.20±0.63 (mean±SD) and 0.68±0.31 before and after stent insertion, and 0.86±0.48 after stent removal. The mean diameter of the strictured esophageal lumen was 3.37±1.23 mm and 25.77±3.89 mm before and after stent insertion, and 16.15±2.96 mm after stent removal. Follow-up time was from 1 week to 96 months (mean 54.26±12.75 months). The complications were chest pain ($n$=37) after stent insertion, and bleeding ($n$=12) and reflux ($n$=13) after stent removal.

**CONCLUSION:** Temporary partially-covered metal stent insertion is one of the best methods for treatment of benign esophageal stricture.

Cheng YS, Li MH, Chen WX, Chen NW, Zhuang QX, Shang KZ. Temporary partially-covered metal stent insertion in benign esophageal stricture. *World J Gastroenterol* 2003; 9(10): 2359-2361

http://www.wjgnet.com/1007-9327/9/2359.asp

## INTRODUCTION

Benign esophageal stricture is a common complication in esophageal diseases. Balloon catheter dilation under X-ray was previously the most common treatment for benign esophageal stricture. Its short-term curative efficacy is good, but it does not last long. The development in stenting techniques has led to the increased application of stents in benign esophageal stricture. However, because of the relatively high incidence of complications and the difficulty of handling those complications, it is preferable to use stents with discretion. Here we report our experiences in using temporary partially-covered metal stent insertion in 83 patients with benign esophageal stricture.

## MATERIALS AND METHODS

### Materials

Eighty-three patients (48 males, 35 females, age 18-82 years) came to our clinic due to dysphagia. A dysphagia score was assessed by the quality of swallowing[1,2]. Grade 0 is for normal swallowing, grade 1 for swallowing most solid food, grade 2 for swallowing semisolids, grade 3 for swallowing liquid food only, and grade 4 for complete dysphagia. The dysphagia score of the 83 patients was 3.20±0.63 (mean±SD), and the causes of stricture were achalasia of cardia ($n$=70), anastomotic stenosis ($n$=5), sclerotic stricture due to ingestion of corrosive agents ($n$=3) and simple sclerosis stricture after radiation therapy for esophageal carcinoma ($n$=5). The diagnoses were made by an upper gastrointestinal contrast examination using barium-meal radiography and gastroscopic assessment. The mean diameter of the strictured esophageal lumen was 3.37±1.23 mm in the 83 patients with partially-covered metal stent insertion.

### Methods

The preparation before stent insertion involved ensuring an empty stomach for at least 4 hours, testing of the bleeding and clotting times, and intramuscular injection of ataractics before interventional procedure. The stent made from nitinol (a nonmagnetic Ni-Ti alloy) by Chinese manufacturers (Zhiye Medical Instruments Corporation, Changzhou, China, and Youyan Yijin Advanced Materials Co.Ltd, Beijing, China) has a length of 4-12 cm and a diameter of 16-30 cm. Its surface is covered with silica gel. The savary conical silica-gel dilator has a diameter of 0.5-2 cm, and contrast medium.

The patients were placed in a sitting position or lying on the side during the stent-placement operation, and false teeth were removed and dental pads were placed. A 260-cm-long exchange guidewire was inserted into the stomach. Along with the guidewire, the nitinol stent was installed in the propeller. After the propeller was pushed to the stricture segment, the mantle annular tube was retracted, after which the stent would automatically expand. After stent expansion, barium-meal radiography was routinely used to examine the stent position and the dilated stricture. Three to seven days after stent deployment, 500-1 000 ml of ice-cold water was poured into the side hole of a gastroscope. A protractor was then used to loosen the stent from its surroundings and was then connected to the stent orifice. The stent orifice shrank when we contracted the protractor, and then the stent was removed along with the gastroscope. After that, a gastroscope was again inserted to examine bleeding and mucosa tearing membranes. The patients were allowed to consume cold food for the first 2 days after interventional procedure, and resumed a normal diet afterwards. Within 1 week, barium-meal examination was used to observe the intraluminal patency and swallowing function.

one-month, 6-months, and 1-year follow-ups were recommended.

## RESULTS

We inserted 85 temporary partially-covered metal stents in 83 patients with esophageal stricture, which were removed 3-7 days later. The placement and removal of stents were successful in 100 % of cases. Immediately after placing the stent, we performed an esophageal contrast examination, which confirmed good stricture patency. The mean diameter of the esophageal lumen was 25.77±3.89 mm after stent insertion, and 16.15±2.96 mm after stent removal. The dysphagia score was 0.68±0.31 after stent insertion, and 0.86±0.48 after stent removal. The mean follow-up time was 54.26±12.75 months (range 1 week-96 months). The complications immediately after stent insertion were chest pain (37 cases). After stent removal, 12 cases had a small amount of bleeding and 13 had reflux. All these complications were managed effectively. Temporary partially-covered metal stent insertion and removal were obviously effective on the esophageal stricture and dysphagia.

## DISCUSSION

The short-term effect of balloon dilation on esophageal benign stricture was good, but its mid-term and long-term curative effect was not ideal. In 1990 Domschke *et al*[1] first reported an expandable stent used in malignant esophageal strictures. Since then different types of stent have been developed and applied to the gastrointestinal system[2]. In 1993 Cwikiel *et al*[3] reported the application of silica-gel-covered stent in benign esophageal strictures. The covering of the stent was found to be effective against recurrence of esophageal stricture[4-39]. Because covered stents would migrate, they have not been widely used for some time. However, patients with uncovered stents exhibited more complications such as reflux, stricture recurrence (hyperplasia of granulation tissue), and pain. There is no effective way to manage these complications, which impacts on the long-term curative effect. The worse thing for cases was that another surgical operation was sometimes required to resect the stricture section and the stent placed previously. In order to solve these problems, we used temporary partially-covered metal stent insertion in benign esophageal stricture[40]. The follow-up time was approximately 8 years. The curative effect was long-lasting, dysphagia was clearly palliated, and complications were reduced and easy to manage. Temporary partially-covered metal stent insertion was effective on benign esophageal stricture. Our follow-up investigations revealed that the mid-term and long-term curative effect of temporary stent insertion was better than that of balloon dilation and permanently uncovered or antireflux covered or partially-covered metal stent[41-48]. Our results demonstrated that it was unnecessary to perform graded diameter increment in such stents, unlike when dilating the balloon catheter. Its necessity could be decided based on the follow-up data. After stent insertion, the complications of pain and reflux due to dilating the esophageal stricture by the stent were mainly caused by the chronic tissue tearing of the layer of esophageal muscle and the damage to the original anatomy of the stomach cardia. The stent continued to expand until it reached to body temperature. Since this took 16-24 hours, the tearing of the esophageal muscle tissue was relatively regular and comparatively less scar tissue was formed. Therefore, the recurrence of stricture was low, unlike in balloon dilation which caused acute and irregular tearing on the layer of esophageal muscle, and a corresponding high recurrence of stricture. This is one reason why the method of temporary partially-covered metal stent insertion in benign esophageal stricture is better than that of balloon dilation. We used uncovered stents in patients with benign esophageal strictures in order to reduce the occurrence rate of stent migration. After stent placement, dilation was excellent and dysphagia disappeared, thus achieving the goal of treatment. However, it was accompanied by new problems such as gastroesophageal reflux and recurrence of stricture (hyperplasia of granulation tissue). The reflux could be treated with drugs, but this took a long time. Recurrence of stricture could be reduced by heat cauterization under gastroscope, but it could easily reccur. When we used antireflux covered stent, compications of gastroesophageal reflux and hyperplasia of granulation tissue were not found, but many unexpected results occurred. Comparative studies and experimental research are recommended to further explore the recurrence mechanisms. Further research and development of new stents that are biodegraded by esophageal organism within 2 months after insertion would improve their curative effect, and provide a new therapeutic method for benign esophageal stricture[49]. With further developments in molecular biology, application of gene therapy in the treatment of benign esophageal stricture is expected.

## REFERENCES


1 **Domschke W**, Foerster EC, Matek W, Rodl W. Self-expanding mesh stent for esophageal cancer stenosis. *Endoscopy* 1990; **22**: 134-136

2 **Song HY,** Choi KC, Kwon HC, Yang DH, Cho BH, Lee ST. Esophageal strictures: treatment with a new design of modified Gianturco stent, Work in progress. *Radiology* 1992; **184**: 729-734

3 **Cwikiel W,** Willen R, Stridbeck H, Lillo-Gil R, von Holstein CS. Self-expanding stent in the treatment of benign esophageal strictures: experimental study in pigs and presentation of clinical cases. *Radiology* 1993; **187**: 667-671

4 **Song HY,** Do YS, Han YM, Sung KB, Choi EK, Sohn KH, Kim HR, Kim SH, Min YI. Covered, expandable esophageal metallic stent tubes: experiences in 119 patients. *Radiology* 1994; **193**: 689-695

5 **Foster DR.** Use of a Strecker oesophageal stent in the treatment of benign oesophageal stricture. *Australas Radiol* 1995; **39**: 399-400

6 **Strecker EP**, Boos I, Vetter S, Strohm M, Domschke S. Nitinol esophageal stents: new designs and clinical indications. *Cardiovasc Intervent Radiol* 1996; **19**: 15-20

7 **Vanderburgh L,** Ho CS. Nonvascular stents. *Prog Cardiovasc Dis* 1996; **39**: 187-200

8 **Moores DW,** Ilves R. Treatment of esophageal obstruction with covered, self-expanding esophageal Wallstents. *Ann Thorac Surg* 1996; **62**: 963-967

9 **Heindel W,** Gossmann A, Fischbach R, Michel O, Lackner K. Treatment of a ruptured anastomotic esophageal stricture following bougienage with a Dacron-covered nitinol stent. *Cardiovasc Intervent Radiol* 1996; **19**: 431-434

10 **Fry SW,** Fleischer DE. Management of a refractory benign esophageal stricture with a new biodegradable stent. *Gastrointest Endosc* 1997; **45**: 179-182

11 **Song HY,** Park SI, Do YS, Yoon HK, Sung KB, Sohn KH, Min YI. Expandable metallic stent placement in patients with benign esophageal strictures: results of long-term follow-up. *Radiology* 1997; **203**: 131-136

12 **Song HY,** Park SI, Jung HY, Kim SB, Kim JH, Huh SJ, Kim TH, Kim YK, Park S, Yoon HK, Sung KB, Min YI. Benign and malignant esophageal strictures: treatment with a polyurethane-covered retrievable expandable metallic stent. *Radiology* 1997; **203**: 747-752

13 **Foster DR.** Self-expandable oesophageal stents in the management of benign peptic oesophageal strictures in the elderly. *Br J Clin Pract* 1997; **51**: 199

14 **Tan BS,** Kennedy C, Morgan R, Owen W, Adam A. Using uncovered metallic endoprostheses to treat recurrent benign esophageal strictures. *Am J Roentgenol* 1997; **169**: 1281-1284

15 **Hramiec JE,** O'Shea MA, Quinlan RM. Expandable metallic esophageal stents in benign disease: a cause for concern. *Surg Laparosc Endosc* 1998; **8**: 40-43

16 **Miller LS,** Jackson W, McCray W, Chung CY. Benign nonpeptic esophageal strictures. Diagnosis and treatment.*Gastrointest Endosc Clin N Am* 1998; **8**: 329-355

17 **Sheikh RA,** Trudeau WL. Expandable metallic stent placement in patients with benign esophageal strictures: results of long-term follow-up. *Gastrointest Endosc* 1998; **48**: 227-229

18 **Wengrower D**, Fiorini A, Valero J, Waldbaum C, Chopita N, Landoni N, Judchack S, Goldin E. EsophaCoil: long-term results in 81 patients. *Gastrointest Endosc* 1998; **48**: 376-382

19 **Cowling MG,** Adam A. Radiological management of oesophageal strictures. *Hosp Med* 1998; **59**: 693-697

20 **Kang SG,** Song HY, Lim MK, Yoon HK, Goo DE, Sung KB. Esophageal rupture during balloon dilation of strictures of benign or malignant causes: prevalence and clinical importance. *Radiology* 1998; **209**: 741-746

21 **Monda LA.** Diagnosis and treatment of esophageal strictures. *Radiol Technol* 1999; **70**: 361-372

22 **Vakil N,** Gross U, Bethge N. Human tissue responses to metal stents. *Gastrointest Endosc Clin N Am* 1999; **9**: 359-365

23 **Sandha GS,** Marcon NE. Expandable metal stents for benign esophageal obstruction. *Gastrointest Endosc Clin N Am* 1999; **9**: 437-446

24 **Boulis NM,** Armstrong WS, Chandler WF, Orringer MB. Epidural abscess: a delayed complication of esophageal stenting for benign stricture. *Ann Thorac Surg* 1999; **68**: 568-570

25 **Pajarinen J**, Ristkari SK, Mokka RE. A report of three cases with an oesophageal perforation treated with a coated self-expanding stent. *Ann Chir Gynaecol* 1999; **88**: 332-334

26 **Cowling MG.** Stenting in the oesophagus.*Hosp Med* 2000; **61**: 33-36

27 **Fiorini A,** Fleischer D, Valero J, Israeli E, Wengrower D, Goldin E.Self-expandable metal coil stents in the treatment of benign esophageal strictures refractory to conventional therapy: a case series. *Gastrointest Endosc* 2000; **52**: 259-262

28 **Chen JS,** Luh SP, Lee F, Tsai CI, Lee JM, Lee YC. Use of esophagectomy to treat recurrent hyperplastic tissue obstruction caused by multiple metallic stent insertion for corrosive stricture. *Endoscopy* 2000; **32**: 542-545

29 **Macdonald S,** Edwards RD, Moss JG. Patient tolerance of cervical esophageal metallic stents. *J Vasc Interv Radiol* 2000; **11**: 891-898

30 **Lee JG,** Hsu R, Leung JW. Are self-expanding metal mesh stents useful in the treatment of benign esophageal stenoses and fistulas? An experience of four cases. *Am J Gastroenterol* 2000; **95**: 1920-1925

31 **Song HY,** Jung HY, Park SI, Kim SB, Lee DH, Kang SG, Il Min Y. Covered retrievable expandable nitinol stents in patients with benign esophageal strictures: initial experience. *Radiology* 2000; **217**: 551-557

32 **Cordero JA Jr,** Moores DW. Self-expanding esophageal metallic stents in the treatment of esophageal obstruction. *Am Surg* 2000; **66**: 956-959

33 **Morgan R,** Adam A. Use of metallic stents and balloons in the esophagus and gastrointestinal tract. *J Vasc Interv Radiol* 2001; **12**: 283-297

34 **Ackroyd R**, Watson DI, Devitt PG, Jamieson GG. Expandable metallic stents should not be used in the treatment of benign esophageal strictures. *J Gastroenterol Hepatol* 2001; **16**: 484-487

35 **Catnach S,** Barrison I. Self-expanding metal stents for the treatment of benign esophageal strictures. *Gastrointest Endosc* 2001; **54**: 140

36 **McManus K**, Khan I, McGuigan J. Self-expanding oesophageal stents: strategies for re-intervention. *Endoscopy* 2001; **33**: 601-604

37 **Power C,** Rynne M, O'Gorman T, Maguire D, McAnena OJ. An unusual complication following intubation of a benign oesophageal stricture. *Endoscopy* 2001; **33**: 642

38 **Lee SH.** The role of oesophageal stenting in the non-surgical management of oesophageal strictures. *Br J Radiol* 2001; **74**: 891-900

39 **Dormann AJ,** Deppe H, Wigginghaus B. Self-expanding metallic stents for continuous dilatation of benign stenoses in gastrointestinal tract - first results of long-term follow-up in interim stent application in pyloric and colonic obstructions. *Z Gastroenterol* 2001; **39**: 957-960

40 **Chen WX,** Cheng YS, Yang RJ, Li MH, Zhuang QX, Chen NW, Xu JR, Shang KZ. Interventional therapy of achalasia with temporary metal internal stent dilatation and its intermediate and long term follow-up. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 896-899

41 **Moses FM,** Wong RK. Stents for Esophageal Disease. *Curr Treat Options Gastroenterol* 2002; **5**: 63-71

42 **Wang YG,** Tio TL, Soehendra N. Endoscopic dilation of esophageal stricture without fluoroscopy is safe and effective. *World J Gastroenterol* 2002; **8**: 766-768

43 **Profili S,** Meloni GB, Feo CF, Pischedda A, Bozzo C, Ginesu GC, Canalis GC. Self-expandable metal stents in the management of cervical oesophageal and/or hypopharyngeal strictures. *Clin Radiol* 2002; **57**: 1028-1033

44 **Therasse E,** Oliva VL, Lafontaine E, Perreault P, Giroux MF, Soulez G. Balloon dilation and stent placement for esophageal lesions: indications, methods, and results. *Radiographics* 2003; **23**: 89-105

45 **Petruzziello L**, Costamagna G. Stenting in esophageal strictures. *Dig Dis* 2002; **20**: 154-166

46 **Cheng YS,** Yang RJ, Li MH, Shang KZ, Chen WX, Chen NW, Chu YD, Zhuang QX. Interventional procedure for benign or malignant stricture or obstruction of upper gastrointestinal tract. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 1354-1360

47 **Chen WX,** Cheng YS, Yang RJ, Li MH, Shang KZ, Zhuang QX, Chen NW. Metal stent dilation in the treatment of benign esophageal stricture by interventional procedure:a follow-up study. *Shijie Huaren Xiaohua Zazhi* 2002; **10**: 333-336

48 **Cheng YS,** Shang KZ. Interventional therapy in dysphagia. *Shijie Huaren Xiaohua Zazhi* 2002; **10**: 1312-1314

49 **Fry SW,** Fleischer DE. Management of a refractory benign esophageal stricture with a new biodegradable stent. *Gastrointest Endosc* 1997; **45**: 179-182


**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2362-2365
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Antiproliferative effect of octreotide on gastric cancer cells mediated by inhibition of Akt/PKB and telomerase

Shan Gao, Bao-Ping Yu, Yan Li, Wei-Guo Dong, He-Sheng Luo

**Shan Gao, Bao-Ping Yu, Wei-Guo Dong, He-Sheng Luo,** Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
**Yan Li,** Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China
**Correspondence to:** Dr. Bao-Ping Yu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China. yubaoping62@yahoo.com.cn
**Telephone:** +86-27-88075814
**Received:** 2003-03-20 **Accepted:** 2003-04-14

## Abstract

**AIM:** To investigate the antiproliferative effect of octreotide, a long-acting analogue of somatostatin, on gastric cancer cell line SGC7901 and its possible molecular mechanisms.

**METHODS:** Gastric cancer cell line SGC7901 employed in the study was treated with 0.008, 0.04, 0.2, 1, 5 and 25 $\mu g \cdot ml^{-1}$ of octreotide respectively for 24 h to evaluate the antiproliferative effect of somatostatin analog on the tumor cells by MTT assay method. To elucidate the underlying mechanism, the cells were exposed to 1 $\mu g \cdot ml^{-1}$ of octreotide for 0, 12, 24 and 48 h, when their Akt/PKB and telomerase activities were respectively determined using PCR-ELSIA and nonradioactive protein kinase assay protocols. The same experimental procedures were also performed in the control cells that were treated with corresponding vehicles instead of somatostatin analog.

**RESULTS:** After exposed to octreotide for 24 h at the concentrations of more than 1 $\mu g \cdot ml^{-1}$, SGC7901 cells exhibited a dose-dependent inhibition of growth with the inhibiting rate to be as high as 34.66 % when 25 $\mu g \cdot ml^{-1}$ of octreotide was applied. The Akt/PKB and telomerase activity of SGC7901 cells was significantly inhibited when the cells were exposed to 1 $\mu g \cdot ml^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts ($P<0.01$), both of which exhibited in a time-dependent manner.

**CONCLUSION:** The antiproliferative effect of octreotide on SGC7901 cells might be mediated by the inhibition of Akt/PKB and telomerase.

Gao S, Yu BP, Li Y, Dong WG, Luo HS. Antiproliferative effect of octreotide on gastric cancer cells mediated by inhibition of Akt/PKB and telomerase. *World J Gastroenterol* 2003; 9(10): 2362-2365

http://www.wjgnet.com/1007-9327/9/2362.asp

## INTRODUCTION

Gastric cancer continues to be one of the most common malignancies in human worldwide[1-4]. In China, its average rate of annual mortality is estimated to be as high as 16 per 100 000 population that accounts for the leading cause of death among malignant tumors[5-7]. Due to the limited efficacy and considerable toxicity of conventional chemotherapy, some novel cytotoxic and noncytotoxic agents such as certain dietary substances, natural hormones and synthetic compounds have been proposed to stop or reverse the process of carcinogenesis[8], in which increasing attention has been paid to somatostatin and its analogs that have been reported to exert antineoplastic effects in a wide range of tumor types such as carcinoid, osteosarcoma, leukemia, and cancers of thyroid, breast, lung, liver, pancreas, colon, as well as gastric carcinomas[9-19]. However, detailed mechanisms underlying the antineoplastic actions of somatostatin and its analogs that remain to be clarified have limited the therapeutic efficacy of these agents in the treatment of clinical malignancies[20, 21].

