

# Strengthen international academic exchange and promote development of gastroenterology

MA Lian-Sheng and PAN Bo-Rong

Approved by the China state Committee of Science and Technology in January 1998 (Document No. 027), China National Journal of New Gastroenterology has been renamed as World Journal of Gastroenterology and will be published bimonthly and distributed world wide. This is an important turning point in the publication of this Journal.

Since the Journal was established on October 1, 1995, 229 articles, including 8 editorials, 165 original articles (72.0%), 5 reviews, 6 case reports and 45 brief reports have been published in 9 issues of 3 volumes. Seventy-four papers were funded by the National Natural Science Foundation of China, accounting for 44.8%. Through its content, the Journal reflected the achievements in China's gastroenterology made in this period and promoted the international academic exchange in the field of digestion. The Journal was distributed worldwide, covering 32 provinces and autonomous regions domestically and many developed countries as well, including UK, USA, Japan, France, Germany, etc. The Journal has been listed in 8 major indexes of China.

The policy of the World Journal of Gastroenterology is to strengthen the global academic exchange in the field of digestion of both modern medicine and traditional medicine, enhance the development of gastroenterology, and make contributions to protecting the people's health in the world.

The principle and standard of the publication are high quality and practical. Being

a peer-reviewed periodical, the Journal will publish the new achievements, in both experimental researches and clinical studies of digestion of modern medicine and traditional medicine. Priorities are especially given to the original articles of international competitive significance, editorials on the hot topics of gastroenterology, and original articles on the major projects funded by the nation. The Journal is also featured by columns of commentary, reviews and brief reports about new methods, new technics, new experience or information.

We greatly appreciate the extensive concern, and opinions and suggestions about the Journal from the gastroenterologists in the world.

On the occasion of the renaming of the Journal, we would like to extend our sincere gratitude to all our readers, members of the international advisory board and the editorial board, and leaders at different levels.

We are especially grateful to Prof. WU Jie-Ping, Vice-Chairman of the Standing Committee of the National People's Congress of the PRC and academician of Chinese Academy of Engineering and Chinese Academy of Sciences, for inscribing the Chinese name of the Journal. We also owe the smooth publication of the Journal to the great help by Prof. XU Jia-Yu, the Honorary Editor-in-Chief (Ruijin Hospital, Shanghai Second Medical University), Prof. LU Han-Ming (Xinhua Hospital, Shanghai Second Medical University), Prof. WU Xie-Ning (Shanghai First People's Hospital), Prof. MA Jing-Yun, the Associate Editor-in-Chief (senior editor of the Chinese Medical Association) and so on. Let us go forward hand in hand and strive to make this Journal an important forum for international academic exchange and contribute to the development of global gastroenterology.

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Received 1998-02-12

# Significance of vascular endothelial growth factor messenger RNA expression in gastric cancer

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**Subject headings** stomach neoplasms; RNA, messenger; neoplasm metastasis; gene expression; endothelial growth factor

## Abstract

**AIM** To study VEGF mRNA expression in gastric carcinoma and to clarify the association of its expression with the clinicopathologic features of the disease.

**METHODS** *In situ* hybridization (ISH) and histochemistry were used to examine and analyze the expression of VEGF mRNA and antigen, and microvessel count (MVC) in 28 cases of gastric carcinomatous tissue in combination with clinical materials.

**RESULTS** Nineteen of 28 gastric carcinomas were positive for VEGF mRNA. VEGF mRNA was mainly expressed in malignant cells and not in normal epithelium of gastric mucosa. Its expression was further increased in tumor cells adjacent to tumor necrosis zones, where stromal cells expressed VEGF mRNA occasionally. There was a close correlation between MVC and VEGF mRNA positivity ( $P < 0.005$ ). High VEGF mRNA levels were significantly associated with serosal invasion, lymph node metastasis and TNM staging ( $P < 0.05$ , respectively).

**CONCLUSION** VEGF mRNA expression is associated with tumor invasion and metastasis by stimulating angiogenesis in gastric carcinoma.

## INTRODUCTION

Solid tumors are composed of two distinct but interdependent compartments, the malignant cells themselves and the vascular and connective tissue stroma that they induce and in which they are dispersed. Stroma provides the vascular supply that tumors require for obtaining nutrients, gas exchange, and waste disposal. Thus, any increase in tumor mass, either primary or metastatic, must be accompanied by stroma formation<sup>[1]</sup>.

The mechanism by which tumors induce stroma has caused considerable attention in recent years. Most work have focused on one aspect of stroma generation, angiogenesis, and have called attention to a variety of tumor-secreted "angiogenesis factor", particularly VEGF, that is likely to play an important role in both angiogenesis and other aspects of tumor stroma generation. VEGF is an  $M_r$  34 000 - 42 000 KD, disulfide-linked glycoprotein synthesized by several human and animal cell types, both normal and neoplastic<sup>[2-4]</sup>. VEGF was originally recognized for its ability to increase the permeability of the microvasculature to circulating macromolecules. More recently, VEGF has also been shown to be a selective endothelial cell mitogen, and therefore has been alternatively called VEGF<sup>[5-7]</sup>. By alternating splicing of mRNA, four different molecular species with 121, 165, 189 and 206 amino acids were determined.

VEGF was originally discovered as a tumor-secreted protein, and its role in tumor development were investigated<sup>[5,8]</sup>. It is considered to play an important role in tumor biology in at least two ways: as a vascular permeability factor and/or endothelial growth factor. As a potent permeability factor, VEGF promotes extravasation of plasma fibrinogen, leading to the formation of a fibrin network which serves as a substratum for cell migration during angiogenesis. While as an endothelial growth factor, VEGF stimulates endothelial cell proliferation and is likely to induce the formation of new blood vessels<sup>[9]</sup>.

VEGF is synthesized and secreted by a variety of tumor cells in tissue culture and by several transplanted animal tumors *in vivo*<sup>[7,10]</sup>. Elevated expression of VEGF in human tumors have been reported in most tumors. However, there has been few reports on the VEGF expression in gastric carcinoma.

In this study, we examined the VEGF expression in gastric carcinoma by means of *in situ*

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**Received** 1997-10-06

hybridization (ISH) and compared it with the pathological features of gastric carcinoma in order to determine whether VEGF is overexpressed in gastric carcinoma compared with the normal stomach tissues and its role in the invasion and metastasis of gastric carcinoma.

## MATERIALS AND METHODS

### *Subjects and tissue samples*

Twenty-eight patients with gastric carcinoma who underwent surgical resections at Ruijin Hospital were studied, including 19 men and 9 women, aged from 38 to 78 (mean age  $56.2 \pm 11.9$ ) years. None had received chemotherapy or radiotherapy before surgical resection. A tumor sample and a normal part of the stomach were obtained during surgical resection. The samples were each divided into two pieces. One was subjected to fixation in 10% formalin for histological examination and immunohistological test, and the other was used for ISH, i.e., tissues were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C and were then transferred to 30% sucrose in PBS, pH 7.4, overnight at 4°C. The tissues were then frozen in OCT (Miles Inc.) and stored at -70°C until use for VEGF mRNA expression analysis. Pathological features of the patients were obtained from the pathological reports and clinical records. Histological analysis of the tumor was done by experienced pathologists without knowing the analytical results. The differentiation grading was based on the predominant findings.

### *Preparation of single-stranded RNA probe for ISH*

The pBluescript II SK+ construct with the VEGF insert (a gift from Dr. Yamauchi, Japan) was linearized with *Bam* H I and transcribed *in vitro* from the T<sub>7</sub> polymerase promoter to yield the antisense RNA probe. The same construct was linearized with *Eco* R I and transcribed from the T<sub>3</sub> polymerase promoter to yield the sense (control) probe. The transcription reaction was performed in the presence of 0.35mM digoxigenin-UTP (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) to yield digoxigenin-labeled RNA probe.

### *In situ hybridization*

*In situ* hybridization was performed using a digoxigenin nucleic acid detection kit with some modifications. Frozen sections were cut at 10 μm, fixed for 20min in 4% paraformaldehyde/0.1M PBS, and rinsed in PBS. Specimens were then treated with 0.1% proteinase K, fixed in 4% paraformaldehyde/0.1M PBS again, acetylated in 0.1M triethanolamine and 0.25% acetic anhydride, and dehydrated through a graded ethanol series. Sections were prehybridized for 2 hours at 53°C with

50 μl of prehybridization fluid containing 50% formamide, 0.3M NaCl, 10mM Tris-HCl(pH 8.0), 1mM EDTA, 10% dextran sulfate, 1 × Denhardt's solution (0.02% Ficoll, 0.02% BSA, 0.02% polyvinyl pyrrolidone), and 0.025% salmon sperm DNA in a humidified chamber. After the prehybridization, hybridization was performed in buffer containing prehybridization fluid and 1.0 mg · L<sup>-1</sup> of either antisense or sense probe at 53°C for 16 hours. Next, sections were washed at 37°C for 30min in 4×SSC (150mM NaCl and 15mM sodium citrate) and 30min in 2 × SSC/0.05%SDS, digested with RNase (20 mg · L<sup>-1</sup>) at 37°C for 15min, and washed again at 37°C for 30min in 2 × SSC/0.05% SDS, and washed at 37°C for 30min in 1 × SSC, 0.5 × SSC, respectively. Then, blocking was performed in blocking buffer containing 1% BSA for 30min. After the blocking buffer was removed, the solution containing 1 : 1000 sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase and 1% BSA was placed on each section. Sections were placed in a humidified chamber and incubated at room temperature for 1 hour. After the sections were rinsed, a coloring reaction was performed using 225 μg · mL<sup>-1</sup> nitroblue tetrazolium salt and 175 mg · L<sup>-1</sup> 5-bromo-4-chloro-3-indolyl-phosphate. When the color reaction was appropriate, stop it by washing in buffer solution. The section was washed, dehydrated and mounted.