Recent advances in molecular biological science have revealed that some molecules such as telomerase and protein kinase B or Akt (PKB/Akt) are involved in the process of carcinogenesis. Telomerase is an enzyme that replaces repetitive (TTAGGG)n sequences on the ends of chromosomes that would otherwise be lost during successive cell divisions. Its activation is closely linked to the attainment of cellular immortality and considered to be an important step in tumorigenesis[22-26]. Another molecule PKB/Akt, a serine/threonine kinase, has also recently been a focus of intense research. It appears that Akt/PKB lies in the crossroads of multiple cellular signaling pathways and acts as a transducer of many functions initiated by growth factor receptors. It is particularly important that Akt/PKB can mediate cell survival and serve as a critical factor in the genesis of malignancies[27-30]. Thus, an investigation to clarify how the antiproliferative effects on these couple of molecules might be helpful in elucidating antineoplastic mechanisms of somatostatin analogs, which is no doubt of both theoretical and practical importance.

## MATERIALS AND METHODS

### *Materials*

Human gastric cancer cell line SGC7901 was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Somatostatin analogue octreotide was provided by Sandoz Corp, RPMI1640 and fetal bovine serum (FBS) were from Gibco. 3-(4,5-dimethyl thiazol-2- yl)-2, 5-diphenyl tetrazolium bromide (MTT), telomerase TRAP PCR-ELISIA kit and Akt/PKB kinase assay kit were purchased from Sigma, Roche Molecular Biochemicals and CST respectively. Microtiter plate reader was made by Digiscan, Austria ASYS Hitech.

### *Cell culture*

SGC7901 cells were maintained in flasks with RPMI-1640 supplemented with 10 % heat- inactivated FBS, 100 $U \cdot ml^{-1}$ penicillin, 100 $U \cdot ml^{-1}$ streptomycin and 20 mM sodium bicarbonate at 37 ℃ in a humidified atmosphere containing 5 % of $CO_2$. The culture medium change was performed every 3 days.

### *MTT assay*

A MTT assay was conducted to determine the cell proliferation. In brief, SGC-7901 cells were seeded at $5\times10^3 \cdot$ $wells^{-1}$ in a 96-

well plate and incubated in 200 μl of culture medium overnight. Then the cells were made quiescent by serum deprivation and treated with 0.008, 0.04, 0.2, 1, 5, 25 $\mu g \cdot ml^{-1}$ of octreotide respectively as the different observatory groups. The cells treated with equal amount of normal saline instead of octreotide served as the control. After cultivated for 24 h, 20 μl of stock MTT solution (2.5 $mg \cdot ml^{-1}$) was added to cells for each well which were further incubated at 37 ℃ for 4 hours. The culture medium was carefully removed followed by adding of 150 μl of DMSO to each well and vibrating culture well for 10 min. The absorbance of samples was measured at a wavelength of 490 nm with a microtiter plate reader. The negative control was conducted using only cell-free culture medium and each assay was performed in triplicate. The inhibiting rate of cell growth was expressed as (A-B)/A×100 %, where A is the absorbance value from the controls and B is that from the experimental cells.

### *Immunoprecipitation kinase assay*

Akt/PKB activity was examined by the immunoprecipitation kinase assay. Briefly, $5\times10^5$ of SGC7901 cells were seeded in a 25-$cm^2$ flask and cultured for 3 days. When being confluent at 70 %, cells nominated as group A were incubated in serum-free RPMI-1640 for 24 h and then treated with 1 $\mu g \cdot ml^{-1}$ octreotide for further cultivation of 0-, 12-, 24- and 48-h respectively. Same procedures were also performed in group B cells that served as the control except being treated with equal volume of normal saline instead of octreotide. Confluent cells were washed three times in cold PBS, then lysed in buffer containing 20 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.5), l50 $mmol \cdot L^{-1}$ NaCl, 1 % Triton X-l00, 1 $mmol \cdot L^{-1}$ EDTA, 1 $mmol \cdot L^{-1}$ EGTA, 2.5 $mmol \cdot L^{-1}$ sodium pyrophosphate, 1 $mmol \cdot L^{-1}$ β-glycerophosphate, 1 $mmol \cdot L^{-1}$ $Na_3VO_4$, 1 $\mu g \cdot ml^{-1}$ leupeptin and 1 $mmol \cdot L^{-1}$ phenyl-methyl-sulfonyl fluoride (PMSF) for 10 min at 4 ℃. Samples were microcentrifuged for 10 min and 200 μl of the cell lysate was incubated with 20 μl of resuspended immobilized Akt antibody slurry at 4 ℃ for 2 h to immunoprecipitate Akt/PKB, the latter was next used to phosphorylate a specific substrate, GSK-3 fusion protein (Ser21/9). That is, 1 μg of GSK-3 fusion protein was incubated with immunoprecipitated Akt/PKB in the presence of ATP for 30 min at 30 ℃ followed by adding 20 μl of 3×SDS sample buffer to terminate the reaction. Samples were then boiled for 5 min, separated by 12 % SDS-PAGE and transferred onto nitrocellulose membrane. Nonspecific reactivity was blocked by 5 % fat-free milk in TBST (10 mM Tris-HCl, pH7.5, l50 mM NaCl, 0.05 % Tween 20) for 1 h. The membrane was incubated with a 1:1 000 dilution of phospho-GSK-3 α/β antibody for 2 h, then with HRP-conjugated anti-rabbit (1:2 000) and anti-biotin antibody (1:1 000) for 1 h at room temperature. Finally, reactive protein was revealed by incubating with 10 ml of LumiGLO for 1 min and exposed to x-ray film for 1-2 min at room temperature.

### *Determination of telomerase activity*

The telomerase activity was determined by using TRAP-ELSIA. $5\times10^5$ of SGC7901 cells were seeded in a 25-$cm^2$ flask and cultured for 3 days. When being confluent at 70 %, cells nominated as group A were incubated in serum-free RPMI-1640 for 24 h and then treated with 1 $\mu g \cdot ml^{-1}$ octreotide for further cultivation of 0-, 12-, 24- and 48-h respectively. Same procedures were also performed in group B cells that served as the control except being treated with equal volume of DMSO instead of octreotide. Confluent cells were harvested and centrifuged at 3 000 g for 10 min at 4 ℃. After resuspended, $5\times10^3$ cells were lysed in an Eppendorf tube, whose extract was next used to amplify telomeric repeat. The telomerase activity was determined with PCR-ELISA according to the manufacturer' s protocol. Sample absorbance was measured with a microtiter plate reader at the wavelength of 450/690 nm within 30 min after addition of the stop reagent.

### *Statistical analysis*

Experimental data were analyzed by ANOVA and Student' s *t* test for multiple comparisons between groups. The data were finally expressed as mean ± standard error of the mean. *P* value less than 0.05 was considered statistically significant.

## RESULTS

### *Antiproliferative effect of octreotide on SGC7901 cells*

When exposed to octreotide for 24 h at the concentration of more than 1 $\mu g \cdot ml^{-1}$, SGC7901 cells exhibited a dose-dependent inhibition of growth with the inhibiting rate to be as high as 34.66 % when 25 $\mu g \cdot ml^{-1}$ of octreotide was applied (Table 1).

**Table 1** Antiproliferative effect of octreotide on SGC7901 cells

| Octreotide (μg / ml) | $OD_{490}$ | IR (%) |
|---|---|---|
| Control | 0.528±0.032 | / |
| 25 | 0.345±0.041 | 34.66[b] |
| 5 | 0.436±0.022 | 21.21[b] |
| 1 | 0.465±0.019 | 11.93[a] |
| 0.2 | 0.493±0.008 | 6.63 |
| 0.04 | 0.510±0.031 | 3.41 |
| 0.008 | 0.504±0.065 | 0.76 |

[a]$P<0.05$, [b]$P<0.01$ *vs* control.

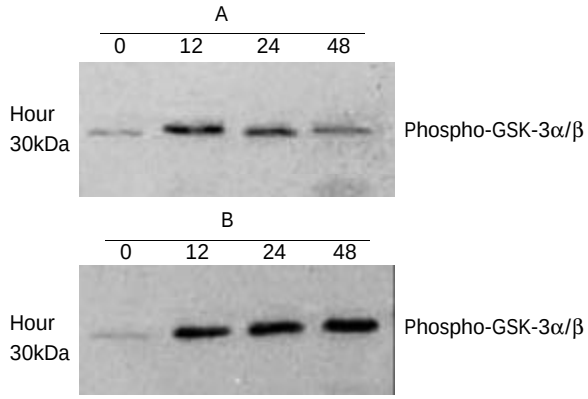



**Figure 1** Inhibitory effects of octreotide on Akt / PkB activity in SGC7901 cells. The Akt / PKB activity in group A SGC7901 cells was significantly inhibited after the cells were exposed to 1 $\mu g \cdot ml^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts (Figure 1B), which manifested in a time-dependent manner as shown in Figure 1A.

### *Inhibition of Akt/PKB activity*

The Akt/PKB activity in group A SGC7901 cells was significantly inhibited after the cells were exposed to 1 $\mu g \cdot ml^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts (Figure 1B), which exhibited in a time-dependent manner as shown in Figure 1A.

### *Inhibition of telomerase activity*

Just like that of the Akt/PKB, the telomerase activity in group A SGC7901 cells was also significantly inhibited after the cells were exposed to 1 $\mu g \cdot ml^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts ($P<0.01$), which exhibited in a time- dependent manner as shown in Figure 2A.

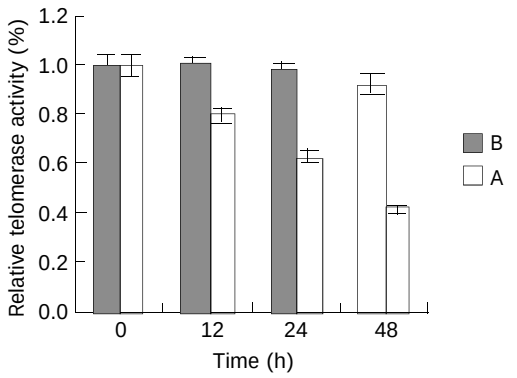



**Figure 2** Inhibitory effects of octreotide on telomerase activity in SGC7901 cells. The telomerase activity in group A SGC7901 cells was significantly inhibited after the cells were exposed to 1 μg· ml$^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts ($P$<0.01), which exhibited in a time-dependent manner as shown in Figure 2A.

## DISCUSSION

Over the past decade, impressive antineoplastic effects of somatostatin and its analogs have been reported in plenty of tumor models and cancer cell types[9-19]. A more recent research has further provided considerable information regarding the mechanisms underlying the antiproliferative and apoptosis-inducing actions of these compounds. These included both "direct" mechanisms that were the sequellae of binding of somatostatin analogs to their corresponding receptors present on neoplastic cells and "indirect" mechanisms that might be the result of reduced or inhibited secretion of growth-promoting hormones and growth factors that stimulated the growth of various types of malignancies[21,31-33]. Apart from these discoveries, some biological actions such as the inhibition of angiogenesis and the negative influence on the immune system were also considered to be the important factors contributing to the antineoplastic effects of somatostatin analogs[21, 34], which therefore suggested that the latter might be involved in an even wider mechanism.

In the present study, we observed the inhibitory effects of octreotide on gastric cancer SGC7901cells, as well as on the activities of Akt/PKB and telomerase. As to our knowledge, it is one of the fewer reports in recent years that concerned about this subject. It revealed that after exposed to octreotide for 24 h at the concentration of more than 1 μg· ml$^{-1}$, SGC7901 cells exhibited a dose-dependent inhibition of growth with the inhibiting rate to be as high as 34.66 % when 25 μg· ml$^{-1}$ of octreotide was applied. Furthermore, in a previous study, we also noted that this inhibitory action could not be abrogated by supplementing 10 % FBS to the cultured cells, which only prolonged the time course of cell death (data to be published). It indicated the mitogenic action provided by serum growth factors in the culture medium could be overcome by octreotide. Akt/PKB and telomerase are the enzymes that have recently been considered to be closely related to carcinogenesis[22-30]. Whether the activation of Akt/PKB was one of the critical factors in cell to determine either a survival or an apoptotic message was conducted on the signal passway[35]. Amplification of genes encoding Akt/PKB isoforms has been found in several types of human cancers[36]. In addition, Akt/PKB has been reported to promote tumor progression and invasiveness by enhancing angiogenesis[28]. So far as the telomerase is concerned, its activity has been detected in over 90 % of human cancers[37-41], In gastric cancer, telomerase was activated very early in the process of this disease[42], which suggested the activation of telomerase might be also a determining factor contributing to the tumorigenesis. The relationship between Akt/PKB and telomerase has also been noted by Kang and his coworkers[43] with the founding that Akt kinase enhanced human telomerase activity through phosphorylation of hTERT subunit as one of its substrate proteins in SK-MEL28 cells. In the present study, we found that both activities of Akt/PKB and telomerase in SGC7901 cells were significantly inhibited after the cells were exposed to 1 μg· ml$^{-1}$ of octreotide for 12, 24 and 48 h compared with that of their control counterparts. Based on these findings, it can be concluded that the antiproliferative effects of somatostatin analogs on SGC7901 cells might be mediated at least in part via a mechanism of inhibition of Akt/PKB and then telomerase. Our further study should aim at elucidating the components on the signal conduction passway in up and downstreams of these events.

## REFERENCES

1 **Newnham A,** Quinn MJ, Babb P, Kang JY, Majeed A. Trends in oesophageal and gastric cancer incidence, mortality and survival in England and Wales 1971-1998 / 1999. *Aliment Pharmacol Ther* 2003; **17:** 655-664
2 **Mathers CD,** Shibuya K, Boschi-Pinto C, Lopez AD, Murray CJ. Global and regional estimates of cancer mortality and incidence by site: I. Application of regional cancer survival model to estimate cancer mortality distribution by site. *BMC Cancer* 2002; **2:** 36
3 **Le Vu B,** de Vathaire F, de Vathaire CC, Paofaite J, Roda L, Soubiran G, Lhoumeau F, Laudon F. Cancer incidence in French Polynesia 1985-1995. *Trop Med Int Health* 2000; **5:** 722-731
4 **Mohandas KM,** Jagannath P. Epidemiology of digestive tract cancers in India. VI. Projected burden in the new millennium and the need for primary prevention. *Indian J Gastroenterol* 2000; **19:** 74-78
5 **Sun X,** Mu R, Zhou Y, Dai X, Qiao Y, Zhang S, Huangfu X, Sun J, Li L, Lu F. 1990-1992 mortality of stomach cancer in China. *Zhonghua Zhongliu Zazhi* 2002; **24:** 4-8
6 **Xue FB**, Xu YY, Wan Y, Pan BR, Ren J, Fan DM. Association of *H. pylori* infection with gastric carcinoma: a Meta analysis. *World J Gastroenterol* 2001; **7**: 801-804
7 **Cao WX,** Ou JM, Fei XF, Zhu ZG, Yin HR, Yan M, Lin YZ. Methionine-dependence and combination chemotherapy on human gastric cancer cells *in vitro*. *World J Gastroenterol* 2002; **8:** 230-232
8 **Morse MA**, Stoner GD. Cancer chemoprevention: principles and prospects. *Carcinogenesis* 1993; **14**: 1737-1746
9 **Scarpignato C,** Pelosini I. Somatostatin analogs for cancer treatment and diagnosis: an overview. *Chemotherapy* 2001; **47**(Suppl 2): 1-29
10 **Kinova S,** Duris I, Kratochvilova E, Ondrejka P, Payer J. Carcinoid tumors-somatostatine in the diagnosis and therapy. *Bratisl Lek Listy* 2002; **103:** 108-112
11 **Ain KB,** Taylor KD, Tofiq S, Venkataraman G. Somatostatin receptor subtype expression in human thyroid and thyroid carcinoma cell lines. *J Clin Endocrinol Metab* 1997; **82:** 1857-1862
12 **Khanna C**, Prehn J, Hayden D, Cassaday RD, Caylor J, Jacob S, Bose SM, Hong SH, Hewitt SM, Helman LJ. A randomized controlled trial of octreotide pamoate long-acting release and carboplatin versus carboplatin alone in dogs with naturally occurring osteosarcoma: evaluation of insulin-like growth factor suppression and chemotherapy. *Clin Cancer Res* 2002; **8**: 2406-2412
13 **Marschke RF Jr,** Grill JP, Sloan JA, Wender DB, Levitt R, Mailliard JA, Gerstner JB, Ghosh C, Morton RF, Jett JR. Phase II study of high-dose somatostatin analogue in patients either previously treated or untreated who have extensive-stage small cell lung cancer. *Am J Clin Oncol* 1999; **22:** 15-17
14 **Setyono-Han B,** Henkelman MS, Foekens JA, Klijn GM. Direct inhibitory effects of somatostatin (analogues) on the growth of human breast cancer cells. *Cancer Res* 1987; **47:** 1566-1570
15 **Wang XB,** Wang X, Zhang NZ. Inhibition of somatostatin analog Octreotide on human gastric cell MKN45 growth *in vitro*. *Shijie Huaren Xiaohua Zazhi* 2002; **10:** 40-42
16 **Gencosmanoglu R,** Bugra D, Bulut T, Yamaner S, Cevikbas U, Bilir A. The inhibitory effect of octreotide on experimental colorectal carcinogenesis. *Surg Today* 2002; **32:** 249-256

17 **Lee JU,** Hosotani R, Wada M, Doi R, Koshiba T, Fujimoto K, Miyamoto Y, Tsuji S, Nakajima S, Hirohashi M, Uehara T, Arano Y, Fujii N, Imamura M. Antiproliferative activity induced by the somatostatin analogue, TT-232, in human pancreatic cancer cells. *Eur J Cancer* 2002; **38:** 1526-1534

18 **Giannetti N,** Enjalbert A, Krantic S. Somatostatin analog SMS 201995 inhibits proliferation in human leukemia T-cell line: relevance of the adenylyl cyclase stimulation. *J Cell Biochem* 2000; **78:** 666-673

19 **Yuen MF,** Poon RT, Lai CL, Fan ST, Lo CM, Wong KW, Wong WM, Wong BC. A randomized placebo-controlled study of long-acting octreotide for the treatment of advanced hepatocellular carcinoma. *Hepatology* 2002; **36:** 687-691

20 **Jenkins SA,** Kynaston HG, Davies ND, Baxter JN, Nott DM. Somatostatin analogs in oncology: a look to the future. *Chemotherapy* 2001; **47**(Suppl 2): 162-196

21 **Froidevaux S,** Eberle AN. Somatostatin analogs and radiopeptides in cancer therapy. *Biopolymers* 2002; **66:** 161-183

22 **Watanabe N.** Telomerase, cell immortality and cancer. *Hokkaido Igaku Zasshi* 2001; **76:** 127-132

23 **Yang SM,** Fang DC, Luo YH, Lu R, Battle PD, Liu WW. Alterations of telomerase activity and terminal restriction fragment in gastric cancer and its premalignant lesions. *J Gastroenterol Hepatol* 2001; **16:** 876-882

24 **Zhan WH,** Ma JP, Peng JS, Gao JS, Cai SR, Wang JP, Zheng ZQ, Wang L. Telomerase activity in gastric cancer and its clinical implications. *World J Gastroenterol* 1999; **5:** 316-319

25 **Miyachi K,** Fujita M, Tanaka N, Sasaki K, Sunagawa M. Correlation between telomerase activity and telomeric-repeat binding factors in gastric cancer. *J Exp Clin Cancer Res* 2002; **21:** 269-275

26 **Nowak J,** Januszkiewicz D, Lewandowski K, Nowicka-Kujawska K, Pernak M, Rembowska J, Nowak T, Wysocki J. Activity and expression of human telomerase in normal and malignant cells in gastric and colon cancer patients. *Eur J Gastroenterol Hepatol* 2003; **15:** 75-80

27 **Semba S,** Moriya T, Kimura W, Yamakawa M. Phosphorylated Akt / PKB controls cell growth and apoptosis in intraductal papillary-mucinous tumor and invasive ductal adenocarcinoma of the pancreas. *Pancreas* 2003; **26:** 250-257

28 **Lee SH,** Kim HS, Park WS, Kim SY, Lee KY, Kim SH, Lee JY, Yoo NJ. Non-small cell lung cancers frequently express phosphorylated Akt; an immunohistochemical study. *APMIS* 2002; **110:** 587-592

29 **Lawlor MA,** Alessi DR. PKB / Akt: a key mediator of cell proliferation, survival and insulin responses? *J Cell Sci* 2001; **114** (Pt 16): 2903-2910

30 **Nicholson KM,** Anderson NG. The protein kinase B / Akt signalling pathway in human malignancy. *Cell Signal* 2002; **14:** 381-395

31 **Hocker M,** Wiedenmann B. Therapeutic and diagnostic implications of the somatostatin system in gastroenteropancreatic neuroendocrine tumour disease. *Ital J Gastroenterol Hepatol* 1999; **31** (Suppl 2): S139- S142

32 **Bousquet C,** Puente E, Buscail L, Vaysse N, Susini C. Antiproliferative effect of somatostatin and analogs. *Chemotherapy* 2001; **47**(Suppl 2): 30-39

33 **Zalatnai A,** Pogany V. Somatostatin analogs in the treatment of pancreatic cancer: utopia or feasible alternative? *Orv Hetil* 2000; **141:** 2333-2338

34 **Wang C,** Tang C. Inhibition of human gastric cancer metastasis by ocreotide *in vitro* and *in vivo*. *Zhonghua Yixue Zazhi* 2002; **82:** 19-22

35 **Itoh N,** Semba S, Ito M, Takeda H, Kawata S, Yamakawa M. Phosphorylation of Akt / PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. *Cancer* 2002; **94:** 3127-3134

36 **Hill MM,** Hemmings BA. Inhibition of protein kinase B / Akt. Implications for cancer therapy. *Pharmacol Ther* 2002; **93:** 243-251

37 **Hao ZM,** Luo JY, Cheng J, Wang QY, Yang GX. Design of a ribozyme targeting human telomerase reverse transcriptase and cloning of it's gene. *World J Gastroenterol* 2003; **9:** 104-107

38 **Kim NW,** Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266:** 2011-2015

39 **Feng RH,** Zhu ZG, Li JF, Liu BY, Yan M, Yin HR, Lin YZ. Inhibition of human telomerase in MKN-45 cell line by antisense hTR expression vector induces cell apoptosis and growth arrest. *World J Gastroenterol* 2002; **8:** 436-440

40 **Yakoob J,** Hu GL, Fan XG, Zhang Z. Telomere, telomerase and digestive cancer. *World J Gastroenterol* 1999; **5:** 334-337

41 **Tatsumoto N,** Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, Yokoyama T. High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res* 2000; **6:** 2696-2701

42 **Hiyama E,** Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y, Kodama T, Piatyszek MA, Shay JW, Matsuura Y. Telomerase activity in gastric cancer. *Cancer Res* 1995; **55:** 3258-3262

43 **Kang SS,** Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* 1999; **274:** 13085-13090


**Edited by** Zhu L and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2366-2369
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Molecule action mechanisms of NM-3 on human gastric cancer SGC-7901 cells *in vivo* or *in vitro*

Jin-Shui Zhu, Bo Shen, Jin-Lian Chen, Guo-Qiang Chen, Xiao-Hu Yu, Hua-Fang Yu, Zu-Ming Zhu

**Jin-Shui Zhu, Bo Shen, Jin-Lian Chen, Guo-Qiang Chen, Xiao-Hu Yu, Hua-Fang Yu, Zu-Ming Zhu,** Affiliated Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Guo-Qiang Chen,** Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai 200233, China
**Supported by** Shanghai Natural Science Foundation, No.02ZB14072
**Correspondence to:** Jin-Shui Zhu, Department of Gastroenterology, Affiliated Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China. zhujs1803@hotmail.com
**Telephone:** +86-21-64369181 Exit 8351 **Fax:** +86-21-54778507
**Received:** 2003-05-12 **Accepted:** 2003-06-02

## Abstract

**AIM:** To study the molecule action mechanisms of NM-3 on the growth of human gastric cancer SGC-7901 cells *in vivo* or *in vitro*.

**METHODS:** SGC-7901 from human non-differentiated gastric cancer cell line was cultured with NM-3 at 100 mg/ml for 24 h. We observed its inhibitory rate and the density of micro-vascular growth in grafted mice with human gastric cancer SGC-7901. The apoptosis of human gastric cancer SGC-7901 was revealed in NM-3 treatment group by using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-fluorescene nick end labeling (TUNEL) method and flow cytometry analysis.

**RESULTS:** The growth of SGC-7901 cells was markedly inhibited compared with control growp, which was smaller than that in normal saline control group (4.17 g±0.22 g *vs* 9.45 g±1.38 g, $P<0.01$). The level of apoptosis of human gastric cell line SGC-7901 was obviously increased in NM-3 treatment group at 1 mg.$L^{-1}$ for 24 h. NM-3 inducing apoptotic index in NM-3 plus carboplatin group was 3.5 times that of carboplatin control group (TUNEL: 27.98±6.12 % *vs* 12.94±2.12 %, FACScan: 26.86±5.69 % *vs* 11.86±1.09 %, $P<0.01$). Western blot analysis showed that the apoptotic index of human gastric cancer was elevated for 12, 24 and 36 h with an evident time-effect relationship in groups at 100 mg.$L^{-1}$. NM-3 enhanced the inhibitive effects and sensitivity of chemotherapy for human gastric cancer in nude mice. These results suggested that NM-3 played a key inhibitive role in the growth of grafted human gastric cancer in nude mice.

**CONCLUSION:** NM-3 can inhibit the growth of human gastric cancer cell line SGC-7901, and enhance the sensitivity of carboplatin on SGC-7901 and induced its apoptosis.

ZhuJS, Shen B, Chen JL, Chen GQ, Yu XH, Yu HF, Zhu ZM. Molecule action mechanisms of NM-3 on human gastric cancer SGC-7901 cells *in vivo* or *in vitro*. *World J Gastroenterol* 2003; 9(10):2366-2369
http://www.wjgnet.com/1007-9327/9/2366.asp

## INTRODUCTION

Apoptosis plays a key role in the proliferation and turnover of malignant tumor cells. It has been known that its extent is often enhanced in gastric cancer by some anti-cancer drugs, such as chemotherapeutic drugs, hormones or immune agents, micro-vascular growth inhibitors have been proved to have some inhibitory effects on maligant tumors,especially on gastric tumors. But it has not been clear whether NM-3 is a micro-vascular inhibitive agent for solid tumor growth[1-3], it might suppress gastric cancer cell proliferation and cause tumor cell loss and nuclear condensation *in vitro*. Up to date, NM-3 is considered as the newest micro-vasular inhabitor[1-4]. Combined with carboplatin, it can soften hard lumps and dissolve phlegm, enhance apoptosis of human gastric cancer xenografts in nude mice. On the other hand, NM-3 can enhance the sensitivity of chemotherapeutic drugs on human gastric cancer. Based on previous studies, NM-3 exerts its effects on solid tumor growth by promoting apoptosis of human gastric cancer cell SGC-7901 and increasing the suppressive effects of carboplatin.

## MATERIALS AND METHODS

### *Materials*

A human gastric cancer cell line SGC-7901 grafted onto nude mice was used as the animal model, the age of these 60 mice was 6-7 weeks old female balb/c-nu/nu mice (weight 18-22 g) and a human gastric cancer cell line SGC-7901 was obtained from Shanghai Tumor Institute (No: 01842). The animals were subcutaneously xerografted under abdominal skin with the SGC-7901 cell line. The tumor transplantation procedure was described previously. The animal model and SGC-7901 cell line were obtained from Shanghai Experimental Animal Centre, Chinese Academy of Sciences.

NM-3 was composed of 2-c8-hydroxy-6-methoxy-1-oxo-1 h-2-benzopyran-3-yd, concentrantion of NM-3 was 100 mg· $L^{-1}$, the concentration of carboplatin was 100 mg· $L^{-1}$.

### *Methods*

Experimental schedule: After grafting, these nude mice were randomly divided into 3 groups: control group and two experimental groups assigned to receive NM-3 or carboplatin respectly. Each experimental mouse in two experimental groups was given a 0.5 ml dose of NM-3 drug via intra-abdominal injection or gastric perfusion (empty control group) once every three days over a 40-day period beginning at 1st day after being xerografted. The control animals received normal saline according to the same schedule by gastric perfusion. The animals were killed 41 days after being xerografted.

In our study, NM-3 induced gastric cancer cell apoptosis, and enhanced the chemotherapeutic sensitivity of human gastric cancer cell line SGC-7901 on carboplatin *in vitro*. Apoptosis induced by NM-3 needed further investigation.

Therapeutic effects on human gastric cancer cell growth were assessed. Tumor size was measured twice a week by multiplying two perpendicular diameter and tumor weight was determined immediately by electron balance after the animals were killed. Apoptotic cells and apoptotic index were

determined by the terminal deoxynucleotidyl transferase-mediated deoxy-uridine triphosphate-fluorescene nick end labeling (TUNEL) method and flow cytometry analysis. Morphological atterations were observed with electron microscope.

Flow cytometry analysis: Propidium iodide (PI) staining was used for flow cytometric detection of apoptosis. $1\times10^6$ cells from each of the samples were treated with RNase and stained with PI.The apoptotic cells labled by DNA strand were measured with a flow cytometer (FACS Calibur,Becton Dickinson,U.S.A).The data from $1\times10^6$ cells/sample were collected,stored,and analyzed using CELLQUES' T and MODFITLT for macV1.01 software[4-12].

### *Statistical analysis*

The results were expressed as $\bar{x}\pm s$, Student' s *t* test was used. *P* value <0.05 was concidered significantly.

## RESULTS

### *NM-3 inhibited growth of micro-vascular of tumor in nude mice with human gastric cancer SGC-7901*

NM-3 group decreased signicantly the neo-microvascular density (1.17±0.05 $mm^3$) of gastric cancer tumor implanted onto nude mice was significantly decreased in NM-3 group compared with that in saline group (5.37±1.12 $mm^3$) and carboplatin group (4.72±1.18 $mm^3$, $P>0.05$). The micro-vascular density in NM-3 combinated with carboplatin group (1.18±0.05 $mm^3$) was not significantly different from that in NM-3 group ($P>0.05$, Table 1).

**Table 1** Growth of neo-microvascular around gastric tumor supressed by NM-3 ($\bar{x}\pm s$)

| Treatment | *n* | Density ($mm^3$) |
|---|---|---|
| Carboplatin | 10 | 4.72±1.18[a] |
| NM-3 plus carboplatin | 10 | 1.18±0.05[b] |
| Saline | 10 | 5.37±1.72 |

[a]$P>0.05$, [b]$P<0.01$ *vs t* test in saline control group.

### *NM-3 enhanced sensitivity of carboplatin on human gastric cancer induced by apoptosis of human gastric cancer cell in vitro or in vivo*

The apoptotic indix (AI) of SGC-7901 induced by carboplatin was enhanced in NM-3 group. The apoptotic indix (TUNEL: 27.98±6.12 %, FACScan: 26.86±5.69 %) was markedly increasd in that of carboplatin group by using either TUNEL method or flow cytometry analysis compared with the carboplatin group (TUNEL: 12.94±2.12 %, $P<0.01$; FACScan: 11.86±1.09 %, $P<0.01$). The apoptotic indix in NM-3 group (TUNEL: 16.47±4.13 % FACScan: 15.97±1.49 %) was higher than that in normal saline group (TUNEL 1.83±0.12 %, $P<0.01$; FACScan: 1.06±0.09 %, $P<0.01$, Table 2).

**Table 2** Apoptotic indix (AI) of human gastric cancer line SGC-7901 enhanced by NM-3 *in vitro* ($\bar{x}\pm s$)

| Treatment | *n* | AI (TUNEL)% | AI (FACScan) % |
|---|---|---|---|
| NM-3 | 10 | 16.47±4.13[b] | 15.97±2.49[b] |
| Carboplatin | 10 | 12.94±2.12[b] | 11.86±1.09[b] |
| NM-3 plus carboplatin | 10 | 27.98±6.12[b] | 26.86±5.69[b] |
| Saline | 10 | 1.83±0.12 | 1.06±0.09 |

[a]$P>0.05$, [b]$P<0.01$ *vs t* test, in saline control group.

### *NM-3 effected on growth of xerografted human gastric cancer cell line SGC-7901 in nude mice*

The tumor weight of nude mice in NM-3 group (4.17±0.22 g) was obviously lower than that in normal saline controls (9.45±0.38 g), the tumor size of the prior group (0.68 g±0.07$cm^3$, $P<0.05$) was smaller than that in normal saline control group (8.94±1.46 $cm^3$). The tumor weight and size of nude mice in NM-3 combinated with carboplatin group were 2.78±0.18 g and 0.34±0.02 $cm^3$ respectively. However, those in NM-3 group were not significantly different compared with carboplatin group (4.46±0.23 g, 0.71±0.08 $cm^3$) 7 weeks later. Tumor growth was (size and weight) markedly inhibited by treatment with NM-3 ($P<0.01$). The tumor inhibitory rate of single NM-3 on tumors was 67.7 %, that in NM-3 combinated with carboplatin group was up to 98.7 % (Table3).

**Table 3** Growth of xerografted human gastric tumor affected by NM-3 in nude mice ($\bar{x}\pm s$)

| Treatment | *n* | Weight (g) | Size ($cm^3$) |
|---|---|---|---|
| NM-3 | 10 | 4.17±0.22[a] | 0.68±0.07[a] |
| Carboplatin | 10 | 4.46±0.23[a] | 0.71±0.08[a] |
| NM-3 plus carboplatin | 10 | 2.78±0.18[b] | 0.34±0.02[b] |
| Saline | 10 | 9.45±1.38 | 8.94±1.46 |

[a]$P<0.05$, [b]$P<0.01$ *vs t* test, in saline control group.

## DISCUSSION

Gastric cancer remains one of the most common causes of cancer-related death in the world. At present, gastric cancer is still diagnosed at its advanced stage in most patients throughout the world. Even with curative resection, they remain at a high risk of relapse[13-32]. Thus, there is a great need for effective adjuvant therapy for patients with gastric cancer. Our previous clinic paired comparative studies suggested that NM-3 had therapeutic effects on advanced gastric cancer. It could increase the surviving period of the patients, improve the life quality and increase the metastasis and recurrence after operation because of its lower toxic side-effect compared with intravenous chemical therapy[33-47]. Up to date, the effect of NM-3 on human gastric cancer has not been reported in the world. So we thought it is worth to make a further research on its anti-cancer mechanisms.

Gastric cancer is not only a disease with abnormal cell proliferation and differentiation, but also a disease with abnormal apoptosis. Enhanced apoptosis in human gastric cancer cells could be observed after treatmemt with 5-fluorouracil, cisplatin, arsenous oxide, *etc*. These data suggest that it is a therapeutic method for patients with gastric cancer to induce apoptosis of cancer cells[64]. The present study indicated that tumor growth was significantly inhibited by treatment with carboplatin or NM-3. The results obtained by TUNEL method and cytometry analysis suggested that gastric cancer cells were suppressed *in vivo*, NM-3 was related to the induction of apoptosis of human gastric cancer cell line SGC-7901. These data suggest that NM-3 can inhibit gastric cell proliferation. So inhibition of gastric cancer induced by NM-3 is also related to the suppression of its proliferation.

Apoptosis is a complex and active cellular process, whereby individual cells are triggered to undergo self-destruction in a manner that would neither injures neghiboring cells nor elicites any inflammatory reation. Various triggering factors initiate corresponding proteo-lysis cascade reaction depending on mitochondrion or APO 1\FAS\CD95 receptors mediate apoptotic pathways. There are oncogenes and tumor suppressor gene products in the regulation and execution of apoptosis.

It has been proved[14-22] that p53, Rb, myc, ras, raf, play important roles in apoptosis and are thus named the guardians of genomes[23-44]. They monitor the state of DNA and cell cycle is blocked in case of DNA damage. This takes place through the induction of CIP/Swaf/p21. In the absence of phosphorylated active cyclin-dependent kinases, the cell cycle remains inactive (unphosphorylated).

This leads to activation of DNA repair machinery. If DNA repair fails, p53 will take over again and trigger apoptosis in a process that involves upregulation of the apoptosis-inducing bax and down-regulation of the apoptotic bal-2[45-60]. We also detected apoptosis-inhibiting member of the bcl-2 family[61-64]: bcl-2 mRNA. The interaction between NM-3 and gastric cancer cell SGC-7901 induced its apoptosis of gastric cancer cells, but further *in vivo or in vitro* studies are needed.

## REFERENCES


1 **Richardson M,** Gunawan J, Hatton MW, Seidlitz E, Hirte HW, Singh G. Malignant ascites fluid (MAF), including ovarian-cancer-associated MAF, contains angiostatin and other factor(s) which inhibit angiogenesis.*Gynecol Oncol* 2002; **86:** 279-287

2 **Basaki Y,** Chikahisa L, Aoyagi K, Miyadera K, Yonekura K, Hashimoto A, Okabe S, Wierzba K, Yamada Y. Gamma-Hydroxybutyric acid and 5-fluorouracil, metabolites of UFT, inhibit the angiogenesis induced by vascular endothelial growth factor. *Angiogenesis* 2001; **4:** 163-173

3 **Yamamoto S**, Yasui W, Kitadai Y, Yokozaki H, Haruma K, Kajiyama G, Tahara E. Expression of vascular endothelial growth factor in human gastric carcinomas. *Pathol Int* 1998; **48:** 499-506

4 **Li C**, Guo B, Bernabeu C, Kumar S. Angiogenesis in breast cancer: The role of transforming growth factor beta and CD105. *Microsc Res Tech* 2001; **52:** 437-449

5 **Zhai Y,** Yu J, Iruela-Arispe L, Huang WQ, Wang Z, Hayes AJ, Lu J, Jiang G, Rojas L, Lippman ME, Ni J, Yu GL, Li LY. Inhibition of angiogenesis and breast cancer xenograft tumor growth by VEGI, a novel cytokine of the TNF superfamily. *Int J Cancer* 1999; **82:** 131-136

6 **Masood R**, McGarvey ME, Zheng T, Cai J, Arora N, Smith DL, Sloane N, Gill PS. Antineoplastic urinary protein inhibits Kaposi's sarcoma and angiogenesis *in vitro* and *in vivo*. *Blood* 1999; **93:** 1038-1044

7 **Zhai Y,** Ni J, Jiang GW, Lu J, Xing L, Lincoln C, Carter KC, Janat F, Kozak D, Xu S, Rojas L, Aggarwal BB, Ruben S, Li LY, Gentz R, Yu GL. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*. *FASEB J* 1999; **13:** 181-189

8 **Tsunemi T,** Nagoya S, Kaya M, Kawaguchi S, Wada T, Yamashita T, Ishii S. Postoperative progression of pulmonary metastasis in osteosarcoma. *Clin Orthop* 2003; **407:** 159-166

9 **Thomas JP,** Arzoomanian RZ, Alberti D, Marnocha R, Lee F, Friedl A, Tutsch K, Dresen A, Geiger P, Pluda J, Fogler W, Schiller JH, Wilding G. Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol* 2003; **21:** 223-231

10 **Anderson KC.** Moving disease biology from the laboratory to the clinic. *Semin Oncol* 2002; **29:** 17-20

11 **Folkman J**. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 2002; **29:** 15-18

12 **Ferrara N.** Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol* 2002; **29:** 10-14

13 **Jain RK**. Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. *Semin Oncol* 2002; **29:** 3-9

14 **Gee MS**, Procopio WN, Makonnen S, Feldman MD, Yeilding NM, Lee WM. Tumor vessel development and maturation impose limits on the effectiveness of anti-vascular therapy. *Am J Pathol* 2003; **162:** 183-193

15 **Koyanagi S,** Tanigawa N, Nakagawa H, Soeda S, Shimeno H. Oversulfation of fucoidan enhances its anti-angiogenic and antitumor activities. *Biochem Pharmacol* 2003; **65:** 173-179

16 **Buchler P**, Reber HA, Buchler M, Shrinkante S, Buchler MW, Friess H, Semenza GL, Hines OJ. Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer. *Pancreas* 2003; **26:** 56-64

17 **Chlenski A,** Liu S, Crawford SE, Volpert OV, DeVries GH, Evangelista A, Yang Q, Salwen HR, Farrer R, Bray J, Cohn SL. SPARC is a key Schwannian-derived inhibitor controlling neuroblastoma tumor angiogenesis.*Cancer Res* 2002; **62:** 7357-7363

18 **Davis PD**, Dougherty GJ, Blakey DC, Galbraith SM, Tozer GM, Holder AL, Naylor MA, Nolan J, Stratford MR, Chaplin DJ, Hill SA. ZD6126: a novel vascular-targeting agent that causes selective destruction of tumor vasculature. *Cancer Res* 2002; **62:** 7247-7253

19 **Cline EI**, Bicciato S, DiBello C, Lingen MW. Prediction of *in vivo* synergistic activity of antiangiogenic compounds by gene expression profiling. *Cancer Res* 2002; **62:** 7143-7148

20 **Giguere CM**, Bauman NM, Smith RJ. New treatment options for lymphangioma in infants and children. *Ann Otol Rhinol Laryngol* 2002; **111:** 1066-1075

21 **Manley PW**, Furet P, Bold G, Bruggen J, Mestan J, Meyer T, Schnell CR, Wood J, Haberey M, Huth A, Kruger M, Menrad A, Ottow E, Seidelmann D, Siemeister G, Thierauch KH. Anthranilic acid amides: a novel class of antiangiogenic VEGF receptor kinase inhibitors. *J Med Chem* 2002; **45:** 5687-5693

22 **Ranieri G**, Gasparini G. Angiogenesis and angiogenesis inhibitors: a new potential anticancer therapeutic strategy.*Curr Drug Targets Immune Endocr Metabol Disord* 2001; **1:** 241-253

23 **Lewy-Trenda I**. [Neoplasms and angiogenesis]. *Pol Merkuriusz Lek* 2002; **13:** 225-228

24 **Yoo GH**, Piechocki MP, Ensley JF, Nguyen T, Oliver J, Meng H, Kewson D, Shibuya TY, Lonardo F, Tainsky MA. Docetaxel induced gene expression patterns in head and neck squamous cell carcinoma using cDNA microarray and PowerBlot. *Clin Cancer Res* 2002; **8:** 3910-3921

25 **Lenzi R,** Rosenblum M, Verschraegen C, Kudelka AP, Kavanagh JJ, Hicks ME, Lang EA, Nash MA, Levy LB, Garcia ME, Platsoucas CD, Abbruzzese JL, Freedman RS. Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Mullerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. *Clin Cancer Res* 2002; **8:** 3686-3695

26 **Casassus P,** Caillat-Vigneron N, Martin A, Simon J, Gallais V, Beaudry P, Eclache V, Laroche L, Lortholary P, Raphael M, Guillevin L, Lortholary O. Treatment of adult systemic mastocytosis with interferon-alpha: results of a multicentre phase II trial on 20 patients. *Br J Haematol* 2002; **119:** 1090-1107