### *Microvessel count*

The methods of microvessel staining and counting were as described previously<sup>[11]</sup>.

### *Statistical analysis*

The clinicopathological features between VEGF expression and nonexpression groups were analyzed by Fisher's test. The correlation between MVC and VEGF expression was evaluated with Student's *t* test.

## RESULTS

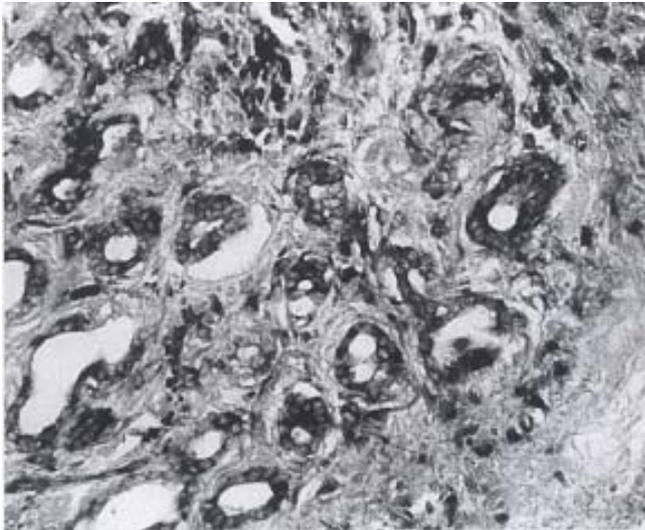
### *Expression of VEGF mRNA in gastric cancer*

Twenty-eight cases of human gastric carcinoma were studied by ISH with both antisense and sense (control) probes to VEGF. VEGF mRNA was expressed in the malignant epithelium of 19 of the 28 patients (19/29, 67.9%). Generally, VEGF mRNA was distributed homogeneously and intensely throughout the tumor. VEGF was expressed by tumor cells under microscopy (Figure 1). However, it is noteworthy that in the periphery of necrotic foci of the gastric cancer, labeling with the VEGF antisense probe was distinctly intensified in tumor epithelium adjacent to the necrotic foci. The tumor stroma cells also labeled for VEGF mRNA. Stromal labeling was distinctly focal as compared to the labeling regularly observed over tumor cells and usually occur red in stroma immediately adjacent to

the foci of overt tissue necrosis. Labeled stromal cells included fibroblasts and smooth muscle cells. However, vascular endothelium did not express detectable VEGF mRNA. No specific cellular labeling was seen with the VEGF antisense in normal epithelium of gastric mucosa from these cases, nor did tumors label with the VEGF sense probe.

#### Comparison of clinicopathological findings and VEGF mRNA expression

VEGF mRNA expression in gastric carcinoma was compared with the clinical and histological features of the tumors. The comparison between VEGF mRNA expression groups and nonexpression groups is shown in Table 1.



**Figure 1** ISH with antisense probe to VEGF of a gastric adenocarcinoma ( $\times 200$ ).

**Table 1** Comparison of clinicopathological features of gastric carcinoma between VEGF mRNA expression groups and nonexpression groups

Characteristics	VEGF mRNA(+)	VEGF mRNA(-)	P
Vascularity			
MVC	18.1 $\pm$ 7.8	12 $\pm$ 5.9	<0.005
Serosal invasion			
Positive	16	3	<0.005
Negative	3	6	
Lymph node metastasis			
Positive	17	4	<0.05
Negative	2	5	
Histologic type			
Differentiated	2	2	<0.05
Undifferentiated	17	7	
Other metastasis			
Positive	3	0	<0.05
Negative	16	9	
TNM staging			
I	1	4	
II	9	3	<0.05
III	6	2	
IV	3	0	

VEGF mRNA expression was significantly correlated with serosal invasion and lymph node metastasis, although no correlation was found between its expression and histologic type, and other metastasis. Incidences of VEGF mRNA expression were increased as the pathologic stage progressed.

#### Correlation between VEGF mRNA expression and MVC

Factor VIII was stained in tumor vascular endothelial cell. Microvessel counts varied from 3 to 38 counts/ $\times 200$  field (average, 15.6 counts). Table 1 shows the correlation between the MVC and VEGF mRNA status. The MVC in VEGF mRNA positive tumors was significantly higher than that in VEGF nonexpression tumors.

#### Relationship between VEGF antigen and its mRNA expression

VEGF antigen was mainly identified in the cytoplasm of cancer cells. Expression rate of VEGF antigen was 46.4% (13/28). Although it was lower than that of VEGF mRNA, there was a significant correlation between their staining intensity ( $r = 0.487$ ,  $P < 0.05$ ).

#### DISCUSSION

Brown *et al*<sup>[12]</sup> examined 2 cases of gastric adenocarcinomas on VEGF mRNA and observed a detectable signal for VEGF in all them using ISH. In this paper, 19 of the 28 primary human gastric carcinoma expressed VEGF mRNA as judged by ISH, the expression rate was 67.9%. VEGF mRNA was mainly detected in malignant glands but not in normal epithelium. This is different from the phenomena that VEGF mRNA expression was detected both in tumor and normal tissue in liver<sup>[13]</sup> and lung<sup>[14]</sup>, and it is suggested that the VEGF expression seems to be a characteristic of the malignant phenotype in gastric carcinoma.

It is worth notice that there are some differences in the strongly stained areas between VEGF mRNA and antigen. Tumor cells stained strongly for VEGF antigen were observed more often in the invasion front than in the tumor center, inversely, VEGF mRNA was expressed more often in the tumor center adjacent to the zones of tumor necrosis than in the invasion front. VEGF mRNA expression elevation around the necrotic area of the tumor has been reported in several other human cancers. Rapid cell proliferation in the center of a tumor can lead to increased interstitial pressure, which may lead to compression closure of capillaries and consecutive tumor necrosis. Tumor hypoxia has been reported to increase expression of VEGF mRNA in a variety of cultured tumor cells<sup>[15]</sup>. Therefore, it is likely that, in the development of necrosis, hypoxia may increase the VEGF mRNA

expression.

Some studies had demonstrated the correlation between the expression of VEGF mRNA and tumor invasion and metastasis. Recently, Zhang *et al.*<sup>[16]</sup> reported that the transfection of this growth factor gene into a tumor cell line expressing a low level of VEGF proteins altered tumor cells more angiogenesis and more progressive in the nude mouse model without change of cell growth rate *in vitro*. In this study, we observed a significant difference of MVC between VEGF mRNA expression group and nonexpression group. The incidences of serosal invasion and lymph node metastasis in tumor which expressed VEGF mRNA were higher than that of tumors with non-VEGF mRNA expression. The tumors with liver or ovarian metastasis are VEGF mRNA positive. Moreover, the rate of VEGF mRNA expression in the advanced tumors is higher than that in early stage tumors. All these results indicate that VEGF mRNA may play an important role in tumor angiogenesis, invasion and metastasis of gastric carcinoma.

VEGF mRNA expression was observed in 19 cases, but still was not detected in 9 cases. This suggests that angiogenesis is not simply controlled by the presence of VEGF but is mediated by several angiogenic inducers. To date, many angiogenic factors have been found, including basic fibroblast growth factor, transforming growth factor- $\beta$ , and tumor necrosis factor  $\alpha$ . In fact, there has been a lot of reports on bFGF expression in gastric carcinoma. Therefore, it is likely that vascularization in some tumors is related to other angiogenic factor. If VEGF is indeed responsible for gastric cancer angiogenesis, therapeutic strategies to inhibit its activity using either specific antibodies or antisense RNA may allow clinicians to treat not only the malignant cells within a tumor but also the vascular supply of the tumor. In

conclusion, the identification of VEGF that correlates with angiogenesis in gastric cancer may provide a basis for targeting this angiogenic factor to inhibit vascularization of tumors.

## REFERENCES

- 1 Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992;267(12): 10931 - 10934
- 2 Berse B, Brown LF, Van De Water L, Dvorak HF, Senger DR. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differently in normal tissues, macrophages, and tumors. *Mol Biol Cell*, 1992;3 (3):211 - 210
- 3 Senger DR, Galli SJ, Dvorak AM, Peruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, 1983;219(4356):983-985
- 4 Dvorak HF, Orenstein NS, Carvalho AC, Churchill WH, Dvorak AM, Galli SJ. Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. *J Immunol*, 1979;122(2):166 - 174
- 5 Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun*, 1989;161(7):851 - 858
- 6 Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, 1989;246 (4426):1306 - 1309
- 7 Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino TJ *et al.* Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest*, 1989;84(12):1470 - 1478
- 8 Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature*, 1992;359 (3682):845 - 848
- 9 Senger DR, Van DeWater L, Brown LF, Nagy JJ, Yeo KT, Yeo TK *et al.* Vascular permeability factor in tumor biology. *Cancer Metastasis Rev*, 1993;12 (3):303 - 324
- 10 Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A *et al.* Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: Concentration in tumor blood vessels. *J Exp Med*, 1991;174(4):1275 - 1278
- 11 Maeda K, Chung YS, Takatsuka S, Onoda N, Sawada T *et al.* Tumor angiogenesis and tumor cell proliferation as prognostic indicators in gastric carcinoma. *Brit J Cancer*, 1995;72(2):319 - 323
- 12 Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Senger DR *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptor in adenocarcinomas of the gastrointestinal tract. *Cancer Res*, 1993;53 (19):4727-4735
- 13 Suzuki K, Hayashi N, Miyamoto Y, Yamamoto M, Ohkawa K, Ito Y *et al.* Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res*, 1996;56(13):3004 - 3009
- 14 Ohta Y, Endo Y, Tanada M, Shimizu J, Oda M, Hayashi Y, Watanabe Y *et al.* Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. *Clin Cancer Res*, 1996;2(8):1411 - 1416
- 15 Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced hypoxia may mediate hypoxia-initiated angiogenesis. *Nature*, 1992;359 (3864):843 - 845
- 16 Zhang HT, Craft P, Scott PAE. Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. *J Natl Cancer Inst*, 1995;87(3):213 - 219

# CCR5 gene expression in fulminant hepatitis and DTH in mice

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**Subject headings** CCR5 gene; DNA, complementary; gene expression; cloning, molecular; hepatitis; hypersensitivity, delayed; disease models, animal; polymerase chain reaction; immunohistochemistry

## Abstract

**AIMS** To isolate mouse CCR5 cDNA (muCCR5) and study its expression *in vivo*.

**METHODS** Marathon PCR was used to isolate muCCR5 cDNA and two animal models were designed to investigate the gene expression *in vivo*, one was mouse fulminant hepatitis induced by *Propionibacterium acnes* (P.acnes) and the other was that with delayed type hypersensitivity reaction (DTH). A specific GST-NH<sub>2</sub>-terminus of muCCR5 fusion protein antibody F(ab')<sub>2</sub> was prepared and clarified. RT-PCR and immunohistochemical analysis were used to observe the expression level of CCR5 gene in mice.

**RESULTS** A positive reaction of mouse macrophage was found in DTH but not expressed in P.acnes induced fulminant hepatitis by RT-PCR and immunohistochemical analysis.

**CONCLUSION** This muCCR5 expression may be involved in an allergic process mediated by cellular immunity but not acute inflammatory reaction induced by P.acnes.

## INTRODUCTION

Leukocytotaxis plays an important role in immune system surveillance and chronic inflammation. Locally produced chemotactic factors are thought to be critical in this directed migration. The chemotactic family can be divided into two subfamilies: ① the 1st subfamily having the first two of the conserved cystein residues separated by another amino acid, the CXC ( $\alpha$ ) chemokines, and the second with CC unseparated the CC ( $\beta$ ) chemokine. The  $\beta$ -chemokine receptors are some G-protein coupled receptors with seven transmembrane domains and share a high degree of amino acid homology in their putative transmembrane domains. Recent researches indicated that the entry of HIV into target cells required the participation of at least two cell surface molecules: one was CD4<sup>+</sup> which was utilized by all HIV strains as the primary virus receptor through a high affinity interaction with the viral envelope protein. However, the CD4<sup>+</sup> alone was not sufficient for virus entry, and some additional cell surface molecules, termed cofactor, for example, CCR5, CCR3, CCR2b and fusin<sup>[1-5]</sup> were found to mediate the entry of HIV-1 into the host cells. CCR5 can express in monocytes, macrophages, and primitive T cells, and bind to  $\beta$ -chemokine RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$ . Expression of CCR5 in conjunction with CD4<sup>+</sup> in a variety of cell types renders them permissive for infection through M-tropic envelope proteins. Meanwhile, CCR5 and CD4<sup>+</sup> are expressed in several cells to mediate the M-tropic HIV strain envelope to form syncytia<sup>[6]</sup>. The M-tropic HIV-1 strain is most sensitive to changes in the first extracellular loop, and therefore, to understand the CCR5 expression is very important. Unfortunately, we found that under the normal condition, mouse CCR5 gene expressed only in a few cell lines and at a very low level *in vivo*. In this study, we used the model of DTH (delayed type hypersensitivity) and a fulminant hepatitis induced by P. acnes for observing the CCR5 expression *in vivo* and analyzed the mechanism of the gene expression.

## MATERIALS AND METHODS

### Cell culture

Human embryonic kidney cell line 293 was cultured in DMEM (Nissui Pharmaceutical Co, LTD,) medium containing 10% fetal calf serum and 50 U/ml penicillin G and 50  $\mu$ g/ml streptomycin.

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**Received** 1997-08-21

### ***Molecular cloning of mouse CCR5 cDNA***

Mouse peritoneal macrophages isolated 3 days after pentose injection, and their total RNA was prepared using RNA 201 B (Cinna/Biotec, Houston, TX). Macrophage total RNA was reverse-transcribed by RT-PCR using a random primer (Takara Shuzo Co. LTD) in the presence of RNase inhibitor (Promega). Corresponding to the sequences of the highly conserved region between the second and fifth transmembrane domains within human MCP-1 and mouse MIP-1 $\alpha$  receptors, the sense primer was (PTM<sub>2</sub>) 5'-GCGAATTCTGGCCAT (CT) TCTGA (CT)CTGCT(CT)TT(CT)CT-3', and the antisense primer was (PTM<sub>3</sub>) 5' GCAAGCTT (GC) A (CT) (GT) GG (AG)TTGA (CT) (AG) CAGCAGTG (AC) GT-3'. 5'-RACE and 3'-RACE reactions were performed to isolate the full-length mouse CCR5 cDNA by means of Marathon cDNA amplification kit (Clontech, CA). In brief, the first PCR reaction was carried out using primer "R1" and the primer adapter 1. The second PCR reaction was performed with the internal primer "R2" of CCR5 and the other primer adaptor 2. The specific primers of mouse CCR5 were as follows: (R1) 5'-GGATCAGGCTCAAGATGACC-3', (F1) 5'-ACACTCAGTATCATTCTGG-3'. PCR products were digested with appropriate restriction enzymes and subcloned into pBluescript SKII<sup>+</sup> (Stratagene). DNA sequencing reaction was performed by a PCR procedure employing fluorescent dideoxynucleotides and analyzed by a model 373A automated sequencer (Applied Biosystem).

### ***Construction of expression vector for mouse CCR5 and preparation of stable transfectant***

For construction of the expression vector of mouse CCR5, the coding region of mouse CCR5 gene was amplified by PCR with specific primers and cloned into pcDNA3 (Invitrogen Corporation). The 5' primer for PCR was designed to generate Kozak sequence, and the constructs were introduced into a human embryonic kidney cell line 293 by the calcium phosphate coprecipitation method modified by Chen<sup>[7]</sup>. Transfected cells were selected in the presence of a neomycin analogue, G-418 (Life Technologies, Inc), at a concentration of 500 ng/L in complete medium.

### ***Preparation of GST proteins fused with extracellular domains of mouse CCR5***

For preparing a recombinant GST protein fused with NH<sub>2</sub>-terminal portion of muCCR5 cDNA, the NH<sub>2</sub>-terminal extracellular binding domain encoding Met1-Leu38 from the ORF region of muCCR5 cDNA was obtained by polymerase chain reaction, and then cloned into EcoRI and BamHI restriction sites of the GST-fusion protein expression vector pGEX-IN. The recombinant DNA was transferred into *E. coli* HB 101 competent cells (Toyobo competent).

The expression and purification of a GST fusion protein were induced with 0.1 M IPTG (isopropylthio- $\beta$ -galactoside, Wako Pure Chemical Industries, Ltd) for 5 hours, put on glutathione-sepharose 4B affinity column (Pharmacia Biotech AB Upsala, Sweden), and then eluted with 5mM of reduced glutathione.

### ***Preparation of polyclonal antibodies F(ab')<sub>2</sub> of GST protein fused with NH2-terminal portion of muCCR5***

Two New Zealand white rabbits were immunized with 100  $\mu$ g of the GST-NH<sub>2</sub>-terminal of muCCR5 fusion protein in CFA (Iatron, Tokyo, Japan); first time, the footpads were injected, and other 9 times at biweekly intervals were given s.c.. One week after the final immunization, rabbits were bled, sera were obtained, and fractionated into IgG by a protein A agarose column (Pharmacia-Biotech, Upsala, Sweden). A portion of the IgG fraction was further digested with pepsin (Sigma Chemical, St. Louis, MO) and the F(ab')<sub>2</sub> fragment was obtained by sequential chromatographies using a protein A affinity and a sepharose 12gel filtration column as previously described<sup>[8]</sup>.