27 **Hata-Sugi N**, Kawase-Kageyama R, Wakabayashi T. Characterization of rat aortic fragment within collagen gel as an angiogenesis model; capillary morphology may reflect the action mechanisms of angiogenesis inhibitors. *Biol Pharm Bul* 2002; **25:** 446-451

28 **Matsunaga T,** Weihrauch DW, Moniz MC, Tessmer J, Warltier DC, Chilian WM. Angiostatin inhibits coronary angiogenesis during impaired production of nitric oxide. *Circulation* 2002; **105:** 2185-2191

29 **Chew LJ**, Pan H, Yu J, Tian S, Huang WQ, Zhang JY, Pang S, Li LY. A novel secreted splice variant of vascular endothelial cell growth inhibitor. *FASEB J* 2002; **16:** 742-744

30 **Kalmes A,** Daum G, Clowes AW. EGFR transactivation in the regulation of SMC function. *Ann N Y Acad Sci* 2001; **947:** 42-54

31 **Yu J**, Tian S, Metheny-Barlow L, Chew LJ, Hayes AJ, Pan H, Yu GL, Li LY. Modulation of endothelial cell growth arrest and apoptosis by vascular endothelial growth inhibitor. *Circ Res* 2001; **89:** 1161-1167

32 **Wong ST,** Baker LP, Trinh K, Hetman M, Suzuki LA, Storm DR, Bornfeldt KE. Adenylyl cyclase 3 mediates prostaglandin E(2)-induced growth inhibition in arterial smooth muscle cells. *J Biol Chem* 2001; **276:** 34206-34212

33 **Stepan H,** Leitner E, Bader M, Walther T. Organ-specific mRNA distribution of C-type natriuretic peptide in neonatal and adult mice. *Regul Pept* 2000; **95:** 81-85

34 **Damon DH.** Adrenoceptor-mediated modulation of endothelial-dependent vascular smooth muscle growth. *J Auton Pharmacol* 2000; **20**: 47-54

35 **Veyssier-Belot C**, Cacoub P. Role of endothelial and smooth muscle cells in the physiopathology and treatment management of pulmonary hypertension. *Cardiovasc Res* 1999; **44:** 274-282

36 **Hishikawa K**, Oemar BS, Tanner FC, Nakaki T, Fujii T, Luscher TF. Overexpression of connective tissue growth factor gene induces apoptosis in human aortic smooth muscle cells. *Circula-*

*tion* 1999; **100:** 2108-2112
37 **Shukunami C**, Iyama K, Inoue H, Hiraki Y. Spatiotemporal pattern of the mouse chondromodulin-I gene expression and its regulatory role in vascular invasion into cartilage during endochondral bone formation. *Int J Dev Biol* 1999; **43:** 39-49
38 **Nakano N**, Higashiyama S, Takashima S, Tsuruoka N, Klagsbrun M,Taniguchi N. Purification and characterization of a novel vascular endothelial cell growth inhibitor secreted by macrophage-like U-937 cells. *J Biochem* 1999; **125:** 368-374
39 **Masood R,** McGarvey ME, Zheng T, Cai J, Arora N, Smith DL, Sloane N, Gill PS. Antineoplastic urinary protein inhibits Kaposi's sarcoma and angiogenesis *in vitro* and *in vivo*. *Blood* 1999; **93:** 1038-1044
40 **Zhai Y**, Ni J, Jiang GW, Lu J, Xing L, Lincoln C, Carter KC, Janat F, Kozak D, Xu S, Rojas L, Aggarwal BB, Ruben S, Li LY, Gentz R, Yu GL. VEGI, a novel cytokine of the tumor necrosis factor family, is an angiogenesis inhibitor that suppresses the growth of colon carcinomas *in vivo*. *FASEB J* 1999; **13:** 181-189
41 **Van Rees BP**, Caspers E, Zur Hausen A, Van Den Brule A, Drillenburg P, Weterman MA, Offerhaus GJ. Different pattern of allelic loss in Epstein-Barr virus-positive gastric cancer with emphasis on the p53 tumor suppressor pathway. *Am J Pathol* 2002; **161:** 1207-1213
42 **Hiyama T**, Tanaka S, Kitadai Y, Ito M, Sumii M, Yoshihara M, Shimamoto F, Haruma K, Chayama K. p53 Codon 72 polymorphism in gastric cancer susceptibility in patients with *Helicobacter pylori*-associated chronic gastritis. *Int J Cancer* 2002; **100:** 304-308
43 **Murakami K**, Fujioka T, Kodama M, Honda S, Okimoto T, Oda T, Nishizono A, Sato R, Kubota T, Kagawa J, Nasu M. Analysis of p53 mutations and *Helicobacter pylori* infection in human and animal models. *J Gastroenterol* 2002; **37:** 1-5
44 **Chang MS**, Kim HS, Kim CW, Kim YI, Lan Lee B, Kim WH. Epstein-Barr virus, p53 protein, and microsatellite instability in the adenoma-carcinoma sequence of the stomach. *Hum Pathol* 2002; **33:** 415-420
45 **Moritani S,** Sugihara H, Kushima R, Hattori T. Different roles of p53 between Epstein-Barr virus-positive and -negative gastric carcinomas of matched histology. *Virchows Arch* 2002; **440**: 367-375
46 **Shigeishi H**, Yokozaki H, Oue N, Kuniyasu H, Kondo T, Ishikawa T, Yasui W. Increased expression of CHK2 in human gastric carcinomas harboring p53 mutations. *Int J Cancer* 2002; **99:** 58-62
47 **Kubicka S**, Claas C, Staab S, Kuhnel F, Zender L, Trautwein C, Wagner S, Rudolph KL, Manns M. p53 mutation pattern and expression of c-erbB2 and c-met in gastric cancer: relation to histological subtypes, *Helicobacter pylori* infection, and prognosis. *Dig Dis Sci* 2002; **47:** 114-121
48 **Fox JG,** Sheppard BJ, Dangler CA, Whary MT, Ihrig M, Wang TC. Germ-line p53-targeted disruption inhibits helicobacter-induced premalignant lesions and invasive gastric carcinoma through down-regulation of Th1 proinflammatory responses. *Cancer Res* 2002; **62:** 696-702
49 **Iwamatsu H,** Nishikura K, Watanabe H, Ajioka Y, Hashidate H, Kashimura H, Asakura H. Heterogeneity of p53 mutational status in the superficial spreading type of early gastric carcinoma. *Gastric Cancer* 2001; **4:** 20-26
50 **Jenkins GJ**, Morgan C, Baxter JN, Parry EM, Parry JM. The detection of mutations induced in vitro in the human p53 gene by hydrogen peroxide with the restriction site mutation (RSM)assay. *Mutat Res* 2001; **498:** 135-144
51 **Li HL**, Chen DD, Li XH, Zhang HW, Lu YQ, Ye CL, Ren XD.Changes of NF-kB, p53, Bcl-2 and caspase in apoptosis induced by JTE-522 in human gastric adenocarcinoma cell line AGS cells: role of reactive oxygen species. *World J Gastroenterol* 2002; **8:** 431-435
52 **Satomi D**, Takiguchi N, Koda K, Oda K, Suzuki H, Yasutomi J, Ishikura H, Miyazaki M. Apoptosis and apoptosis-associated gene products related to the response to neoadjuvant chemotherapy for gastric cancer. *Int J Oncol* 2002; **20:** 1167-1171
53 **Wu YL**, Sun B, Zhang XJ, Wang SN, He HY, Qiao MM, Zhong J, Xu JY. Growth inhibition and apoptosis induction of Sulindac on Human gastric cancer cells. *World J Gastroenterol* 2001; **7:** 796-800
54 **Wang J,** Chi DS, Kalin GB, Sosinski C, Miller LE, Burja I, Thomas E. *Helicobacter pylori* infection and oncogene expressions in gastric carcinoma and its precursor lesions. *Dig Dis Sci* 2002; **47:** 107-113
55 **Zhang TC**, Cao EH, Qin JF. Opposite biological effects of arsenic trioxide and arsacetin involve a different regulation of signaling in human gastric cancer MGC-803 cells. *Pharmacology* 2002; **64:** 160-168
56 **Monden N**, Abe S, Hishikawa Y, Yoshimura H, Kinugasa S, Dhar DK, Tachibana M, Nagasue N. The role of P-glycoprotein in human gastric cancer xenografts in response to chemotherapy. *Int J Surg Investig* 1999; **1:** 3-10
57 **Wacheck V**, Heere-Ress E, Halaschek-Wiener J, Lucas T, Meyer H, Eichler HG, Jansen B. Bcl-2 antisense oligonucleotides chemosensitize human gastric cancer in a SCID mouse xenotransplantation model. *J Mol Med* 2001; **79:** 587-593
58 **Enomoto A**, Esumi M, Yamashita K, Takagi K, Takano S, Iwai S. Abnormal nucleotide repeat sequence in the TGF-betaRII gene in hepatocellular carcinoma and in uninvolved liver tissue. *J Pathol* 2001; **195:** 349-354
59 **Shyu RY**, Lin DY, Reichert U, Jiang SY. Synthetic retinoid CD437 induces cell-dependent cycle arrest by differential regulation of cell cycle associated proteins. *Anticancer Res* 2002; **22:** 2757-2764
60 **Li Y,** Lu YY. Isolation of diallyl trisulfide inducible differentially expressed genes in human astric cancer cells by modified cDNA representational difference analysis. *DNA Cell Biol* 2002; **21:** 771-780
61 **Potthoff A**, Ledig S, Martin J, Jandl O, Cornberg M, Obst B, Beil W, Manns MP, Wagner S. Significance of the caspase family in *Helicobacter pylori* induced gastric epithelial apoptosis.*Helicobacter* 2002; **7:** 367-377
62 **Nitti D**, Belluco C, Mammano E, Marchet A, Ambrosi A, Mencarelli R, Segato P, Lise M. Low level of p27(Kip1) protein expression in gastric adenocarcinoma is associated with disease progression and poor outcome. *J Surg Oncol* 2002; **81:** 167-175
63 **Chen Y,** Wu Q, Song SY, Su WJ. Activation of JNK by TPA promotes apoptosis via PKC pathway in gastric cancer cells. *World J Gastroenterol* 2002; **8:** 1014-1018
64 **Liu JR**, Chen BQ, Yang YM, Wang XL, Xue YB, Zheng YM, Liu RH. Effect of apoptosis on gastric adenocarcinoma cell line SGC-7901 induced by cis-9, trans-11-conjugated linoleic acid. *World J Gastroenterol* 2002; **8:** 999-1004

**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2370-2373
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Selection and evaluation of three interventional procedures for achalasia based on long-term follow-up

Ying-Sheng Cheng, Ming-Hua Li, Wei-Xiong Chen, Ni-Wei Chen, Qi-Xin Zhuang, Ke-Zhong Shang

**Ying-Sheng Cheng, Ming-Hua Li, Qi-Xin Zhuang, Ke-Zhong Shang,** Department of Radiology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Wei-Xiong Chen, Ni-Wei Chen,** Department of Gastroenterology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China
**Supported by** the National Key Medical Research and Development Program of China during the 9th Five-year Plan Period (No.96-907-03-04), Shanghai Nature Science Funds (No.02Z1314073), Shanghai Medical Development Funds (No.00419)
**Correspondence to:** Dr. Ying-Sheng Cheng, Department of Radiology, Sixth People' s Hospital, Shanghai Jiaotong University, Shanghai 200233, China. chengys@sh163.net
**Telephone:** +86-21-64368920 **Fax:** +86-21-64701361
**Received:** 2003-05-13 **Accepted:** 2003-06-02

## Abstract

**AIM:** To determine the best method out of the three types of interventional procedure for achalasia based on a long-term follow-up.

**METHODS:** The study cohort was comprised of 133 patients of achalasia. Among them, 60 patients were treated under fluoroscopy with pneumatic dilation (group A), 8 patients with permanent uncovered or antireflux covered metal stent dilation (group B), and 65 patients with temporary partially covered metal stent dilation (group C).

**RESULTS:** One hundred and thirty dilations were performed on the 60 patients of group A (mean 2.2 times per case). The mean diameter of the strictured cardia was 3.3±2.1 mm before dilation and 10.6±3.8 mm after dilation. The mean dysphagia score was 2.7±1.4 before dilation and 0.9±0.3 after dilation. Complications in group A were chest pain ($n$=30), reflux ($n$=16), and bleeding ($n$=6). Thirty-six patients (60 %) in group A exhibited dysphagia relapse during a 12-month follow-up, and 45 patients (90 %) out of 50 exhibited dysphagia relapse during a 36-month follow-up. Five uncovered and 3 antireflux covered expandable metal stents were permanently placed in the 8 patients of group B. The mean diameter of the strictured cardia was 3.4±1.9 mm before dilation and 19.5±1.1 mm after dilation. The mean dysphagia score was 2.6±1.3 before dilation and 0.4±0.1 after dilation. Complications in group B were chest pain ($n$=6), reflux ($n$=5), bleeding ($n$=3), and hyperplasia of granulation tissue ($n$=3). Four patients (50 %) in group B exhibited dysphagia relapse during a 12-month follow-up, and 2 case (66.7 %) out of 3 patients exhibited dysphagia relapse during a 36-month follow-up. Sixty-five partially covered expandable metal stents were temporarily placed in the 65 patients of group C and withdrawn after 3-7 days via gastroscopy. The mean diameter of the strictured cardia was 3.3±2.3 mm before dilation and 18.9±3.5 mm after dilation. The mean dysphagia score was 2.4±1.3 before dilation and 0.5±0.2 after dilation. Complications in group C were chest pain ($n$=26), reflux ($n$=13), and bleeding ($n$=8). 6 patients (9.2 %) out of 65 exhibited dysphagia relapse during a 12-month follow-up, and 8 patients (14.5 %) out of 55 exhibited dysphagia relapse during a 36-month follow-up. All the stents were inserted and withdrawn successfully. The follow-up in groups A-C lasted 12-96 months.

**CONCLUSION:** Temporary partially covered metal stent dilation is one of the best methods with interventional procedure for achalasia in terms of long-term follow-up.

Cheng YS, Li MH, Chen WX, Chen NW, Zhuang QX, Shang KZ. Selection and evaluation of three interventional procedures for achalasia based on long-term follow-up. *World J Gastroenterol* 2003; 9(10):2370-2373
http://www.wjgnet.com/1007-9327/9/2370.asp

## INTRODUCTION

Achalasia is the most common primary motility disorder of the esophagus. Three interventional procedures are used clinically for achalasia, namely pneumatic dilation, permanent metal stent dilation, and temporary metal stent dilation. These methods provide excellent immediate therapeutic efficacy, but their long-term results are unknow[1,2]. Therefore, we formulated several treatment plans for patients with achalasia from July 1994 to May 2002 and evaluated them in terms of long-term follow-up.

## MATERIALS AND METHODS

### *Materials*

The subjects were 133 patients (77 males, 56 females; aged 12-84 years, mean 48.3 years) with symptoms of dysphagia. A dysphagia score was assessed by the quality of swallowing[1,2]: grade 0 for normal swallowing, grade 1 for swallowing most solid food, grade 2 for swallowing semisolids, grade 3 for swallowing liquid food, and grade 4 for complete dysphagia. Based on different methods of interventional procedure, the patients were divided into three groups as follows. In 60 patients with pneumatic dilation (group A), the mean dysphagia score was 2.7±1.4, and the mean diameter of the narrowest region of the cardia was 3.3±2.1 mm. In 8 patients with permanent uncovered or antireflux covered metal stent dilation (group B), the mean dysphagia score was 2.6±1.3, and the mean diameter of the narrowest region of the cardia was 3.4±1.9 mm. In 65 patients with temporary partially covered metal internal stent dilation (group C), the mean dysphagia score was 2.4±1.3, and the mean diameter of the narrowest region of the cardia was 3.1±2.3 mm. The course of disease in all the patients was 1-10 years (mean 5.4±4.4 years). All the patients were examined by barium-meal radiography of the upper digestive tract and gastroscope or esophageal intracavity manometric method.

### *Methods*

Preoperative preparation involved an empty stomach for at least 4 hours and examination of the bleeding and clotting times. The device used was an SY dumbbell-like catheter (manufactured in Jinan, Shandong, China). The metal stent

for achalasia was a nitinol stent (developed by Zhiye Medical Equipment Research Institute, Changzhou, China, and Youyan Yijin Advanced Materials Co.Ltd, Beijing, China). Uncovered and antireflux covered metal stents were used in group B, partially covered metal stents were used in group C. The body of partially covered stents was covered with intracavity silica gel. The areas within 2 cm of both ends of partially covered stents were not covered. Stents were 6-10 cm in length and 16-30 mm in diameter.

The patients in which pneumatic dilation was used were placed in lying on the side or sitting position. Topical anesthesia of the pharynx was administered before the procedure. A guidewire was inserted through the mouth and passed through the stricture section under fluoroscopy. A catheter with a diameter of 28 mm was passed through the region of achalasia of the esophagus via the guidewire, which aligned the center of saccule with the most strictured point. The saccule was injected using an injector with dilated contrast medium or gas. Under fluoroscopy, and according to the pain reaction of the patient, pressurization was applied to gradually dilation of the saccule. The back of saccule was dumbbell-shaped. When further pressurization flattened the surface of saccule or when the pressure did not change as pressurization was applied, pressurization was suspended and the piston was closed off. The pressure of saccule was maintained for 5-30 min, after which the piston was released. After the pressure of saccule had been reduced for 5 min, pressurization was again applied. Typically each treatment involved 3-5 dilations, after which the catheter was withdrawn. The second and third treatments with graded pneumatic dilation were carried out using dilators with diameters of 30 mm and 32 mm, respectively, in some of the patients every 2 weeks until clinical symptoms disappeared and the patients returned to a nomal diet.

When stents were placed in groups B and C, the sites of thoracic vertebra and spine were determined by barium-meal radiography to facilitate the stent placement. Patients were placed in a sitting position or lying on the side, and false teeth were removed and a teeth bracket was mounted. A 260-cm-long exchange guidewire was firstly led into the stomach. The stent was installed on the propeller whose front end was covered with sterilized liquid paraffin. Guided by the guidewire, the propeller on which the stent was mounted was moved through the segment with lesions. Under fluoroscopic control, the outer sheath was slowly withdrawn and the stent expanded under its own tension. After a stent was placed, esophageal radiography was performed to observe the patency of the esophagus. In group C, 500-1 000 ml of ice-cold water was injected 3-7 days after stent placement via a bioptic hole under gastroscope, which caused the stent to retract and reduce its diameter. Bioptic pliers were then used to withdraw the stent with the help of a gastroscope. Gastroscopy was performed again to detect complications, such as bleeding, mucosa tearing, and esophageal perforation. The patients returned to the ward and consumed cold drinks and snacks for 2 days before returning to a normal diet. It was preferable for patients to eat solid food since the natural expansion of food reduced retraction of the esophagus.

The criteria for therapeutic efficacy were as follows: the diameter of the narrowest region of the esophagus before and after dilation, and the dysphagia score before and after dilation. Postoperative treatment of pneumatic dilation, barium-meal radiography of the esophagus was performed immediately after interventional procedure to check the esophagus patency and perforation and submucous hematomas. Patients drank fluids for 2 h after interventional procedure and were treated with antibiotics, antacid drugs, and analgesics. In groups B and C, after stent placement, barium-meal radiography was used to observe the patency of the esophagus. Patients ate semisolid food on the day after interventional procedure and were treated with antibiotics and antacid drugs. In group C, esophageal radiography was performed within 1 week after stent removal to observe the patency of the esophagus. The follow-up time was 1 month, 6 months, 1 year and 3 years by telephone or clinic visit.

All the data were expressed as the mean±SD, and the paired *t*-test was used for statistical comparisons before and after interventional procedure within a group.

## RESULTS

The 60 patients in group A involved 130 dilations (mean 2.2 times per case), of which 29 patients had three graded dilations of increasing diameter, 12 patients had two graded dilations of increasing diameter, and 19 patients had one dilation. In 8 patients of group B, 5 uncovered and 3 antireflux covered stents were successfully placed. In group C, 65 partially covered stents were placed and removed under gastroscope guidance 3-7 days after intervertional procedure. The success rate of stent placement and removal was 100 %. The differences in the cardia diameter before and after the three methods of interventional procedure and the dysphagia scores (Table 1) were statistically significant ($P<0.01$). The incidence of complications in the three interventional procedures is presented in Table 2 and the rate of dysphagia recurrence during follow-up is shown in Table 3. The follow-up period for the three interventional procedures was 12-96 months.

**Table 1** Diameter of the narrowest cardia region before and after treatment with three interventional procedures, and dysphagia score

| Group | Diameter of cardia before and after treatment (mm) | | Dysphagia score before and after treatment (grade) | |
|---|---|---|---|---|
| A | 3.2±2.1 | 10.6±3.8[b] | 2.7±1.4 | 0.9±0.3[b] |
| B | 3.4±1.9 | 19.5±1.1[b] | 2.6±1.3 | 0.4±0.1[b] |
| C | 3.1±2.3 | 18.9±3.5[b] | 2.4±1.3 | 0.5±0.2[b] |

[b]$P<0.01$ *vs* before and after treatment.