### ***DTH reaction and RT-PCR amplification of muCCR5***

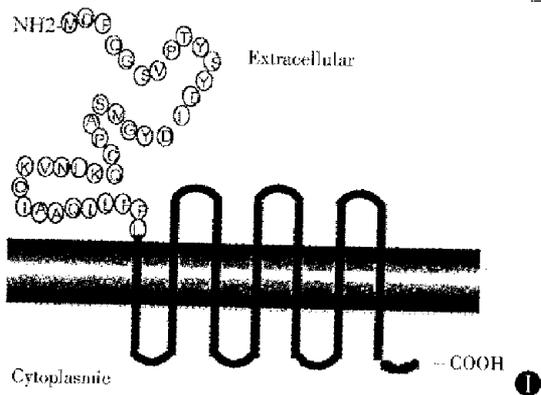
BALB/c female mice of 6 - 7 weeks were used. The hair of abdomen were shaved, 5% Picryl Chloride 100  $\mu$ l was painted on the surface of abdomen, after 5 days 1% picryl chloride was painted on the right ear, 24 and 48 hours later the effect of DTH was evaluated, and then the tissues were taken for preparing total RNA with RNA 201. RT-PCR was run according to Normua<sup>[9]</sup> (94°C for 1min, 55°C for 1min, and 72°C for 1.5min, with a total of 30 cycles).

### ***Induction of fulminant hepatitis in mice***

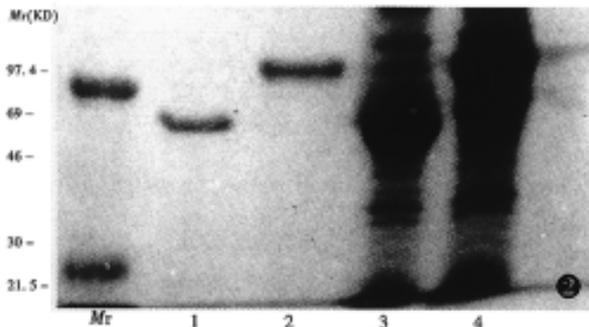
Mice were injected with 1mg of heat-killed *P.acnes* into the tail vein. Seven days later the indicated dosages of LPS were administered intravenously<sup>[10]</sup>. At 3 hours from LPS challenge, three to four mice were killed to obtain the liver. Liver tissues were fixed in 4% paraformaldehyde for 2 hours before being transferred to 70% ethanol and subsequent paraffin embedding.

### ***Immunohistochemical analysis of liver and spleen tissues***

For immunohistochemical analysis, paraffin-embedded tissues were dewaxed with histo-clear (National Diagnostics, Tokyo, Japan) and dehydrated through graded concentrations of ethanol. After being treated with trypsin and blocked with 1% skim milk, the tissue sections were covered with 40ng/L of anti-muCCR5 antibody F

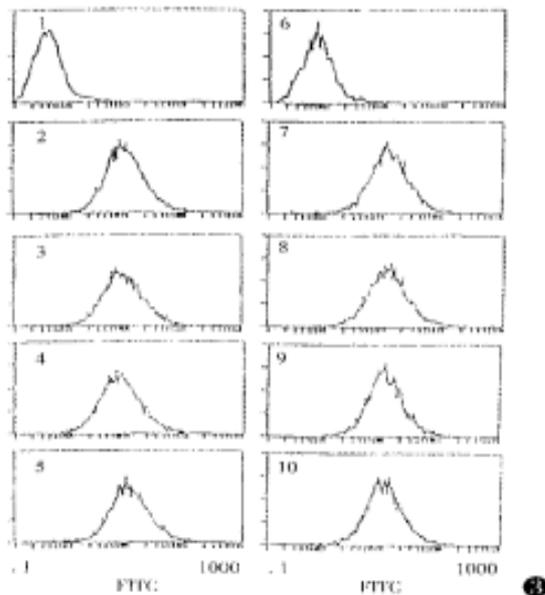


**Figure 1** The first extracellular loop amino acid sequence and transmembrane structure of muCCR5. The amino acid fragment from M to L is located in the first loop of extracellular and it is the binding domain of mouse CCR5 for ligand (MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES).



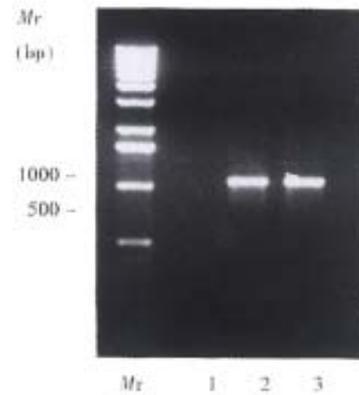
**Figure 2** Analysis of recombinant GST-muCCR5-NH2 terminal fusion protein.

Mr: MW marker, 1, 2: products purified by glutathione sepharose, 3, 4: products not purified, 1, 3: expression products induced by IPTG



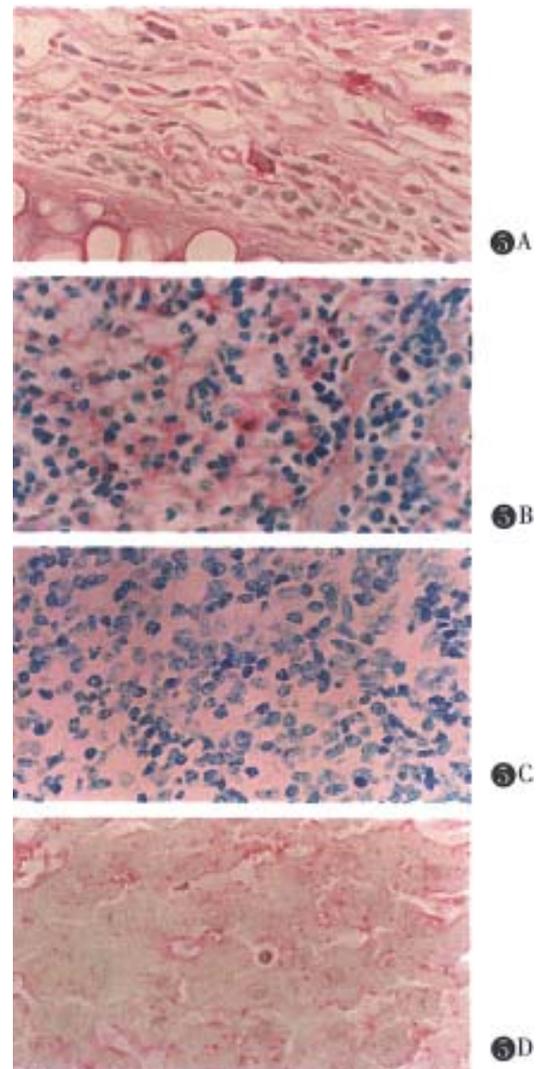
**Figure 3** Flow cytometric analysis of specificity of anti-muCCR5 antibody F(ab')<sub>2</sub>.

F(ab')<sub>2</sub> volume is 40mg/L, blocking reagent volume is 100mg/L.  
 1. 293 cells; 2. transfected cells of muCCR5. Transfected cells were blocked by:  
 3. GST; 4. muIL-8; 5. huFusin; 6. GST-muCCR5-NH2; 7. muCCR1;  
 8. huCCR2; 9. huCCR3 and 10. huCCR4.



**Figure 4** RT-PCR analysis of DTH reactions. muCCR5 specific primers were used to amplify the cDNA from spleen cells.

Lane 1. BALB/C mouse fulminant hepatitis induced by *P. acnes*, Lane 2. 24 hs result after 1% picryl chloride stimulation. Lane 3. 48 hs result after 1% picryl chloride stimulation.



**Figure 5** Immunohistochemical analysis of DTH reaction.

A. Ear: Most of the macrophages in subepidermal interstitial tissue and in the local capillary were obviously positive in their cytoplasm for the staining.  
 B. Spleen: There were plenty of reticular-macrophage in red medulla. A few slightly positive cells, however, could be seen scattered there.  
 C. The negative result was used by anti-GST F(ab')<sub>2</sub>.  
 D. Liver: The cytoplasm of Kupffer cells were slightly stained by the dyes.

(ab')<sub>2</sub> and GST over night at 4°C, incubated with biotinylated swine anti-rabbit IgG, and then reacted with ALP substrate solution (Vector Laboratories, Burlingame, CA) containing 1mM levamisole and solution I, II, III for 40min at room temperature. Finally, the sections were rinsed and counterstained with 1% methyl green.

## RESULTS

### *Molecular cloning of muCCR5 cDNA and preparation of a fusion protein of NH<sub>2</sub>-terminus of muCCR5 with the C-terminus of GST*

A full length cDNA sequence and amino acid sequence of muCCR5 was cloned by Marathon PCR from the poly (A)<sup>+</sup>RNA of mouse peritoneal macrophages. The ORF region of muCCR5 consisted of 355 amino acid, and had a 82% homology with human CCR5. A GST fusion protein of the NH<sub>2</sub>-terminal 38 amino acids of the first loop of extracellular part of muCCR5 was expressed as cytoplasmic protein in *E. coli* (Figure 1). A corresponding fusion protein of 30kD and GST molecule of 26kD were observed on 15% SDS-PAGE with Coomassie blue-staining (Figure 2). In general, 3mg-5mg GST-NH<sub>2</sub>-terminus of muCCR5 fusion protein was produced from 200 ml *E. coli* solution.

### *Determination of the specificity of anti-GST-NH<sub>2</sub>-muCCR5 antibody*

An experiment was performed to examine the cross reaction of the anti-muCCR5 F(ab')<sub>2</sub> with other receptors by means of the muCCR5 293 transfectant, and the results showed that the anti-muCCR5 F(ab')<sub>2</sub> could bind with GST-NH<sub>2</sub>-muCCR5 fusion protein but not human CCR1, CCR2, CCR3, CCR4, Fusin and mouse IL-8 (Figure 3), suggesting the specificity of the anti-muCCR5 F(ab')<sub>2</sub> to muCCR5.

### *RT-PCR analysis*

The expression of muCCR5 mRNA in DTH was confirmed by RT-PCR, with the sense primer 5'-ATGGATTTTCAAGGGTCAGTTC-3' and antisense primer 5'-TCATAAACCAGTAGAACTTC-3'. The results showed that after induction with 1% picryl chloride for 24 and 48 hours, muCCR5 could express in spleen cells and the negative result was found in fulminant hepatitis induced by *P.acnes* (Figure 4).

### *Immunohistochemical analysis*

In the normal mice, CCR5 expression level was very low or nil, and muCCR5 was not expressed in fulminant hepatitis induced by *P.acnes* reaction (unpublished data). The Immunohistochemical analysis revealed that muCCR5 clearly expressed on ear at the point of picryl chloride induction (Figure 5A). A weakly positive result was found in spleen

macrophages in DTH reaction induced by picryl chloride (Figure 5B), but a negative result of DTH reaction induced by picryl chloride was found in normal experiment (Figure 5C) and a liver Kupffer cells also showed a positive result in DTH reaction (Figure 5D).

## DISCUSSION

In this study we first cloned mouse CCR5 full length cDNA sequence from peritoneal macrophages poly (A)<sup>+</sup> RNA with Marathon PCR, then constructed the fusion protein of GST with 5' terminal extracellular binding domain 38 amino acid of muCCR5 and successfully have it expressed in *E. coli*. In the Northern analysis, we could not find expression of muCCR5 in vivo. In order to understand the expression of CCR5 in vivo, two animal models were designed, one was mouse fulminant hepatitis induced by *P.acnes* and the other was that with delayed type hypersensitivity reaction (DTH). In previous studies, *P.acnes* induced transient increase in serum TNF- $\alpha$  levels but not those of IL-1ra, IL-1 and IL-6. However subsequent LPS challenge induced the elevated serum levels of all these cytokines and the peak serum IL-1ra level was 20 times that of serum IL-1 levels. Immunohistochemical analysis demonstrated that IL-1ra was predominantly produced by hepatocytes during the priming phase by *P.acnes* and eliciting phase by LPS challenge<sup>[10]</sup>. The responsiveness of alveolar lymphocytes to recombinant IL-2 was evaluated by <sup>3</sup>H-thymidine uptake in the presence and absence of *P.acnes*. *P. acnes* stimulates IL-2 production and IL-2 receptor induction in alveolar lymphocytes from patients with active sarcoidosis<sup>[11]</sup>. In our study of the expression of muCCR5 in *P.acnes* induction, we did not observe a positive reaction, which indicated that muCCR5 gene expression did not involve an acute inflammatory process. In the DTH reaction, the results showed that muCCR5 had expressed in mouse macrophages. No matter some factors might induce a DTH condition, in this case, cell immunity mediated CCR5 gene expression and HIV-1 in infected macrophages more easily. This is an interesting problem which needs further studies.

There have been some reports about the DTH reaction, for example, RANTES produced by IFN- $\alpha$  and TNF- $\alpha$  induction has already been described in a number of studies and clearly IFN- $\alpha$  and TNF- $\alpha$  have a synergic action. INF-d stimulated macrophage to express RANTES and TNF- $\alpha$  stimulated cells to express RANTES gene. It is well known that RANTES is a ligand of CCR5. In the DTH state, macrophages not only express ligand but also express its receptor. In this case, if the ligand binds to its receptor, what physiological changes will happen? Or the ligand (RANTES) thus produced may induce receptor expression; this is another interesting

problem. Th-2 cytokines such as IL-4 and IL-10, have been demonstrated to inhibit DTH granule formation and increased production of these cytokines was associated with impaired DTH reaction<sup>[11-15]</sup>. Th-2 cytokines may affect CCR5 expression of macrophages, because a high concentration of Th-2 cytokines can alter the recruitment of immune cells to DTH granules at least partly by inhibiting CCR5 expression.

## REFERENCES

- Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M. Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, 1996;381(6584):661 - 666
- Dragic T, Litton V, Alloy GP, Martin SR, Huang Y, Nagashima KA. HIV-1 entry into CD4+ cells is mediated by the receptor CC-CKR-5. *Nature*, 1996;381(6584):667 - 673
- Samson M, Libretto F, Doranz BJ, Rucker J, Liesnard C, Farmer CM. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 receptor gene. *Nature*, 1996;382(6593):722 - 725
- Cocchi F, DeVico AL, Grain Demo A, Arya SK, Gallo RC, Lush P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science*, 1995;270(5243):1811-1815
- Paxton WA, Martin SR, Tse D, O'Brien TR, Skurnick J, VanDervanter NL. Relative resistance to HIV-1 infection of CD4+ lymphocytes from persons who remain despite multiple high-risk sexual exposure. *Nat Med*, 1996; 2 (4): 412 - 417
- Choe H, Farzan M, Sun Y, Sultan N, Rollins B, Ponath PD. The beta-receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell*, 1996;85(7):1135-1148
- Chen C, and Okayama H. High efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol*, 1987;7:2745-2749
- Morohashi H, Miyawaki T, Normura H, Kuno K, Murakami S, Matsushima K. Expression of both types of human interleukin-8 receptors on mature neutrophils, monocytes, and natural killer cells. *J Biol*, 1995;57(1):180-187
- Nomura H, Nielsen BW, Matsushima K. Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. *Intern Immunol*, 1993;5(10):1239 - 1249
- Pour F, Melon S, Coffinur RL. Interleukin-4 and interleukin-10 to inhibit cell-mediated immunity in vivo. *Er J Immunol*, 1993;23(11):3043 - 3049
- Devergne O, Marring-Choke A, Schall TJ, Leger-Raft MB, Sadick M, Peuchmaur M. Production of the RANTES in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. *J Exp Med*, 1994;179(5): 1689-1694
- Tsipopoulos A, Hamid Q, Varney V, Ying S, Moqbel R, Durham SR. Preferential messenger RNA expression of Th1-type cells (IFN-gamma, IL-2+) in classical delayed type (tuberculin) hypersensitivity reactions in human skin. *J Immunol*, 1992;148(7):2058 - 2061
- Devergne O, Emilie D, Peuchmaur M, Craven MC, Agay MF, Galanaud P. Production of cytokines in sacred lymph nodes: preferential expression of interleukin-1 beta and interferon-gamma genes. *Hum Patrol*, 1992;23(3):317-323
- Heinzl FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of evidence for expansion of distinct helper T cell subsets. *J Exp Med*, 1989;169(1):59-72
- Sadick MD, Heinzl FP, Holiday BJ, Pu RT, Dawns RS, Locksley RM. Cure of moun with anti-interleukin 4 monoclonal antibody evidence for a T cell-dependent, interferon gamma-independent mechanism. *J Exp Med*, 1990;171(1):115-127

## Relationship between tumor necrosis factor- $\alpha$ and liver fibrosis

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**Subject headings** tumor necrosis factor/metabolism; laminin/blood; hyaluronic acid/blood; liver cirrhosis/blood

### INTRODUCTION

To investigate the relationship between tumor necrosis factor- $\alpha$  and liver fibrosis in patients with chronic liver disease.

### METHODS

Radioimmunoassay was made in 20 patients with mild chronic hepatitis (CMH), 20 patients with severe chronic hepatitis (CSH), 51 patients with liver cirrhosis (LC) and 32 normal persons to determine the contents of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), laminin (LN) and hyaluronate (HA) in serum. The changes in and relationship between TNF- $\alpha$ , LN and HA were analyzed. The TNF- $\alpha$  and collagen III were determined using

immunohistochemical studies in liver tissues from 32 persons including 7 normal persons, 3 patients with MCH, 5 patients with SCH and 17 with LC.