**Table 2** Incidence of complications following treatment with three interventional procedures (%)

| Group | Pain (*n*) | Reflux (*n*) | Bleeding (*n*) | Hyperplasia of granulation tissue (*n*) |
|---|---|---|---|---|
| A | 50.0 %(30/60) | 26.7 %(16/60) | 10.0 %(6/60) | - |
| B | 62.5 %(5/8) | 62.5 %(5/8) | 37.5 %(3/8) | 37.5 %(3/8) |
| C | 40.0 %(26/65) | 20.0 %(13/65) | 12.3 %(8/65) | - |

**Table 3** Relapse rate of dysphagia during follow-up

| Group | Follow-up >12 months | | | Follow-up >36 months | | |
|---|---|---|---|---|---|---|
| | Follow-up (*n*) | Relapse of dysphagia (*n*) | Relapse rate (%) | Follow-up (*n*) | Relapse of dysphagia (*n*) | Relapse rate (%) |
| A | 60 | 36 | 60% | 50 | 45 | 90 % |
| B | 8 | 4 | 50% | 3 | 2 | 66.7 % |
| C | 65 | 6 | 9.2% | 55 | 8 | 14.5 % |

## DISCUSSION

### *Techniques of interventional procedures*

The techniques used to treat achalasia, such as surgery, bougienage, pneumatic dilation, botulinum toxin injection, permanently uncovered or antireflux covered metal stent dilation and temporary partially covered metal stent dilation, had advantages and drawbacks[1-5]. Bougienage is now uncommon since it has poor therapeutic efficacy and many complications. The use of surgery is declining due to the associated large lesion, a high risk, and high recurrence rate. Pneumatic dilation was first introduced in the plasty of hematostenosis, as its reliable therapeutic efficacy led to its gradual application to other plasty operations. Remarkable results were achieved when it was used in benign gastrointestinal strictures, and later it was widely used in the nonsurgical treatment of achalasia, exhibiting remarkable therapeutic efficacy. Many authors[6-15] have reported that graded dilation is better than single dilation in therapeutic efficacy, and our experience has confirmed this. Botulinum toxin injection in achalasia had a short term therapentic efficacy, dysphagia was relapsed witnin 6 months.

Permanent metal stent dilation is primarily used in the treatment of malignant gastrointestinal stricture and obstruction, and exhibits remarkable palliative therapeutic efficacy. Cwikiel *et al*[1] reported an experimental and clinical study of the treatment of benign esophageal stricture with expandable metal stents. We used uncovered stents in five patients with achalasia in order to reduce the occurrence rate of stent migration. After stent placement, dilation was excellent and dysphagia disappeared, thus achieving the goal of treatment. However, it was accompanied by new problems such as gastroesophageal reflux, recurrence of stricture (hyperplasia of granulation tissue). The reflux could be treated with drugs, but this took a long time. Recurrence of stricture could be reduced by heat cauterization under gastroscope, but it could easily reccur. So we used antireflux covered stent, compication of gastroesophageal reflux and hyperplasia of granulation tissue were not found, but many unexpected results occurred. These difficulties led to the use of a temporary partially covered metal stent dilation. Clinicians and patients have gradually accepted and now prefer to use temporary partially covered metal stent dilation due to its fewer complications and excellent therapeutic efficacy[16-22].

### *Long-term follow-up*

Dysphagia recurred in 60 % of the patients at a 12-month follow-up, and in 90 % of the patients at a 36-months follow-up, demonstrating that pneumatic dilation of achalasia has excellent immediate therapeutic efficacy but its long-term therapeutic efficacy is poor[23-44]. Firstly, this was associated with the diameter of the saccule. Kadakia *et al*[10] suggested that the saccule diameter in pneumatic dilation should be 35-45 mm, but the incidence of complications was very high (e.g., 15 % had esophageal perforation). We used saccules with diameters of 28-32 mm in order to reduce the incidence of serious complications, but their long-term therapeutic efficacy was not satisfactory. Secondly, the therapeutic efficacy was associated with the frequency of dilation. One dilation did not produce excellent therapeutic efficacy, since it was affected by various factors. For example, whether the saccule was correctly located, whether pressure applied to the saccule reached the stipulated index, and variations in the anatomy of the cardia. It was suggested that three graded dilations should be used to achieve the treatment goal. Thirdly, the therapeutic efficacy was associated with the course of the disease. If this was very short, the cardiac muscularis was not fleshy and elastic. If this was very long, the cardiac muscularis was fleshy and not elastic. We used permanently uncovered stent dilation in five patients with achalasia and achieved excellent immediate therapeutic efficacy, but its long-term therapeutic efficacy was poor. This was mainly due to the frequent occurrence of serious gastroesophageal reflux and hyperplasia of granulation tissue. After a 12-month follow-up the stent could not be removed in three patients, and hence we had to resect and reconstruct the esophageal cardia. Therefore, permanently uncovered metal stent dilation was unsuitable for patients with achalasia[45-48]. Temporary partially covered metal stent dilation had excellent immediate and long-term therapeutic efficacy. First, the design of the stent coincided with the specific physiological structure of the cardia and the specific pathological manifestations of achalasia. The epicardia is a part of the expanded esophagus and the lower cardiac part is a very large gastric cavity. If a stent is not well designed, it will lose its therapeutic efficacy, and moreover, the rate of stent migrations will increase. To avoid these problems, we designed a special stent for achalasia. The stent was partially covered with a membrane covering the inner wall of the stent but not covering the area within 2 cm of the stent outlet. The upper outlet of the stent was a large horn, which increased the stability of the stent but made it difficult to extract. Second, the diameter of the stents used in this group was 20-30 mm. By expanding the stent, the cardia could be returned nearly to the maximum diameter of the normally dilated esophageal lumen. The most appropriate stent diameter was that which could expand the cardia stricture while not cause gastroesophageal reflux. This needs to be investigated further. Thirdly, the internal metal stent expansion procedure took a long time, and the stent was placed for 3-7 days. Why the therapeutic efficacy of temporary partially covered stent dilation was better than that of pneumatic dilation? We considered that this was mainly due to the stent expansion which caused chronic tearing of the cardia muscularis. The stent gradually expanded with body temperature, taking 12-24 h to reach 36 ℃, for it to reach the expected diameter. Therefore, the cardia muscularis was torn regularly with relatively few scars formed and a very low incidence of restenosis when it was repaired. In pneumatic dilation the tearing of cardia muscularis was acute and irregular with many scars formed when it was repaired. Therefore, restenosis was common and the long-term therapeutic efficacy was poor. This might explain why the therapeutic efficacy of temporary partially covered metal stent dilation was better in the treatment of achalasia than that of pneumatic dilation.

Developments in biologically degradable stents for the esophagus which are degraded within 2 months, would provide the advantages of a long retention time without the need for stent removal. This would provide another interventional procedure for patients with achalasia. We compared three methods of interventional procedure for patients with achalasia and took the following factors into consideration such as extent of lesion, incidence of complications, therapeutic efficacy, and degree of patient acceptance. We found that in the treatment of benign gastrointestinal stricture, the use of temporary partially covered metal internal stent dilation was preferred due to its superior long-term therapeutic efficacy.

## REFERENCES

1 **Cwikiel W,** Willen R, Stridbeck H, Liool-Gil R, Von Holstein CS. Self-expanding stent in the treatment of benign esophageal stricture:experimental study in pigs and presentation of clinical cases. *Radiology* 1993; **187**: 667-671

2 **Song HY,** Park SI, Do YS, Yoon HK, Sung KB, Sohn KH, Min YI. Expandable metallic stent placement in patients with benign esophageal strictures: results of long-term follow-up. *Radiology* 1997; **203**: 131-136

3 **Kadakia SC,** Wong RK. Graded pneumatic dilation using Rigiflex achalsia dilators in patients with primary esophageal achalasia.

*Am J Gastroenterol* 1993; **88**: 34-38
4 **Vaezi MF,** Richter JE. Current therapies for achalasia: comparison and efficacy. *J Clin Gastroenterol* 1998; **27**: 21-35
5 **Peracchia A,** Bonavina L.Achalasia: dilation, injection or surgery? *Can J Gastroenterol* 2000; **14**: 441-443
6 **Hoogerwerf WA,** Pasricha PJ. Achalasia: treatment options revisited. *Can J Gastroenterol* 2000; **14**: 406-409
7 **Massey BT.** Management of idiopathic achalasia: short-term and long-term outcomes. *Curr Gastroenterol Rep* 2000; **2**: 196-200
8 **Scarpignato C.** New therapeutic modalities for benign oesophageal disease: an overview. *Dig Liver Dis* 2001; **33**: 260-265
9 **Urbach DR,** Hansen PD, Khajanchee YS, Swanstrom LL.A decision analysis of the optimal initial approach to achalasia: laparoscopic Heller myotomy with partial fundoplication, thoracoscopic Heller myotomy, pneumatic dilatation, or botulinum toxin injection. *J Gastrointest Surg* 2001; **5**: 192-205
10 **Kadakia SC,** Wong RK. Pneumatic balloon dilation for esophageal achalasia. *Gastrointest Endosc Clin N Am* 2001; **11**: 325-346
11 **Bittinger M,** Wienbeck M. Pneumatic dilation in achalasia. *Can J Gastroenterol* 2001; **15**: 195-199
12 **Da Silveira EB,** Rogers AI. Achalasia: a review of therapeutic options and outcomes. *Compr Ther* 2002; **28**: 15-22
13 **Trifan A,** Stanciu C. Treatment of achalasia: an update. *Rev Med Chir Soc Med Nat Iasi* 2000; **104**: 11-13
14 **Chen WX,** Cheng YS, Yang RJ, Li MH, Zhuang QX, Chen NW, Xu JR, Shang KZ. Interventional therapy of achalasia with temporary metal internal stent dilatation and its intermediate and long term follow-up. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 896-899
15 **Cheng YS,** Yang RJ, Li MH, Shang KZ, Chen WX, Chen NW, Chu YD, Zhuang QX. Interventional procedure for benign or malignant stricture or obstruction of upper gastrointestinal tract. *Shijie Huaren Xiaohua Zazhi* 2000; **8**: 1354-1360
16 **Chen WX,** Cheng YS, Yang RJ, Li MH, Shang KZ, Zhuang QX, Chen NW. Metal stent dilation in the treatment of benign esophageal stricture by interventional procedure:a follow-up study. *Shijie Huaren Xiaohua Zazhi* 2002; **10**: 333-336
17 **Bansal R,** Nostrant TT, Scheiman JM, Koshy S, Barnett JL, Elta GH, Chey WD. Intrasphincteric botulinum toxin versus pneumatic balloon dilation for treatment of primary achalasia. *J Clin Gastroenterol* 2003; **36**: 209-214
18 **Scatton O,** Gaudric M, Massault PP, Chaussade S, Houssin D, Dousset B. Conservative management of esophageal perforation after pneumatic dilatation for achalasia. *Gastroenterol Clin Biol* 2002; **26**: 883-887
19 **Khan AA,** Shah SW, Alam A, Butt AK, Shafqat F. Efficacy of Rigiflex balloon dilatation in 12 children with achalasia: a 6-month prospective study showing weight gain and symptomatic improvement. *Dis Esophagus* 2002; **15**: 167-170
20 **Sabharwal T,** Cowling M, Dussek J, Owen W, Adam A. Balloon dilation for achalasia of the cardia: experience in 76 patients. *Radiology* 2002; **224**: 719-724
21 **O'Connor JB,** Singer ME, Imperiale TF, Vaezi MF, Richter JE. The cost-effectiveness of treatment strategies for achalasia. *Dig Dis Sci* 2002; **47**: 1516-1525
22 **West RL,** Hirsch DP, Bartelsman JF, de Borst J, Ferwerda G, Tytgat GN, Boeckxstaens GE. Long term results of pneumatic dilation in achalasia followed for more than 5 years. *Am J Gastroenterol* 2002; **97**: 1346-1351
23 **Upadhyaya M**, Fataar S, Sajwany MJ. Achalasia of the cardia: experience with hydrostatic balloon dilatation in children. *Pediatr Radiol* 2002; **32**: 409-412
24 **Vaezi MF,** Baker ME, Achkar E, Richter JE. Timed barium oesophagram: better predictor of long term success after pneumatic dilation in achalasia than symptom assessment. *Gut* 2002; **50**: 765-770
25 **Penagini R,** Cantu P, Mangano M, Colombo P, Bianchi PA. Long-term effects of pneumatic dilatation on symptoms and lower oesophageal sphincter pressure in achalasia. *Scand J Gastroenterol* 2002; **37**: 380-384
26 **Babu R,** Grier D, Cusick E, Spicer RD. Pneumatic dilatation for childhood achalasia. *Pediatr Surg Int* 2001; **17**: 505-507
27 **Becker K,** Biesenbach S, Erckenbrecht JF, Frieling T. Effect of balloon compliance on symptomatic success of pneumatic dilation in achalasia patients. *Z Gastroenterol* 2001; **39**: 831-836
28 **Diener U,** Patti MG, Molena D, Tamburini A, Fisichella PM, Whang K, Way LW. Laparoscopic Heller myotomy relieves dysphagia in patients with achalasia and low LES pressure following pneumatic dilatation. *Surg Endosc* 2001; **15**: 687-690
29 **Swift GL,** Smith PM, McKirdy HC, Lowndes RH. Vector volume analysis of the lower esophageal sphincter in achalasia and the effect of balloon dilation. *Dis Esophagus* 2001; **14**: 54-56
30 **Hep A,** Dolina J, Dite P, Plottova Z, Valek V, Kala Z, Prasek J. Restoration of propulsive peristalsis of the esophagus in achalasia. *Hepatogastroenterology* 2000; **47**: 1203-1204
31 **Hunt DR,** Wills VL, Weis B, Jorgensen JO, DeCarle DJ, Coo IJ. Management of esophageal perforation after pneumatic dilation for achalasia. *J Gastrointest Surg* 2000; **4**: 411-415
32 **Morino M,** Rebecchi F. Pneumatic dilatation and laparoscopic cardiomyotomy in the management of achalasia. *Surg Endosc* 2000; **14**: 870-871
33 **Rajput S,** Nandwani SK, Phadke AY, Bhandarkar PV, Abraham P, Tilve GH.Predictors of response to pneumatic dilatation in achalasia cardia. *Indian J Gastroenterol* 2000; **19**: 126-129
34 **Hamza AF,** Awad HA, Hussein O. Cardiac achalasia in children. Dilatation or surgery? *Eur J Pediatr Surg* 1999; **9**: 299-302
35 **Ponce J,** Juan M, Garrigues V, Pascual S, Berenguer J. Efficacy and safety of cardiomyotomy in patients with achalasia after failure of pneumatic dilatation. *Dig Dis Sci* 1999; **44**: 2277-2282
36 **Gideon RM,** Castell DO, Yarze J. Prospective randomized comparison of pneumatic dilatation technique in patients with idiopathic achalasia. *Dig Dis Sci* 1999; **44**: 1853-1857
37 **Singh V,** Duseja A, Kumar A, Kumar P, Rai HS, Singh K. Balloon dilatation in achalasia cardia. *Trop Gastroenterol* 1999; **20**: 68-69
38 **Khan AA,** Shah SW, Alam A, Butt AK, Shafqat F, Castell DO. Massively dilated esophagus in achalasia: response to pneumatic balloon dilation. *Am J Gastroenterol* 1999; **94**: 2363-2366
39 **Vaezi MF,** Baker ME, Richter JE. Assessment of esophageal emptying post-pneumatic dilation: use of the timed barium esophagram. *Am J Gastroenterol* 1999; **94**: 1802-1807
40 **Metman EH,** Lagasse JP, d'Alteroche L, Picon L, Scotto B, Barbieux JP. Risk factors for immediate complications after progressive pneumatic dilation for achalasia. *Am J Gastroenterol* 1999; **94**: 1179-1185
41 **Lisy J**, Hetkova M, Snajdauf J, Vyhnanek M, Tuma S. Long-term outcomes of balloon dilation of esophageal strictures in children. *Acad Radiol* 1998; **5**: 832-835
42 **Katz PO,** Gilbert J, Castell DO. Pneumatic dilatation is effective long-term treatment for achalasia. *Dig Dis Sci* 1998; **43**: 1973-1977
43 **Khan AA,** Shah SW, Alam A, Butt AK, Shafqat F, Castell DO. Pneumatic balloon dilation in achalasia: a prospective comparison of balloon distention time. *Am J Gastroenterol* 1998; **93**: 1064-1067
44 **Mukherjee S,** Kaplan DS, Parasher G, Sipple MS. Expandable metal stents in achalasia-is there a role? *Am J Gastroenterol* 2000; **95**: 2185-2188
45 **Lee JG,** Hsu R, Leung JW. Are self-expanding metal mesh stents useful in the treatment of benign esophageal stenoses and fistulas? An experience of four cases. *Am J Gastroenterol* 2000; **95**: 1920-1925
46 **De Palma GD,** Catanzano C. Removable self-expanding metal stents: a pilot study for treatment of achalasia of the esophagus. *Endoscopy* 1998; **30**: S95-96
47 **Mukherjee S,** Kaplan DS, Parasher G, Sipple MS. Expandable metal stents in achalasia–is there a role? *Am J Gastroenterol* 2000; **95**: 2185-2188
48 **De Palma GD**, Iovino P, Masone S, Persico M, Persico G. Self-expanding metal stents for endoscopic treatment of esophageal achalasia unresponsive to conventional treatments. Long-term results in eight patients. *Endoscopy* 2001; **33**: 1027-1030

**Edited by** Zhang JZ and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2374-2376
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

# Effects of glycine on plasma and liver tissue changes of TNF-α, ET-1 and nitric oxide contents in rats with obstructive jaundice

He-Qing Fang, Ying-Bin Liu, Hai-Jun Li, Shu-You Peng, Yu-Lian Wu, Bin Xu, Jian-Wei Wang, Jiang-Tao Li, Xin-Bao Wang

**He-Qing Fang, Ying-Bin Liu, Hai-Jun Li, Shu-You Peng, Yu-Lian Wu, Bin Xu, Jian-Wei Wang, Jiang-Tao Li, Xin-Bao Wang,** Department of General Surgery, Second Affiliated Hospital, Medical College of Zhejiang University, Hangzhou 310009, Zhejiang Province, China
**Correspondence to:** Dr. Ying-Bin Liu, Department of General Surgery, Second Affiliated Hospital, Medical College of Zhejiang University, Hangzhou 310009, Zhejiang Province, China. laoniulyb@163.com
**Telephone:** +86-571-87783585
**Received:** 2003-06-05 **Accepted:** 2003-07-22

## Abstract

**AIM:** To evaluate the effect of glycine on plasma and liver tissue changes of tumor necrosis factor-α (TNF-α), endothelin-1 (ET-1) and nitric oxide (NO) contents in rats with obstructive jaundice.

**METHODS:** Ninety healthy Wistar rats of both sexes weighing 275±25 g were randomly divided into sham-operated, bile duct-ligated, and bile duct-ligated plus glycine-treated groups, the latter was performed with 5 % glycine solution substituting for tap water drunk *ad libium* for 5 days before and 6 days after operation. Blood and liver tissue were sampled at the time of sacrifice on the 8th day post operation. Plasma total bilirubin, endotoxin, levels, as well as TNF-α, ET-1 and NO contents in liver tissue were determined.

**RESULTS:** Plasma endotoxin and total bilirubin levels were significantly higher in both bile duct-ligated and bile duct-ligated plus glycine-treated rats than in sham-operated animals ($P$=0.000613, 0.00921 and 0.00737, 0.00841 respectively), whereas they did not display any statistically significant difference between the former groups ($P$=0.417 and 0.374 respectively). Likewise, TNF-α, ET-1 and NO contents in both plasma and liver tissue were significantly increased in both bile duct-ligated and bile duct-ligated plus glycine-treated rats compared with sham-operated animals ($P$=0.00813, 0.00793, 0.00671, 0.00804, 0.00872, and 0.00947 in plasma and 0.00531, 0.00785, 0.00912, 0.00981 and 0.00635 in liver tissue respectively). However, these inflammatory mediators in both plasma and liver tissue were significantly reduced in bile duct-ligated rats fed on 5 % glycine solution compared with that without ($P$=0.00953, 0.00891, 0.0795, 0.00867, 0.0697 and 0.00907 in plasma and liver tissue respectively).

**CONCLUSION:** Reduction of TNF-α, ET-1 and NO contents in plasma and liver tissue of rats fed on glycine may be helpful to alleviate pathological lesions in obstructive jaundice.

Fang HQ, Liu YB, Li HJ, Peng SY, Wu YL, Xu B, Wang JW, Li JT, Wang XB. Effects of glycine on plasma and liver tissue changes of TNF-α, ET-1 and nitric oxide contents in rats with obstructive jaundice. *World J Gastroenterol* 2003; 9(10):2374-2376
http://www.wjgnet.com/1007-9327/9/2374.asp

## INTRODUCTION

Endotoxemia is one of the major causes that can lead to complicated pathophysiologic alterations in the process of obstructive jaundice. Current research has demonstrated that severe endotoxemia in obstructive jaundice could activate immunocompetent cells such as monocytes, macrophages and endothelial cells to produce a variety of cytokines that could contribute to an uncontrollable inflammatory cascade causing multiple organ dysfunction (MODS) or even death[1-3]. Among these cytokines, TNF-α, ET-1 and NO have been considered to be the main effectors in endotoxemia[4-10]. It is also worthy to note that glycine could provide an effective protection against endotoxemia that has been recently published in several repots[11-15]. The present study, therefore, was conducted with the aim to evaluate the effect of glycine on the plasma and liver tissue changes of TNF-α, ET-1 and nitric oxide contents in rats with obstructive jaundice.