### RESULTS

TNF- $\alpha$ , LN and HA levels in serum of CSH and LC patients were significantly higher than those in healthy controls (SCH:  $1.11 \pm 0.59, 130.7 \pm 17.2, 219.1 \pm 121.3$ ; LC:  $0.92 \pm 0.66, 156.8 \pm 31.7, 400.5 \pm 183.7, P < 0.05 - 0.01$ ), which increased gradually, and correlated positively with each other in all patients with liver diseases ( $n = 91, \gamma = 0.3149, P < 0.01$ ). TNF- $\alpha$  contents showed a remarkably positive correlation with HA and LN levels in CMH and CSH (LN:  $n = 40, \gamma = 0.3404, P < 0.05$ ; HA:  $n = 40, \gamma = 0.3847, P < 0.05$ ). The total collagen content of MCH, SCH and LC increased gradually in liver biopsy specimens. The number of TNF- $\alpha$  positive cells increased significantly in liver tissues from patients with SCH and LC (62%; 45%;  $P < 0.01$ ). TNF- $\alpha$  positive cells were mainly located in the periportal areas.

### CONCLUSION

TNF- $\alpha$  may be related to liver fibrosis, and might promote liver fibrosis.

problem. Th-2 cytokines such as IL-4 and IL-10, have been demonstrated to inhibit DTH granule formation and increased production of these cytokines was associated with impaired DTH reaction<sup>[11-15]</sup>. Th-2 cytokines may affect CCR5 expression of macrophages, because a high concentration of Th-2 cytokines can alter the recruitment of immune cells to DTH granules at least partly by inhibiting CCR5 expression.

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- Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M. Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, 1996;381(6584):661 - 666
- Dragic T, Litton V, Alloy GP, Martin SR, Huang Y, Nagashima KA. HIV-1 entry into CD4+ cells is mediated by the receptor CC-CKR-5. *Nature*, 1996;381(6584):667 - 673
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### CONCLUSION

TNF- $\alpha$  may be related to liver fibrosis, and might promote liver fibrosis.

# Induction of apoptosis of lymphocytes in rat mucosal immune system \*

CHEN Xue-Qing, ZHANG Wan-Dai, SONG Yu-Gang, ZHOU Dian-Yuan

**Subject headings** mucosal immune system; cycloheximide; proliferating cell nuclear antigen; immunohistochemistry

## Abstract

**AIM** To undergo apoptosis during negative and positive selection processes in rat mucosal immune system which are implicated in the pathogenesis of various mucosal diseases.

**METHODS** Female Sprague-Dawley rats were given protein synthesis inhibitor, cycloheximide, intravenously or intraperitoneally, an apoptosis was recognized by morphological hallmark under light and electronmicroscopy, and the expression of proliferating cell nuclear antigen was visualized immunohistochemically.

**RESULTS** The apoptosis of mucosal lymphocytes in the digestive tract, as well as in trachea, uterus and lacrimal gland was induced by cycloheximide ( $>1.0$  mg·kg<sup>-1</sup> body weight), which were located mainly in lamina propria and germinal centers of lymphoid nodules. At the same time, a portion of crypt epithelial cells of proliferating zone in small and large intestine, and the epithelial cells in genital tract were also found to undergo apoptosis. Immunostainings showed that apoptotic cells expressed proliferating cell nuclear antigen.

**CONCLUSION** Apoptosis of lymphocytes in mucosal immune system can be induced by cycloheximide. This model will facilitate the understanding of normal mucosal immune system and its role in the pathogenesis of related diseases such as inflammatory bowel diseases.

## INTRODUCTION

Mucosal immune system represents the most important humoral immune system of the body quantitatively<sup>[1,2]</sup>. In adults, about 80% of immunoglobulin producing immunocytes are located in the intestinal lamina propria showing the dominant role of the gut as an activated B-cell organ<sup>[1-3]</sup>. Antigenic priming of organized gut-associated lymphoid tissues, mainly includes the Peyer's patches, and may give rise to specific secretory immunity in not only the gut, but also the respiratory tract, lacrimal, salivary and lactating mammary glands<sup>[2]</sup>. The main function of the mucosal immune system is to protect the host against pathogenic bacteria and viruses to which the mucosal surfaces are exposed. The mucosal immunocytes play an important role in the pathogenesis of inflammatory bowel diseases (IBD), gluten enteropathy and Sjögren's syndrome, although the mechanisms have not been elucidated yet<sup>[4,5]</sup>.

Apoptosis (programmed cell death) is a process genetically controlled leading to cell suicide<sup>[6]</sup>. Peripheral T and B lymphocytes, as well as their precursors in the central lymphoid organs, may undergo apoptosis during the negative and positive selection processes. Abnormality of apoptotic machinery within the cell results in the development of various types of diseases. Resistance to apoptosis may be coupled to autoimmune diseases, and leukemia. In contrast, elevated apoptotic decay of lymphocyte has been shown to be involved in lymphopenia caused by human immunodeficiency virus infection<sup>[7]</sup>. Therefore, it is reasonable to expect that induction of apoptosis in immune system will facilitate better understanding of immune system and its role in related diseases.

In this experiment, we used a protein synthesis inhibitor cycloheximide (CHX) to establish a method for rapid induction of apoptosis of immunocytes in rat mucosal immune system. We focused our research on digestive tract as well as on trachea, uterus and lacrimal glands.

## MATERIALS AND METHODS

### Reagent

Cycloheximide was purchased from Serva. The monoclonal antibody against proliferating cell nuclear antigen (PCNA) used for immunostaining were from ZYMED Lab, Inc. VECTASTAIN® ABC kits were products of VECTOR. 3'-

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\*The research was financially supported by Ph. D. grant from First Military Medical University, Guangzhou.

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Received 1997-07-22

diaminobenzidine hydrochloride (DAB) and pepsin were purchased from Sigma. All other chemicals were of reagent grade.

### **Animal model**

Female Sprague-Dawley rats (PLA Animal Center of South China, Guangzhou) weighing  $200 \pm 20$  g were maintained on a standard laboratory diet and given water ad libitum. Twenty-four hours before experiment, they were deprived of food, but accessible to water freely. After anaesthetized with ether, the rats were injected with CHX (dissolved in 0.9% sodium chloride) intravenously or intraperitoneally at dosage of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and  $10 \text{ mg} \cdot \text{kg}^{-1}$  body weight. Controls were given an equal amount of saline. Rats were sacrificed at various time points after treatment (1,2,3 and 4 hours).

### **Histologic examination**

The tissues collected from lacrimal gland, uterus, trachea, and digestive tract (including stomach, duodenum, jejunum and ileum and colon, small portions ( $0.5 \text{ cm} \times 0.5 \text{ cm}$ ) were fixed immediately in 4% buffered-paraformaldehyde (pH 7.2) and embedded in paraffin. These were cut at  $4 \mu\text{m}$  thickness and stained with hematoxylin and eosin (H & E). Apoptotic cells were identified morphologically<sup>[6,8]</sup> for shrinkage of the cells, chromatin condensation, formation of apoptotic bodies and absence of inflammation. Apoptosis incidence could be calculated randomly by counting the apoptotic bodies in lamina propria of uterus<sup>[8]</sup>. At least 200 cells per sec were counted, and three to four rats per group were used.

### **Electronmicroscopic examination**

After the rats were sacrificed, the specimens were immediately fixed in 2.5% glutaraldehyde buffered in 0.1 M- PBS (pH 7.2) at  $4^\circ\text{C}$  for at least 2h. Tissues were washed with the same buffer and postfixed in 1% osmium tetroxide in phosphate buffer at  $4^\circ\text{C}$  for 2h, then dehydrated in graded acetone and embedded in Epon resin. After stained with uranyl acetate and lead citrate, the ultrathin sections were examined under the electronmicroscope (JEOL, JEM, 1200-EX, Japan).

### **Immunohistochemistry**

Paraffin sections were deparaffined, rehydrated and digested with 0.001% pepsin in 0.01N hydrochloric acid at  $37^\circ\text{C}$  for 10min, and rinsed in 0.01 M PBS (pH 7.2) for another 10min. The tissues were then incubated in chronological order with a primary anti-PCNA antibody (diluted 1 : 100) at  $4^\circ\text{C}$  overnight, a biotinylated-secondary antibody at  $37^\circ\text{C}$  for 30min, and avidin-biotin complex for development at  $37^\circ\text{C}$  for 45min. During incubation, the sections were washed for 10min in PBS. Staining was developed in DAB-hydrogen dioxide and

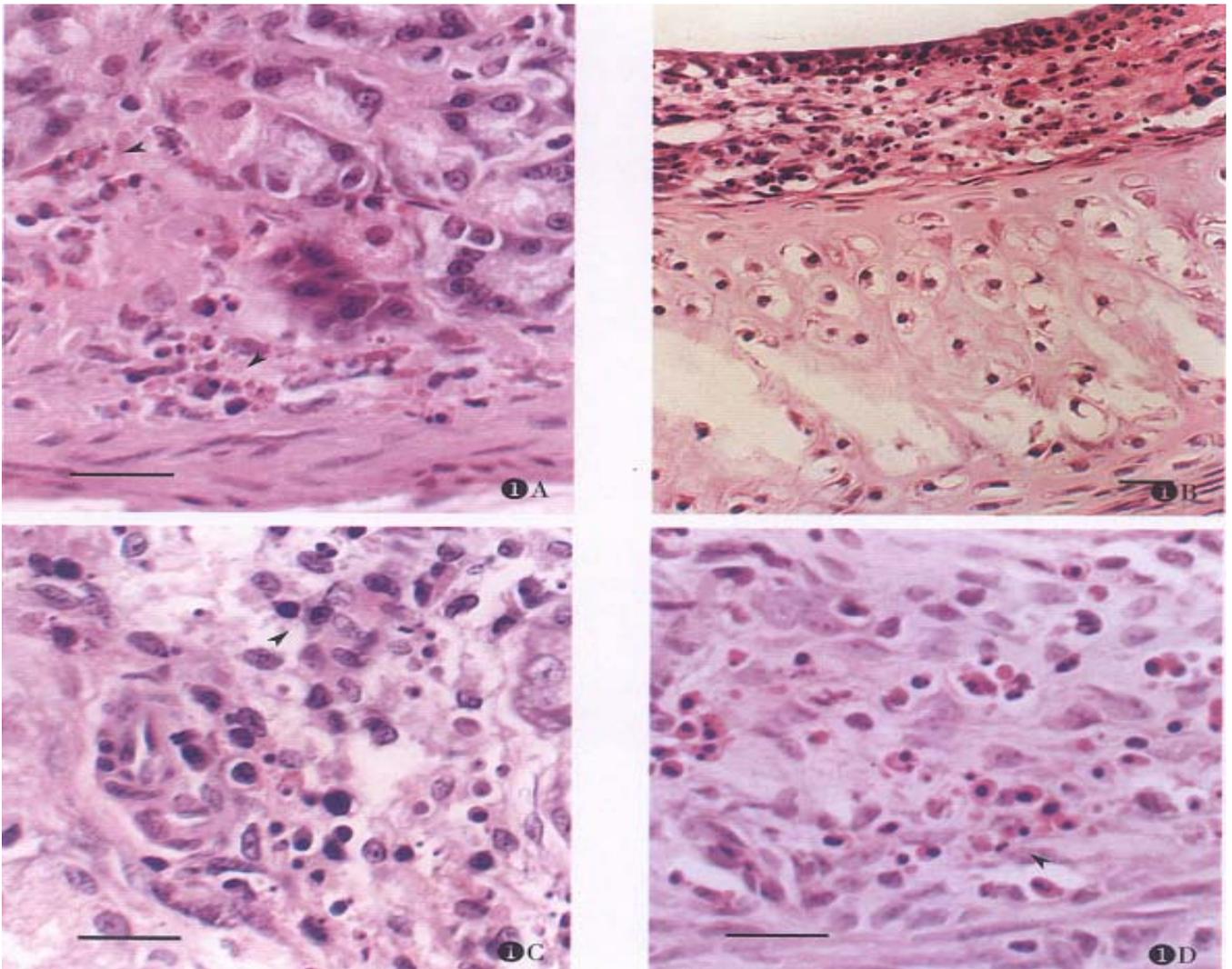
sections were counterstained with hematoxylin. Primary antibody was omitted in negative controls.

### **RESULTS**

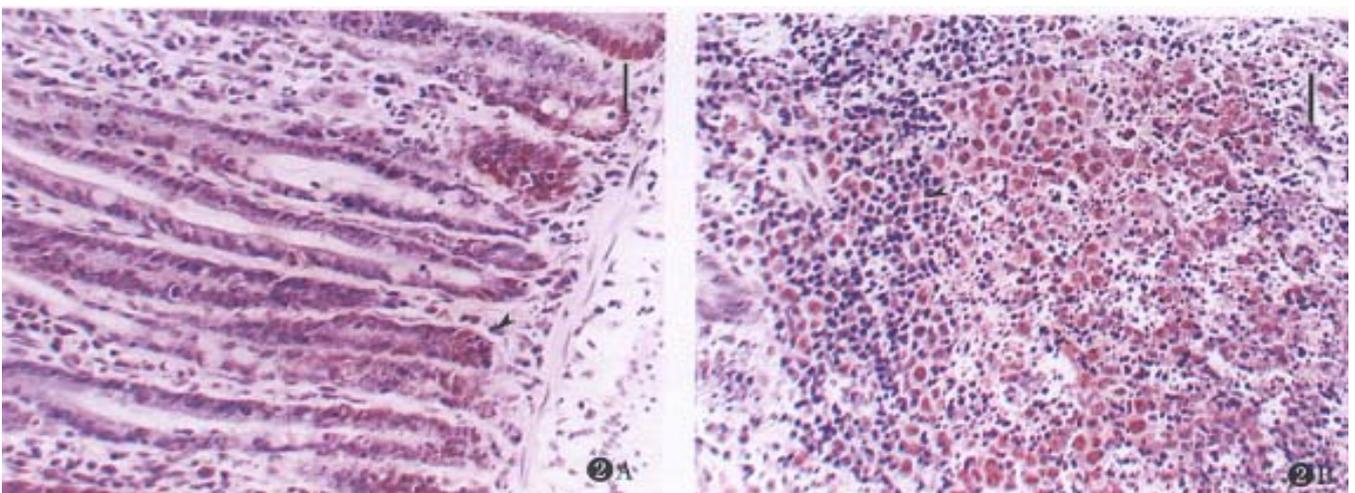
Apoptotic cells were rarely found in normal mucosal of digestive and respiratory tracts, lacrimal gland and reproductive system, even in the germinal centers of lymphoid nodules. After the rats were given CHX, apoptosis was found in the mucosa of these organs with characteristic features, and the apoptotic tissues were devoid of inflammatory response at any time point (Figure 1 A, B, C, D, Figure 2 A, B, Figure 3 B). The induced lymphocyte apoptosis was scattered in lamina propria and clustered in lymphoid nodules. At the same time, the apoptosis of intraepithelial lymphocytes (IEL), particularly in digestive tract, was also observed. Besides, a portion of the mucosal epithelial cells were found dead, including crypt epithelial cells in the small and large intestines (Figure 2 A) and epithelial cells of uterus. However, the epithelial cells lacrimal glands and trachea were still normal (Figure 1 B, C). The apoptosis of epithelium in the digestive tract was within proliferating zone of crypt epithelium (Figure 2 A, B). Despite these changes, the epithelium remained in integrity, and no erosion or ulceration were seen macroscopically. Microscopically, vascular changes were found in lamina propria with capillary dilatation and the vessels packed with erythrocytes, and no thromb was observed.

Apoptosis was defined as condensation and margination of chromatin, contraction of cytoplasm, and cell shrinkage, and the mitochondria could be seen<sup>[6,8]</sup>. In this study, at the early apoptotic stage, the mitochondria was well preserved, and its number increased as well (Figure 3B). Activated macrophages phagocytosed apoptotic bodies (Figure 1B, C). Some apoptotic bodies of lymphocytes and epithelial cells were phagocytosed by the neighboring epithelial cells.

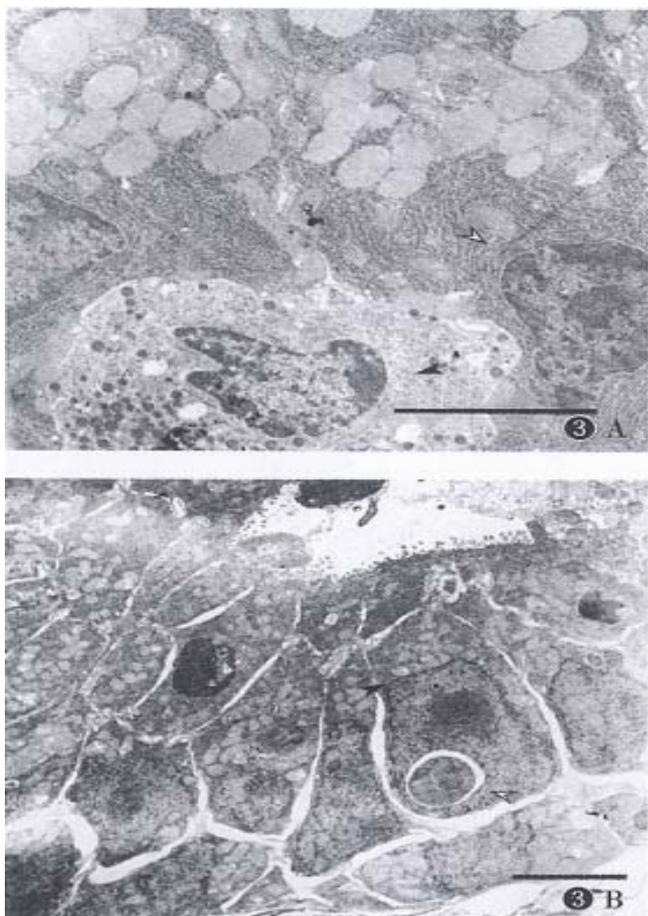
PCNA is an important index proliferating cells<sup>[9]</sup>. The immunostaining showed strong positive expression of PCNA in lymphocytes of germinal centers, crypt epithelial cells of proliferating zone of small and large intestines (Figure 2A,B), and epithelial cells of reproductive system. The scattered lymphocyte in lamina propria in lymphoid nodules, especially in the gut were also positively expressed (Figure 3B). Fragmented nuclei were still found expressed with PCNA. Therefore, the PCNA expression was in consistent with the induced apoptosis. However, some of the lymphocytes and epithelial cells with positive PCNA expression appeared normal morphologically (Figure 3A, B). On the other hand, the cells with negative PCNA expression failed in apoptosis (Figure 3A, B), even with large dosage of CHX ( $>10.0 \text{ mg} \cdot \text{kg}^{-1}$ ).