## MATERIALS AND METHODS

### *Animal model and experimental protocol*

Ninety healthy Wistar rats of both sexes weighing 275±25 g were employed in the study. According to the experimental protocol, the animals were randomly divided into Group A in which the rats were performed a sham operation, Group B in which the rats were operated merely to ligate the common bile duct and Group C in which the rats were treated with both the ligation of common bile duct and a glycine regimen. Before operation, all animals were allowed access to standard rat chow and water *ad libium* for 5 days except that the rats in Group C drank 5 % glycine solution (provided by Shanghai Institute of Biological Products Research) instead of water[2]. After a 12-h fasting period, the animals were weighed and anesthetized with 1 % pentobarbitale sodium (30 mg/kg, ip) and their common bile duct was exposed and ligated to form a complete obstruction of the extrahepatic bile duct except that the common bile duct in rats of Group A was only exposed following laparotomy. When recovered for a period of 24 h, the rats were fed on their corresponding regimens for another 6 days. Rat venous blood was sampled and liver was removed surgically at the time of sacrifice on the 8th day post operation under anesthesia. Blood samples were prepared respectively according to the procedures for determinating different variables and the resulting plasma was stored at -70 ℃ until use. The liver was perfused via a cannula inserted into the portal vein with sterile normal saline to remove residual blood, and then prepared into 10 % tissue homogenate with 1.0 mol· $L^{-1}$ acetic acid and centrifuged at 3 000×g for 40 min. The supernatant was stored at -70 ℃ until analysis.

### *Measurements*

For determination of plasma endotoxin level, 1 ml of blood sample was collected in a pyrogen-free heparinized tube and centrifuged at 500×g for 5 min. The resulting plasma was analyzed with the limulus-amoebocyte-lysate test (LAL) kit according to the protocol of its manufacturer (Shanghai Yihua Medical Science & Technology Ltd.). The endotoxin

concentration was finally expressed as Eu/ml.

Measurement of ET-1 level was performed using 1 ml of blood samples that was collected in a test tube containing 15 μl of 10 % disodium edentate and 20 μl of aprotinin, and centrifuged at 3 000×g for 10 min at 4 ℃. The resulting plasma and liver homogenate were determined with ET-1 radioimmunoassay kit following the protocol of Research Institute of Radioimmunotechnology, General Hospital of PLA. Heparinised blood samples were collected in a separator tube and spun at 4 000 g for 10 min for detection of plasma bilirubin and TNF-α. To measure TNF-α, the resulting plasma and liver homogenate were analysed with a radioimmunoassay kit according to the instructions supplied by Research Institute of Radioimmunotechnology, General Hospital of PLA. Plasma bilirubin was determined with an automatic multifunction-biochemical analyzer.

Measurement of $NO_2^-/NO_3^-$ content two ml of heparinised blood samples was incubated at 37 ℃ for 1 h and then centrifuged at 2 000×g for 5 min. The resulting plasma and liver homogenate were determined with a $NO_2^-/NO_3^-$ assay kit following the procedures of Research Institute of Radioimmunotechnology, General Hospital of PLA.

### *Statistical analysis*

Experimental data were processed by analysis of variance and *t*-tests for comparison between groups. Results were expressed as mean ± SE. $P<0.05$ was selected as the level of significance.

## RESULTS

Poor appetite and jaundice were observed in rats of Groups B and C on the 2nd day after operation, the jaundice was more apparent on the ear and tail end skin. The dilation of proximal bile duct at the site of ligation, deep-brown-coloured liver and significantly elevated plasma total bilirubin ($P$=0.00921 and 0.00841 in Groups B and C respectively as compared to Group A) were also noted in these animals at the time of sacrifice on the 8nd day post operation. Animals in Group A did not exhibit obvious abnormalities. But there was no significant difference of plasma total bilirubin between rats in Groups B and C ($P$=0.374).

Plasma endotoxin levels in rats of Groups B and C were significantly increased compared with that in rats of Group A ($P$=0.000613 and 0.00737 respectively). However, It did not exhibit significant difference between rats in Groups B and C ($P$=0.417).

Plasma levels of TNF-α, ET-1 and NO in Group B rats were significantly elevated compared with that in Group A rats ($P$=0.00813, 0.00793 and 0.00671 respectively), which were significantly improved when the rats were fed on 5 % glycine solution as shown in Table 1 ($P$=0.00953, 0.00891 and 0.0795). The data for TNF-α, ET-1 and NO levels in liver tissue are shown in Table 2. In accordance with their plasma counterparts, significant reduction of these variables was found in bile duct-ligated rats fed on 5 % glycine solution (0.00867, 0.0697 and 0.00907).

## DISCUSSION

Obstructive jaundice is associated with an increased incidence of postoperative complications such as infection, systemic inflammatory response, and even multiple organ failure due to metabolic and hemodynamic disorders, as well as depressed immune function. Current studies have revealed that endotoxemia was one of the major causes leading to high morbidity and mortality in patients with obstructive jaundice[1-3]. Therefore, the therapeutic strategy aimed at reducing plasma endotoxin level and interrupting its biological activities has become a focus of great concern[4].

It has been found that endotoxin could stimulate monocytes and macrophages to produce a variety of cytokines, because the elevated intracellular $Ca^{2+}$ concentration in these cells was resulted from the activation of $Ca^{2+}$ channel by endotoxin[11,12]. It has also been noted that glycine, a nonessential amino acid, could exert protective effects on animals in multiple morbid conditions by minimizing oxidative stress, as well as toxic eicosanoid cytokine production[11,13] Ding *et al*[14,15] demonstrated that the biological effects of endotoxin could be significantly inhibited by glycine via a mechanism of blocking TNF-α production in immunocompetent cells, the latter could play a critical role in the pathogenesis of endotoxin lesions[4-7]. ET and NO, although as the intense vasoconstrictor and vasodilator respectively, were also pleiotropic factors involveded in endotoxin-mediated pathological processes with endotoxin and TNF-α as their potent releasing irritants[3,8-10]. The present study demonstrated that severe endotoxemia could be observed in obstructive jaundice, and the TNF-α, ET and NO contents in plasma and liver tissue were all significantly increased in bile duct-ligated rats.

Persistent severe endotoxemia in obstructive jaundice could stimulate not only Kupffer cells to release TNF-α to insult directly parenchymal cells of the kidney and liver[3,16-18], but also other immunocompetent cells to produce excessive amount of ET-1 and NO[8] to aggravate the disturbance of splanchnic circulation leading to decreased oxygen delivery, and ultimately multiple organ dysfunction syndrome (MODS).

**Table 1** Plasma levels of bilirubin, endotoxin, TNF-α, ET-1 and $NO_2^-/NO_3^-$ in different groups of rats ($\bar{x}\pm s$, $n$=30)

| Group | Bilirubin (μmol/L) | Endotoxin (Eu/ml) | TNF-α (pg/ml) | ET-1 (pg/ml) | $NO_2^-/NO_3^-$ (μmol/L) |
|---|---|---|---|---|---|
| A | 5.80±1.65 | 5.98±1.00 | 61.37±3.08 | 88.79±7.56 | 5.51±0.44 |
| B | 45.45±6.69[a] | 11.65±1.57[a] | 352.52±20.65[a] | 183.24±34.01[a] | 12.06±0.62[a] |
| C | 43.18±6.53[a] | 11.27±1.30[a] | 158.63±7.07[ab] | 120.68±10.99[ab] | 8.55±0.40[ab] |

[a]$P<0.01$ *vs* group A, [b]$P<0.01$ *vs* group B.

**Table 2** Levels of TNF-α, ET-1 and $NO_2^-/NO_3^-$ in liver tissues of different groups of rats ($\bar{x}\pm s$)

| Group | Samples | TNF-α (pg/ml) | ET-1 (pg/ml) | $NO_2^-/NO_3^-$ (μmol/L) |
|---|---|---|---|---|
| A | 10 | 43.51±2.58 | 43.18±4.47 | 2.15±0.18 |
| B | 10 | 298.46±18.74[a] | 124.56±11.67[a] | 10.53±0.87[a] |
| C | 10 | 113.45±6.67[ab] | 81.49±7.39[ab] | 7.23±0.34[ab] |

[a]$P<0.01$ *vs* group A, [b]$P<0.01$ *vs* group B.

Besides, NO itself is also a highly active free radical and can convert further into $NO_2^-$ and peroxynitrite. The more vigorous oxidants could result in cell injury[9]. Although glycine could reduce the plasma and liver tissue endotoxin level in our study, it did prevent TNF-α, ET-1 and NO from excessive production, which might be beneficial to alleviate the organ injury in obstructive jaundice. However, TNF-α, ET-1 and NO levels in both plasma and liver tissue remained elevated to some extent in rats fed on glycine as rats compared with sham-operated rats. To elucidate the mechanism underlying this phenomenon is thus the purpose of our further studies.

## REFERENCES


1 **Greig JD,** Krukowski ZH, Matheson NA. Surgical morbidity and mortality in one hundred and twenty-nine patients with obstructive jaundice. *Br J Surg* 1988; **75**: 216–219

2 **Reynolds JV,** Murchan P, Leonard N, Clarke P, Keane FB, Tanner WA. Gut barrier failure in experimental obstructive jaundice. *J Surg Res* 1996; **62**: 11-16

3 **Inan M,** Sayek I, Tel BC, Sahin-Erdemli I. Role of endotoxin and nitric oxide in the pathogenesis of renal failure in obstructive jaundice. *Br J Surg* 1997; **84:** 943-947

4 **Sheen-Chen SM**, Chen HS, Ho HT, Chen WJ, Sheen CC, Eng HL. Effect of bile acid replacement on endotoxin-induced tumor necrosis factor-alpha production in obstructive jaundice. *World J Surg* 2002; **26**: 448-450

5 **Heller J**, Sogni P, Barriere E, Tazi KA, Chauvelot-Moachon L, Guimont MC, Bories PN, Poirel O, Moreau R, Lebrec D. Effects of lipopolysaccharide on TNF-alpha production, hepatic NOS2 activity, and hepatic toxicity in rats with cirrhosis. *J Hepatol* 2000; **33**: 376-381

6 **O'Neil S,** Hunt J, Filkins J, Gamelli R. Obstructive jaundice in rats results in exaggerated hepatic production of tumor necrosis factor-alpha and systemic and tissue tumor necrosis factor-alpha levels after endotoxin. *Surgery* 1997; **122**: 281-286

7 **Kennedy JA,** Lewis H, Clements WD, Kirk SJ, Campbell G, Halliday MI, Rowlands BJ. Kupffer cell blockade, tumour necrosis factor secretion and survival following endotoxin challenge in experimental biliary obstruction. *Br J Surg* 1999; **86**: 1410-1414

8 **Liu BH**, Xiao N, Chen HS, Zhou JH. Dynamic alteration and interaction of endothelin-1and nitric oxide levels in the plasma or the liver during endotoxemia. *Zhonghua Chuangshang Zazhi* 2001; **17:** 166-168

9 **Wang D**, Zhu JY, Leng XS, Li S, Wang FS, Du RY. The detection of nitric oxide synthase(NOS) *in liver* tissue from cirrhotic patients with portal hypertension and their clinicopathological significance. *Zhonghua Putong Waike Zazhi* 1999; **14:** 31-33

10 **Sarac AM,** Aktan AO, Moini H, Bilsel S, Scapa E. Role of endothelin in obstructive jaundice. *Dig Dis Sci* 1999; **44**: 356-363

11 **Ikejima K,** Iimuro Y, Forman DT, Thurman RG. A diet containing glycine improves survival in endotoxin shock in the rat. *Am J physiol* 1996; **271**(1Pt 1): G97-103

12 **Portoles MT**, Arahuetes RM, Pagani R. Intracellular calcium alterations and free radical formation evaluated by flow cytometry in endotoxin-treated rat liver Kupffer and endothelial cells. *Eur J Cell Biol* 1994; **65**: 200-205

13 **Wheeler MD**, Thurman RG. Production of superoxide and TNF-alpha from alveolar macrophages is blunted by glycine. *Am J Physiol* 1999; **277**(5Pt 1): L952-959

14 **Ding Y,** Lu DX, Sun W, Zhang SM, Li CJ. Observation on the effect of Gly on the TNF and IL-1 production of monocytes stimulated by ET. *Zhongguo Yiaolixue Tongbao* 1999; **15:** 184-185

15 **Sun W,** Lu DX, Ding Y, Li CJ. Effects of glycine on the combining rate of endotoxin to monocytes and endotoxic configuration. *Zhongguo Bingli Shengli Zazhi* 1998; **14:** 359-362

16 **Tomioka M**, Iinuma H, Okinaga K. Impaired Kupffer cell function and effect of immunotherapy in obstructive jaundice. *J Surg Res* 2000; **92**: 276-282

17 **Ding JW**, Andersson R, Soltesz V, Willen R, Bengmark S. Obstructive jaundice impairs reticuloendothelial function and promotes bacterial translocation in the rat. *J Surg Res* 1994; **57**: 238-245

18 **Sheen-Chen SM**, Chau P, Harris HW. Obstructive jaundice alters Kupffer cell function independent of bacterial translocation. *J Surg Res* 1998; **80**: 205-209


**Edited by** Zhu L and Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2377-2378
World Journal of Gastroenterology
 ISSN 1007-9327

• CASE REPORT •

# Potentially fatal haemobilia due to inappropriate use of an expanding biliary stent

Rakesh Rai, John Rose, Derek Manas

**Rakesh Rai, Derek Manas,** Hepatobiliary Surgery and Liver Transplant Unit, Freeman Hospital, Newcastle, UK
**John Rose,** Department of Interventional Radiology, Freeman Hospital, Newcastle, UK
**Correspondence to:** Mr. Rakesh Rai, Flat-2, 108 St. Georges Terrace, Jesmond, Newcastle, NE22DP, UK. rai2000@hotmail.com
**Telephone:** +44-191-2818326
**Received:** 2003-05-12 **Accepted:** 2003-08-19

## Abstract

**AIM:** To highlight the fatal complication caused by expanding biliary stents and the importance of avoiding use of expanding stent in potentially curable diseases.

**METHODS:** Arteriobiliary fistula is an uncommon cause of haemobilia. We describe a case of right hepatic artery pseudoaneurysm causing arteriobiliary fistula and presenting as severe malena and cholangitis, in a patient with a mesh metal biliary stent. The patient had lymphoma causing bile duct obstruction.

**RESULTS:** Gastroduodenoscopy failed to establish the exact source of bleeding and hepatic artery angiography and selective embolisation of the pseudo aneurysm successfully controlled the bleeding.

**CONCLUSION:** Bleeding from the pseudo aneurysm of the hepatic artery can be fatal. Mesh metal stents in biliary tree can cause this complication as demonstrated in this case. So mesh metal stent insertion should be avoided in potentially benign or in curable conditions. Difficulty in diagnosis and management is discussed along with the review of the literature.

Rai R, Rose J, Manas D. Potentially fatal haemobilia due to inappropriate use of an expanding biliary stent. *World J Gastroenterol* 2003; 9(10):2377-2378
http://www.wjgnet.com/1007-9327/9/2377.asp

## INTRODUCTION

With increasing surgical and radiological intervention in the liver and biliary tree, incidence of haemobilia is on the rise. The mesh metal stent insertion can cause haemobilia as described. Thus inappropriate use of mesh metal stents in potentially curable diseases should be avoided.

The diagnosis of haemobilia may be difficult to establish and the bleeding may be fatal. Pseudoaneurysm of the right hepatic artery is an uncommon cause of haemobilia. A proper facility for radiological and surgical intervention is important to achieve success in control of bleeding.

The presentation and management of a case of haemobilia in a patient with mesh metal stent is described.

## CASE REPORT

A 47-year-old lady was referred as an emergency from another hospital with recurrent cholangitis and severe malena.

Two years previously she had presented in the referring hospital with an abdominal mass, vomiting and obstructive jaundice. Further investigation including computed tomography (CT) scan of the abdomen was inconclusive. A laparotomy was carried out which revealed a large nodular mass in the abdomen causing gastric outlet obstruction and compression at the porta hepatis. It was not possible to resect this mass and because there were extensive small bowel adhesions, an enteric or biliary surgical bypass was not considered a safe option. A sample from the lesion was taken for histology.

Postoperatively the patient underwent percutaneous placement of a self expanding mesh metal stent in her bile duct to relieve the jaundice. Histology suggested the presence of a non-Hodgkin' s lymphoma. Radiotherapy and chemotherapy were commenced, which produced a very good response. The patient was asymptomatic for 6 months and during that time a repeated CT scan showed no evidence of intra-abdominal diseases. Six months later the patient presented with obstructive jaundice and ultrasound examination suggested occlusion of the metal stent. On endoscopy the metal stent was protruding into the duodenum, which was associated with duodenal ulceration. A plastic stent was inserted through the metal stent at ERCP.

After a few months the patient developed further cholangitis and had severe malena leading to referral to our unit.

On arrival the patient had symptoms and signs suggestive of cholangitis. The patient was pale and required blood transfusion and was started on antibiotics for cholangitis. After resuscitation, endoscopy examination showed ulcerations in the 2nd part of duodenum adjacent to the metal stent but no active bleeding. On the 2nd day the patient developed more severe malena and it necessitated laparotomy to control the bleeding. At laparotomy there was an inflammatory mass around the porta, and the common bile duct was adherent to the hepatic artery and portal vein. During dissection the common bile duct was entered inadvertently and the blocked plastic stent was therefore removed. As soon as the plastic stent came out, profuse bleeding occurred inside the common bile duct. The source of this bleeding was unclear. As it was impossible to control the bleeding coming from the bile duct urgent on table, hepatic angiography was performed via the right femoral artery. It showed a pseudo-aneurysm of the right hepatic artery which had ruptured into the common bile duct. A successful embolisation of the right hepatic artery aneurysm was carried out. A t-tube was placed in the common bile duct. Within 24 hours of surgery, percutaneous drainage of the obstructed biliary tree was achieved with an external-internal drain. The patient recovered from surgery with no further malena. The biliary drain was internalised using a plastic stent, T- tube was removed prior to discharge.

Over the fourteen months since laparotomy and embolisation of the right hepatic artery pseudo-aneurysm, the patient has been asymptomatic, the biliary drain has remained patent and a recent CT scan showed no evidence of residual lymphoma.

## DISCUSSION

Haemobilia is an uncommon cause of gastrointestinal bleeding

and currently the most common cause of haemobilia in the Western world is as a consequence of percutaneous liver procedures. In a recent review of 222 cases of haemobilia, 147 were iatrogenic in aetiology following hepatobiliary intervention[1]. The origins of haemobilia might be diverse and included the cystic artery, anomalous hepatic artery, and hepatic artery to portal vein fistulas[2,3]. One of the reported causes of haemobilia was pseudoaneurysm of the hepatic artery, and the most common causes of this include laparoscopic cholecystectomy, exploration of the bile duct and other surgical biliary procedures.

Aneurismal disease of the hepatic artery from any cause is rare. Different authors have identified 21 % to 44 % of all splanchnic artery aneurysms as occurring in the hepatic artery[4,5]. Causes included arteriosclerosis (30-50 %), medial degeneration (25 %), trauma (20 %), mycotic infection (10 %), and congenital disorders (15 %)[3]. Eighty percent of hepatic artery aneurysms are extra hepatic and 20 % are intrahepatic. Extra hepatic aneurysms are distributed in the common hepatic artery (60 %), the right hepatic artery (30 %), the left hepatic artery (5 %), and rarely, in both (4 %).

In the present case, the severe bleeding on table was precipitated by removal of the plastic stent from the bile duct. The inflammation surrounding the bile duct and the presence of adhesions between the metal stent and the hepatic artery may have contributed to the formation of the pseudo-aneurysm of the right hepatic artery which was adherent to the metal stent. The previous episodes of malena probably indicated intermittent bleeding from the pseudoaneurysm, which was partially occluded by the plastic stent.

Thus mesh metal stent insertion can lead to this fatal complication. The most important aspect of this case is that the mesh metal stent was inserted before the response to radiotherapy and chemotherapy. The patient had no evidence of residual lymph node after therapy. Thus a long term mesh metal stent was unnecessary and could have been avoided.

The best treatment option for an occluded metal stent is not clear. The options are placement of a new coaxial metal stent, mechanical cleaning of the blocked stent or coaxial insertion of a plastic stent[6]. In a multicentric study of treatment of an occluded mesh metal stent due to tumour overgrowth, all the three methods were found to be equally effective but insertion of a plastic stent within a mesh metal stent appeared to be the most cost effective method[6].

Diagnosing haemobilia can be difficult. Haemobilia can present as upper or lower GI bleeding and the first investigation should be upper gastrointestinal tract endoscopy. If blood is seen coming from the ampulla of Vater, haemobilia is the likely cause of bleeding. But as few as 12 percent of these endoscopies might be diagnostic[7]. Due to intermittent bleeding from the biliary tree the source of bleeding may not be apparent. In a series of 29 patients with haemobilia, 22 patients had a normal endoscopy[8]. In our case as well the bleeding was not seen coming from the ampulla of Vater at the time of endoscopy and in the presence of duodenal ulceration due to the protruding metal stent, the cause of gastrointestinal bleeding was presumed to be duodenal ulcerations.

The choice of further investigation has varied over the years. Goodnight and Blaisdell[9] in 1981 recommended computed tomography (CT) and then angiography. But now angiography is recognised as the investigation of choice after gastrointestinal endoscopy, as it can be diagnostic as well as therapeutic.

Angiography could be expected to detect a vascular abnormality in over 90 % of cases of significant haemobilia[1]. Angiography can not only demonstrate pseudo-aneurysm of the arteries but also demonstrate arterio-biliary and arterio-portal fistulas.

Once the diagnosis of haemobilia is established, the aim is to stop the bleeding. It is important to correct any coagulopathy if present. The bleeding might stop on conservative treatment depending on the cause of bleeding. In a recent review of 171 cases of haemobilia, 73 (43 %) patients required only conservative treatment[1]. In case of continued bleeding, transarterial embolisation has been shown to be successful in 80 % to 100 % of cases[10-14]. Since the reported morbidity and mortality rates of transhepatic arterial embolisation (TAE) were lower than surgery[14], angiographic embolisation should be attempted first. The relative contraindication of arterial embolisation is hepatic sepsis, and in case of portal vein obstruction, arterial embolisation can cause hepatic necrosis.

Surgery is indicated if embolisation has failed or in case of hepatic sepsis. Adequate drainage of the biliary tree by endoscopic, percutaneous route or by surgery is important.

In conclusion, mesh metal stent insertion in the biliary tree can lead to fatal haemobilia. The diagnosis and management of haemobilia can be difficult and need an experienced multi-disciplinary team - mainly hepatobiliary surgeons, endoscopists and interventional radiologists. To avoid this complication unnecessary use of a mesh metal biliary stent in potentially curable diseases should be avoided.

## REFERENCES


1 **Green MH**, Duell RM, Johnson CD, Jamieson NV. Haemobilia. *Br J Surg* 2001; **88**: 773-786

2 **Strickland SK**, Khoury MB, Kiproff PM, Raves JJ. Cystic artery pseudoaneurysm: a rare cause of hemobilia. *Cardiovasc Intervent Radiol* 1991; **14**: 183-184

3 **Fagan EA**, Allison DJ, Chadwick VS, Hodgson HJ. Treatment of haemobilia by selective arterial embolisation. *Gut* 1980; **21**: 541-544

4 **Salam TA**, Lumsden AB, Martin LG, Smith RB. Nonoperative management of visceral aneurysms and pseudoaneurysms. *Am J Surg* 1992; **164**: 215-219

5 **Miani S**, Arpesani A, Giorgetti PL, Rampoldi V, Giordanengo F, Ruberti U. Splanchnic artery aneurysms. *J Cardiovasc Surg* 1993; **34**: 221-228

6 Tham TCK, Carr-Locke DL, Vandervoort J, Wong RCK, Lichtenstein DR, Van Dam JV, Ruymann F, Chow S, Bosco JJ, Qaseem T, Howell D, Pleskow D, Vannerman W, Libby ED. Management of occluded biliary Wallstents. *Gut* 1998; **42**: 703-707

7 **Counihan TC**, Islam S, Swanson RS. Acute cholecystitis resulting from hemobilia after tru-cut biopsy: a case report and brief review of the literature. *Am Surg* 1996; **62**: 757-758

8 **Moodley J**, Singh B, Lalloo S, Pershad S, Robbs JV. Non-operative management of haemobilia. *Br J Surg* 2001; **88**: 1073-1076

9 **Goodnight JE** Jr, Blaisdell FW. Hemobilia. *Surg Clin North Am* 1981; **61**: 973-979

10 L'Hermine C, Ernst O, Delemazure O, Sergent G. Arterial complications of percutaneous transhepatic biliary drainage. *Cardiovasc Intervent Radiol* 1996; **19**: 160-164

11 **Horak D**, Guseinov E, Adamyan A, Titova M, Danilov M, Trostenyuk N, Voronkova O, Gumargalieva K. Poly (2-hydroxyethyl methacrylate) particles for management of hemorrhage of complicated origin: treatment of hemobilia. *J Biomed Mater Res* 1996; **33**: 193-197

12 **Dousset B**, Sauvanet A, Bardou M, Legmann P, Vilgrain V, Belghiti J. Selective surgical indications for iatrogenic hemobilia. *Surgery* 1997; **121**: 37-41

13 **Yoshida J**, Donahue PE, Nyhus LM. Hemobilia: review of recent experience with a worldwide problem. *Am J Gastroenterol* 1987; **82**: 448-453

14 **Richardson A**, Simmons K, Gutmann J, Little JM. Hepatic haemobilia: non-operative management in eight cases. *Aust N Z J Surg* 1985; **55**: 447-451


**Edited by** Wang XL and Zhu LH

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2379-2381
World Journal of Gastroenterology
 ISSN 1007-9327

• CASE REPORT •

# Liver cell adenoma with malignant transformation: A case report

Masahiro Ito, Makoto Sasaki, Chun Yang Wen, Masahiro Nakashima, Toshihito Ueki, Hiromi Ishibashi, Michitami Yano, Masayoshi Kage, Masamichi Kojiro

**Masahiro Ito,** Department of Pathology, National Nagasaki Medical Center, Omura, Japan
**Makoto Sasaki,** Department of Surgery, National Nagasaki Medical Center, Omura, Japan
**Chun Yang Wen, Masahiro Nakashima,** Atomic Bomb Disease Institute, Nagasaki University School of Medicine, Nagasaki, Japan
**Toshihito Ueki, Hiromi Ishibashi, Michitami Yano,** Clinical Research Center, National Nagasaki Medical Center, Omura, Japan
**Masayoshi Kage, Masamichi Kojiro,** Department of Pathology, Kurume University School of Medicine, Kurume, Japan
**Correspondence to:** Masahiro Ito, Director, Department of Pathology, National Nagasaki Medical Center 2-1001-1 Kubara, Omura City, Nagasaki 856-856 Japan. itohm@nmc.hosp.go.jp
**Received:** 2003-07-17 **Accepted:** 2003-08-02

## Abstract

A 57-year-old woman was referred to our hospital because of a liver mass detected by computed tomography. She had taken oral contraceptives for only one month at the age of thirty. Physical examination revealed no abnormalities, and laboratory data, including hepatic function tests, were within the normal range, with the exception of elevated levels of those serum proteins induced by the absence of vitamin K or by raised levels of the antagonist (PIVKA)-II (3 502 AU/ml). Abdominal ultrasonography revealed a hyperechoic mass measuring 10×10 cm in the left posterior segment of the liver. Because hepatocellular carcinoma could not be completely excluded, this mass was resected. The tumor consisted of sheets of uniform cells with clear cytoplasm, perinuclear eosinophilic granules and round nuclei. These histological findings were consistent with liver cell adenoma. Background hepatic tissue appeared normal. After resection of the tumor, serum PIVKA-II fell to within the normal range. An area of hepatocellular carcinoma (HCC) with a mid-trabecular pattern was immunohistochemically found, which was positive for PIVKA-II. Sinusoidal endothelial cells were CD34-positive, containing scattered PIVKA-II positive cells. This tumor was therefore finally diagnosed as liver cell adenoma with focal malignant transformation to HCC.

Ito M, Sasaki M, Wen CY, Nakashima M, Ueki T, Ishibashi H, Yano M, Kage M, Kojiro M. Liver cell adenoma with malignant transformation: A case report. *World J Gastroenterol* 2003; 9(10):2379-2381
http://www.wjgnet.com/1007-9327/9/2379.asp

## INTRODUCTION

Liver cell adenoma (LCA) is a benign neoplasm composed of cells that closely resemble normal hepatocytes. The lesion arises in normal liver. LCA typically develops in the setting of a hormonal or metabolic abnormality which stimulates hepatocyte proliferation. Exogenous steroid hormone ingestion is undoubtedly the most common such stimulus. Hence, oral contraceptive steroids are the cause of most LCAs[1] although some cases are related to glycogen storage diseases. LCA is still uncommon in countries where oral contraceptives are not used[2-4]. Although the incidence is very low, LCA has been reported in men, children, and women not taking oral contraceptives.

We report here a case of LCA with transformation to hepatocellular carcinoma (HCC) in a woman who had received oral contraceptives for only one month 30 years before.

## CASE REPORT

A 57-year-old woman attended hospital because of slight abdominal fullness. She was referred to our hospital because of a liver mass detected by abdominal CT in June 2001. Computed tomography revealed a single mass at the left hepatic lobe measuring 10×10×8 cm. Her past history was unremarkable and she had no history of drug or alcohol abuse. At the age of thirty she had taken oral contraceptives for one month. Physical examination revealed no hepatomegaly or other abnormalities. The results of urinalysis and peripheral blood analysis were normal. Biochemical findings for blood, i.e. AST, ALT, LDH, γ-GTP, cholinesterase, total bilirubin, and total protein, were also normal. Hepatitis B surface (HBs) antigen, anti-HBs antibody, and anti-hepatitis C virus antibody were negative. Although serum protein induced by the absence of vitamin K, or by increased antagonist-II (PIVKA-II) levels, was elevated, serum alpha fetoprotein levels were normal (18 ng/ml). On June 11, PIVKA-II was 234 μAU/ml and increased rapidly to 3 503 μAU/ml on July 19. Levels of other tumor markers, i.e., CEA and CA 19-9 were all within normal limits.

Abdominal ultrasonography revealed a highly echoic lesion, measuring 10×9 cm in the left posterior segment of the liver. Computed tomography of the abdomen confirmed a large low-density mass, measuring 10.5×8.5 cm in size. Celiac angiography demonstrated a large hypervascular mass (Figure 1). On CT angiography, the mass lesion was enhanced heterogeneously during the early phase, and a sharply demarcated tumor stain was noted during the late phase. This abnormal lesion of the liver was also detected as iso-intensity on T1-weighted and hyper- and hypo-intensity on T2-weighted magnetic resonance imaging (MRI). Therefore, a left lobectomy of the liver was performed under the clinical diagnosis of HCC, in August 2001. After resection of the tumor, serum PIVKA-II fell to the normal level.

The surrounding liver tissue revealed no fibrosis or cirrhosis. The tumor was solitary and spherical, and measured 10×10×8 cm (Figure 2A). On the cut surface, the tumor was firm and well encapsulated. The color varied from yellow-white to reddish brown. There were irregular scars and a variegated appearance with hemorrhage. Microscopically, tumor cells were of uniform size but larger and paler than non-tumor hepatocytes in the surrounding tissue, and arranged in small sheets and cords with an occasional acinar pattern (Figure 2B). Background hepatic tissue looked to be normal. Tumor cell cytoplasm was clear and hydropic with eosinophilic granules around the nucleus in each cell. PAS staining revealed abundant accumulation of glycogen in the cytoplasm. The nuclei of tumor cells were uniform and regular, and mitosis was not seen. Thin-walled vascular channels were scattered throughout the tumor. Peliosis hepatic-like sinusoidal dilatation and hemorrhage were present especially in the central part of the tumor (Figure 2C). Portal tracts were absent throughout the tumor. These histological findings indicated LCA, but not simple LCA because of the following

features. Distinct areas with a mid-trabecular pattern were evident (Figure 2D). Reticulin fibers were well represented in each sheet, and most cell plates were of two-cell thickness. However, three or more cell plates were present in some hypercellular areas. Pseudoglandular structures were not encountered. Immunohistochemistry revealed that PIVKA-II (18B7, Eisai Co. Ltd.; Tokyo) was positive both in the adenoma cells and in mid-trabecular areas (Figure 3A). CD34 (Nichirei, Tokyo, Japan) was positive throughout the endothelial cells of the tumor (Figure 3B). Ki-67 (Dako) was positive sporadically in mid-trabecular area and tumor cells with enlarged nuclei. AFP (Dako, Demark), P53 (DO-7, Dako), estrogen receptor (Novocastra, Newcastle, UK), and progesterone receptor (Novocastra) were all negative throughout.

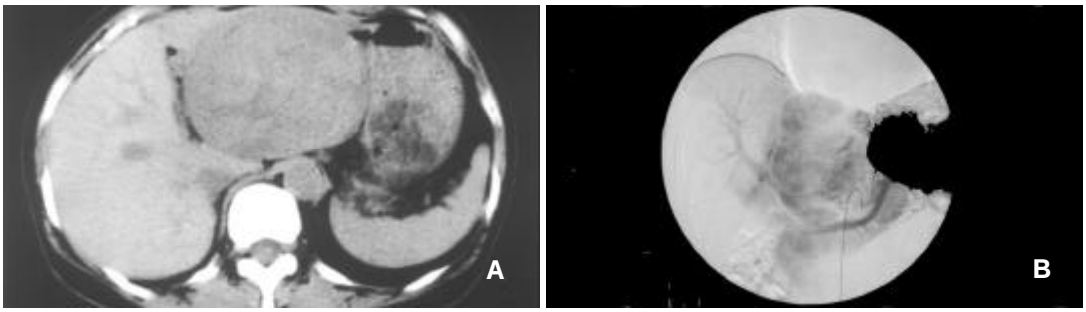



**Figure 1** A: Computed tomography revealing a large tumor in the left lobe. B: Celiac angiography during the arterial phase, showing a large hypervascular lesion.

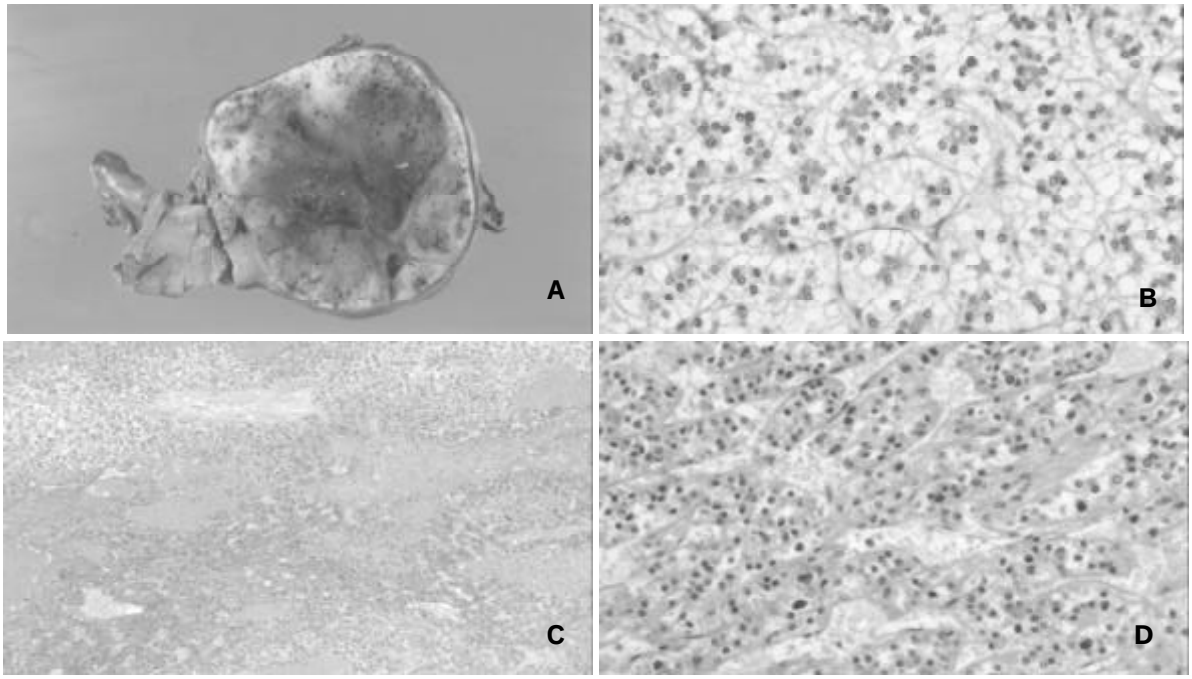



**Figure 2** A: Macroscopic appearance of resected tumor. The tumor was incompletely encapsulated by thin fibrous tissues, and its cut surface was tan-yellowish. Hemorrhage was observed inside part of the tumor. B: Microscopic appearance of tumor. Tumor cells were relatively uniform and had clear eosinophilic cytoplasm with small round nuclei. C: Trabecular structures were sporadically encountered in association with hemorrhage. D: The tumor cells were arranged predominantly in a thin trabecular pattern with moderate nuclear atypia.

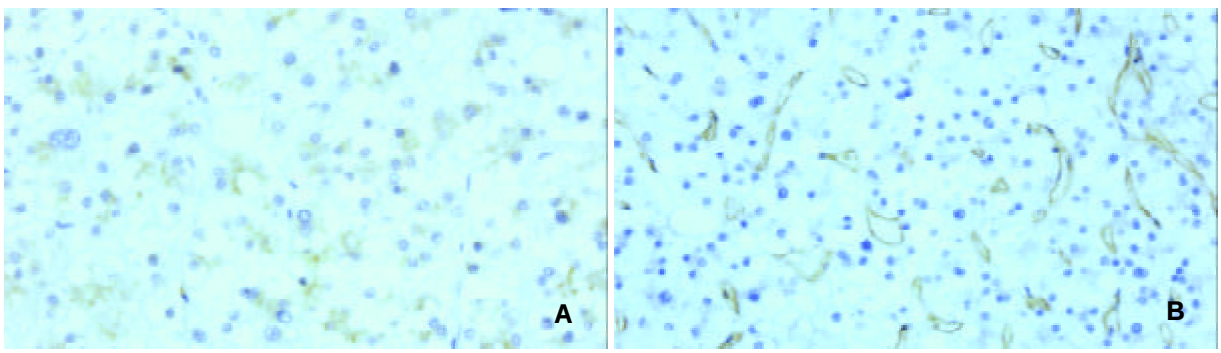



**Figure 3** Immunohistochemistry. A: PIVKA-II. B: CD34. PIVKA-II was positive in the tumor cells with or without nuclear atypism and trabecular structure (A). CD34 was expressed diffusely in the sinusoidal endothelial cells (B).

## DISCUSSION

Oral contraceptive steroids are the cause of most LCAs, as demonstrated by epidemiological case-control studies conducted in the 1970's[5]. The tumor is nearly always found in women aged 15 to 45, with an incidence estimated to be 3 to 4 per 100 000 long-term contraceptive steroid users per year. An incidence of only 1 per million occurs in non-users or women with less than 2 years' exposure to contraceptive steroids. The risk increases with the duration of contraceptive steroid use and with the potency of the preparation. The present case was not related to oral contraceptives although she had a very short period of oral contraceptive use about 30 years before.

Malignant transformation of LCAs is rare, because most are resected on discovery. However, there were a few well documented cases of hepatocellular carcinoma arising in unresected solitary and multiple adenomas[6-8]. This might represent an adenoma-carcinoma progression sequence in hepatocellular neoplasia, similar to that seen in colon cancer[9]. Intratumoral hemorrhage and intraperitoneal tumoral rupture occasionally occurred in LCA, so that surgical excision has been usually advised for LCA to prevent the risk of rupture and hemorrhage, and malignant transformation[10].

Distinguishing LCA from well-differentiated HCC by histopathology is a difficult task in small biopsies and occasionally even in resected tumor specimens. LCA can be differentiated clinically from HCC on the basis of tumor marker abnormalities, such as AFP and PIVKA-II. Gene analysis is one of the useful methods distinguishing these tumors, although it is not practical in ordinary diagnostic procedure. Multiple chromosomal aberrations detected by CGH, including gains or losses in one or more of six chromosomes (*1q, 4q, 8p, 8q, 16p*, and *17p*) have been reported in hepatocellular carcinomas, but not in LCAs[11]. The detection of frequent aberrations supported a diagnosis of carcinoma and made LCA unlikely, as consistently reported by several investigators[12,13].

LCA has a small but not negligible risk of malignant transformation into HCC. LCAs are rare tumors which may be difficult to differentiate from well differentiated HCCs. In the present case the key finding of malignant transformation into HCC was the presence of an irregular mid-trabecular growth pattern as determined histologically. p53 and Ki-67 are useful markers for differentiation of LCA from HCC. p53 protein was detected in the nuclei of tumor cells of 8.5 % to 44 % of HCC. In contrast, immunostaining of p53 was seen in none of the focal nodular hyperplasias examined, and also in none of the LCAs. In addition, mutant p53 expression in HCC was positively correlated with tumor grade[14,15]. Ki-67, assessed using the monoclonal antibody MIB-1, is expressed in the nuclei of cells of HCC, but not in adenoma cells. The nuclei of LCAs are typically uniform and regular, the nuclear/cytoplasmic ratio is normal, and mitosis is not seen. In the present case, p53 was negative throughout the tumor, even in the areas with a trabecular growth pattern that was the key finding of malignant transformation. Ki-67 was sporadically positive in mid-trabecular area and relatively large nuclei, but not many as conventional HCC.

In contrast to the results of Ki-67 and p53 staining, those of CD34 and PIVKA-II supported malignant transformation. CD34 was reported to show diffuse staining of a large number of sinusoids in HCC in contrast to LCA where the staining was focal or identified only marginal sinusoids[16]. However, consideration should be given to the possibility of LCA and focal nodular hyperplasia, which could also exhibit significantly diffuse CD34 reactivity[17]. PIVKA-II levels were elevated in most patients with HCC. Although LCAs with elevated serum levels of PIVKA-II have been sporadically reported, these were relatively low. Diffuse positive reactions of CD34 on sinusoid endothelial cells in the area of suspicious HCC and marked increase of PIVKA-II (3 503 mAU/ml), as seen in the present case, might indicate malignant transformation. The evaluation of a hepatic nodule is a very common clinical problem. Identification of malignant features remains, at times, inconsistent and controversial, and the distinction of HCC from dysplastic nodule can be difficult. Establishment of diagnostic standards for malignant features should be expected. Finally, this case was reviewed by four Western experts, and was diagnosed as LCA with malignant transformation to HCC (personal communication).

## REFERENCES


1 **Edmondson HA**, Hendeson B, Benton B. Liver-cell adenomas associated with use of oral contraceptives. *N Engl J Med* 1976; **294**: 470-472

2 **Alshak NS**, Cocjin J, Podesta L, van de Velde R, Makowka L, Rosenthal P, Geller SA. Hepatocellular adenoma in glycogen storage disease type IV. *Arch Pathol Lab Med* 1994; **118**: 88-91

3 **Kerlin P**, Davis GL, McGill DB, Weiland LH, Adson MA, Sheedy PF 2nd. Hepatic adenoma and focal nodular hyperplasia: clinical, pathologic, and radiologic features. *Gastroenterology* 1983; **84**: 994-1002

4 **Wheeler DA**, Edmondson HA, Reynolds TB. Spontaneous liver cell adenoma in children. *Am J Clin Pathol* 1986; **85**: 6-12

5 **Rooks JB**, Ory HW, Ishak KG, Strauss LT, Greenspan JR, Hill AP**,** Tyler CW Jr. Epidemiology of hepatocellular adenoma. The role of oral contraceptive use. *JAMA* 1979; **242**: 644-648

6 **Foster JH**, Berman MM. The malignant transformation of liver cell adenomas. *Arch Surg* 1994; **129**: 712-717

7 **Janes CH**, McGrill DB, Ludwig J, Krom RA. Liver cell adenoma at the age of 3 years and transplantation 19 years later after development of carcinoma: a case report. *Hepatology* 1993; **17**: 583-585

8 **Scott FR**, El-Refaie A, More L, Scheuer PJ, Dhillon AP. Hepatocellular carcinoma in an adenoma: value of Q Bend 10 immunostaining in diagnosis of liver cell carcinoma. *Histopathology* 1996; **28**: 472-474

9 **Gordon SC**, Reddy KR, Livingstone S, Jeffers LJ, Schiff ER. Resolution of a contraceptive-steroid-induced hepatic adenoma with subsequent evolution into hepatocellular carcinoma. *Ann Intern Med* 1986:**105**: 547-549

10 **Closset J**, Veys I, Peny MO, Braude P, Van Gansbeke D, Lambilliotte JP, Gelin M. Retrospective analysis of 29 patients surgically treated for hepatocellular adenoma or focal nodular hyperplasia. *Hepatogastroenterology* 2000; **47:** 1382-1384

11 **Wilkens L**, Bredt M, Flemming P, Becker T, Klempnauer J, Kreipe HH. Differentiation of liver cell adenomas from well-differentiated hepatocellular carcinomas by comparative genomic hybridization. *J Pathol* 2001; **193**: 476-482

12 **Wilkens L**, Bredt M, Flemming P, Schwarze Y, Becker T, Mengel M, von Wasielewski R, Klempnauer J, Kreipe H. Diagnostic impact of fluorescence in situ hybridization in the differentiation of hepatocellular adenoma and well-differentiated hepatocellular carcinoma. *J Mol Diagn* 2001; **3**: 68-73

13 **Nolte M**, Werner M, Nasarek A, Bektas H, von Wasielewski R, Klempnauer J, Georgii A. Expression of proliferation associated antigens and detection of numerical chromosome aberrations in primary human liver tumours: relevance to tumour characteristics and prognosis. *J Clin Pathol* 1998; **51**: 47-51

14 **Schaff Z**, Sarosi I, Hsia CC, Kiss A, Tabor E. p53 in malignant and benign liver lesions. *Eur J Cancer* 1995; **31A**: 1847-1850

15 **Ojanguren I**, Ariza A, Castella EM, Fernandez-Vasalo A, Mate JL, Navas-Palacios JJ. p53 immunoreactivity in hepatocellular adenoma, focal nodular hyperplasia, cirrhosis and hepatocellular carcinoma. *Histopathology* 1995; **26**: 63-68

16 **Dhillon AP**, Colombari R, Savage K, Scheuer PJ. An immunohistochemical study of the blood vessels with primary hepatocellular tumours. *Liver* 1992; **12**: 311-318

17 **Kong CS**, Appenzeller M, Ferrell LD. Utility of CD34 reactivity in evaluating focal nodular hepatocellular lesions sampled by fine needle aspiration biopsy. *Acta Cytol* 2000; **44**: 218-222


**Edited by** Wang XL

P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(10):2382-2384
World Journal of Gastroenterology
 ISSN 1007-9327

• CASE REPORT •

# Massive gastrointestinal tubercolsis in a young patient without immunosupression

Yasar Settbas, Murat Alper, Yusuf Akcan, Yesim Gurbuz, Sükrü Oksuz

**Yasar Settbas**, Resident, A.I.B.U. Duzce Medical Faculty, Department of Internal Medicine, Duzce, Turkey
**Murat Alper**, Assistant professor, A.I.B.U. Duzce Medical Faculty, Department of Pathology, Duzce, Turkey
**Yusuf Akcan**, Assistant professor, A.I.B.U. Duzce Medical Faculty, Department of Internal Medicine, Duzce, Turkey
**Yesim Gurbuz,** Assistant professor, Kocaeli University Medical Faculty, Department of Pathology, Duzce, Turkey
**Sükrü Oksuz**, Resident, A.I.B.U. Duzce Medical Faculty, Department of Microbiology
**Correspondence to:** Yasar Settbas, M.D., Abant Izzet Baysal University Duzce Medical Faculty Department of internal Medicine, 14450, Konuralp, Duzce, Turkey. ysertbas@hotmail.com
**Telephone:** +90-380-5414107 **Fax**: +90-380-5414105
**Received:** 2002-10-17 **Accepted:** 2002-11-09

## Abstract

Although the lung is the major site for *Mycobacterium tuberculosis* infection, gastrointestinal involvement can be present as part of multiorgan disease process or, less commenly, can be seen as primary gastrointestinal tuberculosis. In the cases where the culture is negative, it can be difficult to differantiate tuberculosis from Crohn's disease based on both the clinical and histological features. When side effects of classic antimycobacteria are encountered, we can initially add ciprofloxacin to the treatment of tuberculosis. We reported a case of 19-yr-old patient, who was treated as Crohn's disease and worsen. We began to tuberculosis treatment, and the patient improved clinically and histologically. The main point in this case is that widespread involvement of gastrointestinal tract can be brought about by non resistant strains of *Mycobacterium tuberculosis* even in immunocompetent patients.

Settbas Y, Alper M, Akcan Y, Gurbuz Y, Oksuz S. Massive gastrointestinal tubercolsis in a young patient without immunosupression. *World J Gastroenterol* 2003; 9(10):2382-2384
http://www.wjgnet.com/1007-9327/9/2382.asp

## INTRODUCTION

*Mycobacterium tuberculosis* is the primary cause of tuberculosis, infects one third of world' s population and causes the most deaths per year of any infectious agents. It is found primarily in devoloping countries, where poor sanitation contributes to its spread. Although pulmonary manifestations predominate in most tuberculosis cases, gastrointestinal involvement may be present as a part of multiorgan disease process or, less commonly as primary gastrointestinal tuberculosis. Gastrointestinal tuberculosis is usually caused by *Mycobacterium tuberculosis* but may also be caused by *Mycobacterium bovis*. Prior to AIDS epidemic, it was seen most commonly in immune competent persons with untreated advanced pulmonary disease[1]. Today, it is most commonly observed in association with immunosupression and, in one series, more than 40 % of patients with gastrointestinal tuberculosis had AIDS[2]. Other groups at highest risk include those who abuse alcohol, injection drug users, those who take chronic steroids, elderly persons. Here, we described a case of gastrointestinal tuberculosis with diffuse involvement of esophagus, stomach, ileum and colon, who did not possess any known immunosuppressive state in his history (Diabetes mellitus, alcohol abuse, protein calori malabsorbtion, AIDS).

## CASE REPORT

A 19 year old male patient presented to the outpatient department with complaints of dispepsia and nausea for two years. Recently, vomiting after meals made the patient fear of eating. He first recieved anti ulcer medication with the diagnosis of peptic ulcer in another center. Since his complaints continued, endoscopic and colonoscopic examinations were performed and several biopsies were obtained. A diagnosis of Crohn' s disease was made at the same center. Lansaprazol 30 mg, mesalazine (500 mg *p.o. t.i.d*), hydrocortisone 10 mg/kg/day were begun. Despite the treatment regimen, no response to mesalazine and hydrocortizone was seen and since his complaints increased, he was admitted to our center with abdominal pain, anorexia, nausea, fatigue, weigth loss (15 kg/6 month). There were no pathologic findings on his chest x-ray. Ultrasound examination of the abdomen revealed unremarkable findings, except some degree of cahexia and apathetic appereance. Laboratory data included hemoglobin 11.5 g/dL, erythrocyte sedimentation rate 10 mm/hr, alanin aminotransferase 30 U/L, aspartat aminotransferase 40 U/L, total protein 4.1 g/dL, albumin 2 g/dL white blood cell count 6 600, platelet count 314 000, HBsAg (-), AntiHBsAg(-), Anti-HCVAg(-), HIV(-) and PPD levels 7 mm. Physical examination did not find any abnormal findings. He had no history of any disease which practically can cause diabetes mellitus, alcoholism and renal insufficiency, which are associated with immunodefficiency.

Endoscopy and colonoscopy were performed due to these complaints in our institution. Endoscopy revealed hyperemia with granulomatous lesions at the lower third of the esophagus. A granular appereance was seen along small curvature of the corpus. Antrum had extensive hyperdmia. Additionally granulomas were seen at bulbar and the second part of duodenum. Subsequently biopsies were obtained from all described areas. A histopathological examination revealed existance of granuloma in the esophagus, stomach and small intestine with caseous necrosis in the stomach (Figures 1-3). Although ileal biopsy revealed edematous *lamina propria* with increase of inflammatory cells, there was a granulation at the ileal biopsy which was done 3 months before the treatment of Crohn' s disease started at another center (Figure 4).

Under the light microscopy of these findings, gastrointestinal tuberculosis was concluded and treated with anti tuberculosis treatment of isoniazid 200 mg/day, rifampicin 450 mg/day, morfazinamid 1 g/day and ethambutol 500 mg/day. During the follow up of the first and second month, retrobulber neuritis and peripheral neuritis were seen as the side effects of ethambutol and isoniazid respectively. At the second month of the treatment, morfazinamid was also stopped. Ciproxin 500 mg two times a day with the combination of rifampicin 450 mg/day was given. A significant improvement in clinical

situation of the patient was observed. He gained 25 kg during the theraphy and got 60 kg. The patient recieved anti tuberculosis treatment for 12 months. Check up endoscopy at the end of treatment revealed normal findings with no granulation and caseous necrosis.

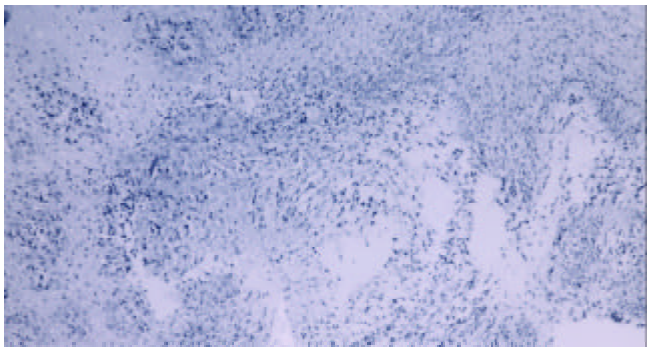


**Figure 1** Granulomatous structures of esophagus (H & E×100).

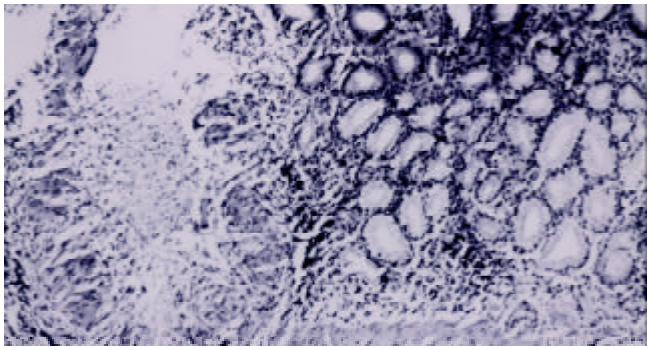


**Figure 2** Granulomatous structures and caseous necrosis in the mucosa of stomach (H & E×100).

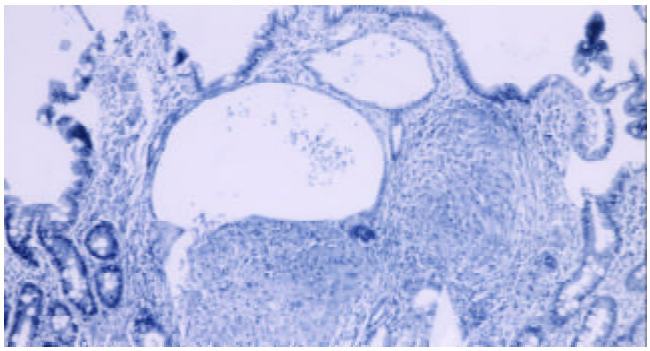


**Figure 3** Granulomatous structures in the mucosa of small intestine (H & E×100).

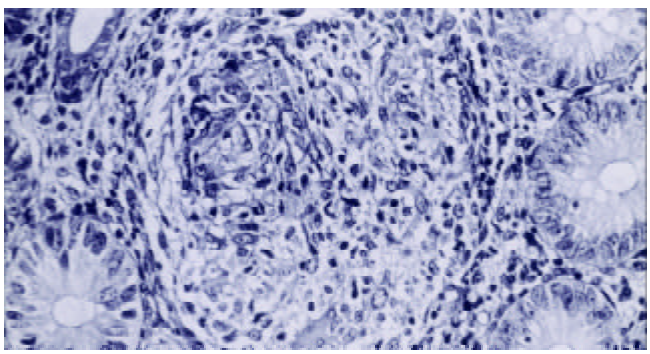


**Figure 4** Granulomatous structures between glands of colon mucosa (H & E×310).

## DISCUSSION

The diagnosis of gastrointestinal tuberrculosis is always difficult. We must keep in mind the possibility of patients with abdominal pain, even without concomitent lung involvement[2,3]. Tuberculosis may affect any part of gastrointestinal tract, but it most commonly involves the terminal ileum and ileocaecal region, as Crohn' s disease did[4]. However it is rare to see the involvement of all parts of the gastrointestinal tract. Therefore, our case is intresting as almost the whole gastrointestinal tract has been affected.

The symptoms of gastrointestinal tuberculosis are nonspecific, and in the absence of pulmonary tuberculosis the diagnosis may be difficult. The most common symptom associated with gastrointestinal tuberculosis is abdominal pain. Other symptoms included diarrhea, fever, anorexia, weight loss, constipation, and hemorrhage[5].

A presumptive diagnosis can be made in the presence of known active pulmonary tuberculosis and based on the clinical and radiological findings in the bowel. Definitive diagnosis is essentially made by histology, with Ziehl-Neelsen staining for acid fast *bacilli* and culture. Colonoscopy and endoscopy of gastrointestinal system are the most useful nonoperative diagnostic tools. A combination of histology and culture can establish the diagnosis up to 80 % of the patients. Altough more expensive PCR of biopsy specimens may facilitate diagnosis since it has higher sensitivity and specifity than routine culture, and results could be obtained in 48 hours instead of weeks[6]. The detection of anti-cord antibodies has recently been shown to facilitate the diagnosis of intestinal tuberculosis from Crohn' s disease[7]. However, the cases, which have negative results of culture and PCR can be seen and the diagnosis is achieved throuh histological assesment and response to antituberculosis treatment. Sometimes we could see the cases that had culture and PCR negative results were diagnosed histologically and recovered by tuberculosis tretment[8]. In our case, we established the diagnosis of tuberculosis by the presence of caseation and granulomatous lesions suggestive of tuberculosis, and essentially by the occurence of dramatic clinical improvement.

The lesions of gastrointestinal tuberculosis have clinically been divided into an acute ulcerative stage and chronic hypertrophic stage of the disease that is characterized by granulomas and extensive fibrosis. Ulcers are typically irregular with a necrotic base, which may extend to perforation. *Fistulae* and anorectal lesions may occur. These features are non-specific, and can also be seen in Crohn' s disease. In contrast, distinguishing histological features of granulomas in intestinal tuberculosis from Crohn' s disease have been described. Caseation, if present, strongly suggests tuberculosis. However central acute necrosis of granulomas may also be seen occasionally in Crohn' s disease.

Tuberculosis should always be considered in the differential diagnosis of patients with obscure abdominal symptoms. Other entities, which are commonly misdiagnosed are Crohn' s disease, lymhoma, carcinoma, diverticular diseases, appendicities and other infections of gastrointestinal tract. Ulcers were more typically linear, and granulomas, when they were found had no caseation necrosis or organisms[9,10].

The prognosis of gastrointestinal tuberculosis depends primarily on the imuune status of the apropriate theraphy in a timely manner. In untreated HIV-infected patients, the disease progresses rapidly and is almost always fatal. If there is suspicious tuberculosis as histologically, treatment should beginn without waiting the result of the culture. The regimen of choice in most of the cases is isoniazid 3 to 5 mg/kg, rifampin 10 mg/kg, pyraazinamid 20-25 mg/kg and ethambutol 15 mg/kg all given once daily. In resistant cases ciprofloxacin can be used. However in our case because of the side affects of

ethambutol and isoniazid, ciprofloxacin was given[11]. After a while of treatment histologic and clinical response was seen. It could be thought that ciprofloxacin could also be used in the treatment of Crohn' s disease[12]. Since the disease was worsen with the use of steroids, it should be thought that the effect of ciprofloxacin was against the tuberculosis.

This case report illustrates that the diagnosis of colonic tuberculosis requires a high index of suspicion. In cases where there is any doubt about the the diagnosis of Crohn' s disease, and even if the histology of colonic biopsies suggests Crohn' s disease, corticosteroids should be withheld due to disastrous results such as dissemination of tuberculosis. On the other hand, if the information not available to differentiate Crohn' s disease from tuberculosis, therapeutic trial of tuberculosis is justified because it whould not aggravate Crohn' s disease. In the cases of side affects of the drugs in tuberculosis, ciprofloxacin can be preferred in the combination with anti-mycobacterials which have no side affects. Tuberculosis of gastrointestinal tract can mostly be seen in immune compromised individuals such as HIV (+) individuals, alcoholic and chronic steroids users,but can also be seen in immunocompatible peoples. Although it' s mostly seen in the ileocecal region of gastrointestinal tract,it must be kept in mind that it can be widespreadly disseminated as in our case.

## REFERENCES


1 **Pettengell KE**, Larsen C, Garb M, Mayet FG, Simjee AE, Piri D. Gastrointestinal tuberculosis in patients with pulmonary tuberculosis. *Q J Med* 1990; **74**: 303-308

2 **Shafer RW**, Kim DS, Weiss JP, Quale JM. Extrapulmonary tuberculosis in patients with human immunodeficiency virus infection. *Medicine* 1991; **70**: 384-397

3 **Perez del Rio MJ**, Fesno Forcelledo M, Diaz Iglesias JM, Veiga Gonzalez M, Alveraz Prida E, Ablanedo Ablanedo P, Herrero Zapatero A. Intestinal Tuberculosis, a difficult suspected diagnosis. *An Med Interna* 1999; **16**: 469-472

4 **Brizi MG**, Celi G, Scaldazza AV, Barbaro B. Diagnostic imaging of abdominal tuberculosis: gastrointestinal tract, peritoneum, lymph nodes. *Rays* 1998; **23**: 115-125

5 **Bouma BJ**, Tygata KM, Shipper HG, Kager PA. Be aware of abdominal tuberculosis. *Neth J Med* 1997; **51**: 119-122

6 **Gan H**, Ouyang Q, Bu H, Li S, Chen D, Li G, Yang X. Value of polymerase chain reaction assay in diagnosis of gastrointestinal tuberculosis and differentiation from Crohn's disease. *Chin Med J* 1995; **108**: 215-220

7 **Kashima K**, Oka S, Tabata A. Detection of anti-cord factor antibodies in intestinal tuberculosis for its differential diagnosis from Crohn's disease and ulcerative colitis. *Dig Dis Sci* 1995; **40**: 2630-2640

8 **Artu P**, Lavergne-Slove, Joly F, Bitoun A, Ramboud JC, Bouhnik Y. Isolated jejunal tuberculosis mimicking Crohn disease. Diagnosis by push video enteroscopy. *Gastroenterol Clin Biol Biol* 1999; **23**: 1086-1089

9 **Jakubowski A**, Elwood RK, Enarson DA. Clinical features of abdominal tuberculosis. *J Infect Dis* 1988; **158**: 687-692

10 **Tabrisky J**, Lindstran RR, Peters R, Lachman RS. Tuberculous enteritis, Review of a protean disease. *Am J Gastroenterol* 1975; **63**: 49-57

11 **Berning SE**. The role of fluoroquinolones in tuberculosis today. *Drugs* 2001; **61**: 9-18

12 **Peppercorn MA**. Is there a role for antibiotics as primary therapy in Crohn's ileitis? *J Clin Gastroenterol* 1993; **17**: 235-237


**Edited by** Xu XQ and Wang XL