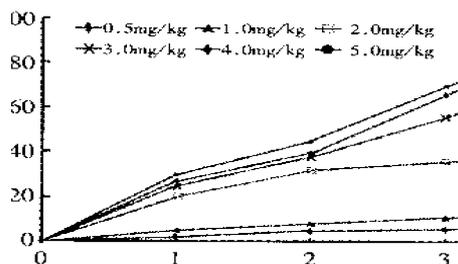
**Figure 1** Occurrence of apoptosis in mucosa 3 hours after injection of CHX ( $3.0 \text{ mg} \cdot \text{kg}^{-1}$ ) (bar =  $20.0 \mu\text{m}$ , H&E). (A) Stomach apoptotic cells (bodies) lie in the lamina propria (arrows) of the mucosa. The parietal cells, chief cells and enterochromaffin-like cells seem not to be affected by treatment of CHX. (D) and (C) apoptosis in lamina propria of uterus and lacrimal gland. The apoptotic cells (bodies) were phagocytised by macrophages. (B) Mucosa apoptosis in trachea.



**Figure 2** Expression of PCNA in mucosa after treated with CHX ( $3.0 \text{ mg} \cdot \text{kg}^{-1}$ ) in 3 hours. (bar =  $20.0 \mu\text{m}$ , stained with hematoxylin). (A) Colon. The apoptosis of crypt epithelial cells on the basement of mucosa were seen with expression of PCNA (arrow). (B) Peyer's patches in ileum. Apoptotic cells around the germinal center with expression of PCNA. The lymphocytes in parafollicular T-cell zone were morphologically normal and were negatively expressed with PCNA (arrow).



**Figure 3** Morphological changes of cells after given CHX (3.0 mg·kg<sup>-1</sup>) 3 hours later (bar = 4.0 μm). (A) Stomach. Normal appearance of chief cell (hollow arrow) and ECL cell (black arrow). (B) Jejunum. Condensed nucleus (hollow arrow) and proliferated mitochondria (black arrow).



**Figure 4** The time- and dosage-dependent effect of CHX on apoptotic bodies (cells) formation in rat uterus.

To estimate the effects of CHX on the induced apoptosis, apoptosis incidence could be calculated by counting apoptotic bodies and apoptotic cells of lamina propria in uterus. The incidence of apoptosis was dose-dependent (Figure 4). CHX 1.5 mg·kg<sup>-1</sup> was nonlethal, and only inhibited the protein synthesis by 98% to 90% 1 - 3 hours after treatment<sup>[8]</sup>. Initial intraperitoneal injection of CHX at a dosage of 1.0 mg·kg<sup>-1</sup> showed insignificant effects on apoptosis induction. Apoptosis was achieved significantly in 2h and reached its maximum 4h after a relatively large

dosage of >2.0 mg·kg<sup>-1</sup> body weight. In this study, the mortality rate of the rats was 33.3% (2/6) with 5.0 mg·kg<sup>-1</sup>, and up to 83.3% (5/6) with 10.0 mg·kg<sup>-1</sup> 18 h after CHX. CHX, when given intravenously or intraperitoneally, had the same effects on incidence of apoptosis.

**DISCUSSION**

In this study, mucosal lymphocytes in the immune system and a small portion of mucosal epithelial cells of the digestive and genital tracts could be induced to apoptosis by protein synthesis inhibitor CHX in rats. The apoptosis identified by morphologic features under light and electron-microscopy, could exhibit obviously in 2 to 4 hours after the rats were treated with relatively high dosage (>1.0 mg·kg<sup>-1</sup>) of CHX, no cell necrosis or inflammatory changes were observed.

Lymphocytes primed by antigens in intestinal mucosal lymphoid follicles may transit through lymphatics and mesenteric lymph nodes, enter into circulation, and subsequently “homing” back to the intestinal lamina propria and other mucosal sites of respiratory tract, the female genital tract<sup>[1,2]</sup>. Peyer’s patches are inductive sites and lamina propria and IEL are effector sites. These lymphoid tissues contain more immunocytes than any other tissues of the body<sup>[1,2]</sup>. Peyer’s patches can be divided into three distinct regions: the specialized dome epithelium, the B-cell zone, and the parafollicular T-cell zone. In our experiment, most of lymphocytes in lamina propria and in the germinal centers of Peyer’s patches underwent apoptosis, together with induction of IEL apoptosis in the digestive tract. Only a few lymphocytes in the parafollicular T cell zone of Peyer’s patches were triggered to death by apoptosis. PCNA was expressed in the apoptotic cells in Peyer’s patches and lamina propria PCNA. It has been demonstrated that elevated PCNA appear in the nucleus during late G1 phase immediately before the onset of DNA synthesis, and peaked during S phase<sup>[9]</sup>. These indicate that most of the apoptotic cells induced by CHX in common immune system were activated B and T lymphocytes (at least IEL in gut mucosa)<sup>[1,2,10,11]</sup>.

Epithelial cells are constantly exfoliating to lumen and replaced by cells migrating from the proli-ferating zone, and homeostasis of this process is thought to be controlled by apoptosis<sup>[6]</sup>. In our study, CHX induced crypt epithelial cells of proli-ferating zone in the intestines and in the genital tract to apoptosis. These apoptotic cells were positively expressed with PCNA. Recent studies showed that passage through S phase is required for apoptosis, and S phase is the critical period to determine cell fate either to replicate or to die<sup>[12]</sup>. It seems likely that inhibition of protein synthesis accelerates the process of cell death by inhibition of *de novo* micromolecular synthesis<sup>[13,14]</sup>.

Alternatively, inhibition of “protective protein” synthesis of cells initiated apoptosis. Paradoxically, a number of epithelial cells at proliferating stage, examined by light microscopy, were not triggered to apoptosis by CHX in this experiment, which is consistent with the aforementioned results of lymphocytes. There might be three explanations: one is that these “normal cells” appear “normal” at the early stage of apoptosis, the second one is that the propensity of these cells to undergo apoptosis are cell-cycle-phase directed, suggesting that CHX may only interfere with a certain phase of proliferating cell cycle, and the third explanation is that the phenotype of apoptosis is not critically dependent on protein synthesis and the endonuclease involved in DNA fragmentation is already in place<sup>[15]</sup>.

Although lymphocytes and epithelial cells underwent apoptosis after rats were given CHX, the monocytes/macrophages in common immune system were not affected. It has been observed that increased macrophages actively moved to and appeared in tissues where apoptotic cells and apoptotic bodies were formed in clusters. They phagocytosed the apoptotic cells and apoptotic bodies. This phenomenon renders the apoptosis distinctly different from necrosis and easily recognizable in tissue slides. In addition, before the DNA fragmentation and formation of apoptotic bodies, electronmicroscopy showed that the morphologic changes of the cells in apoptosis would take place in chronological order separating from their neighbors, increasing number of mitochondria, condensed nucleus and cytoplasm. It suggested that the increased mitochondria within cytoplasm can be an early hallmark of apoptosis. Cytoplasm of apoptotic cells was strongly stained with eosin (Figure 1B). It indicates that proliferating mitochondria may be protective/compensatory to apoptosis in consideration of some “protective protein” like bcl-2 protooncogen protein synthesized in mitochondria<sup>[16]</sup>.

It is reported that CHX can induce apoptosis in hepatic cells<sup>[8]</sup>, intestinal crypt cell of proliferating cell types *in vivo*<sup>[17]</sup>. The former represents the first *in vivo* evidence of apoptosis induced by CHX in a quiescent organ. However, our findings indicated that CHX mainly affected activated lymphocytes and epithelial cells of proliferating phase, and we failed to observe that quiescent epithelial cells and endocrine cells succumb to apoptosis by CHX. Therefore, susceptibility to apoptosis is dependent on cell types. In case of stomach the parietal cell, chief cell and ECL cell were morphologically normal (Figure 1A, Figure 3A), and some parietal cells were found in mitosis phase. Proliferating cells were frequently encountered in the isthmus region of stomach<sup>[18]</sup>. Although these negative findings were observed in our study, it is conceivable these proli-

ferating cells may undergo apoptosis induced by CHX.

In summary, CHX injection can severely damage the body immune system of rats, especially the mucosal immune system, but it influences the mucosal epithelial and endocrine cells only slightly though injury of epithelial cells may damage the integrity of epithelium at the early stage (unpublished data). In fact injection of CHX (2.0 mg·kg<sup>-1</sup>) could cause diarrhea in rats for 3 days possibly due to enterobacterial infection, or it might be correlated with sIgA deficiency and malabsorption<sup>[19]</sup>. We also used this model to study mucosal lymphocytes involved in the mechanisms of gastric acid production (unpublished data). Therefore, this model will be useful in investigating the role of mucosal immune system in the pathogenesis of related diseases such as IBD, celiac diseases, and Sjögren's syndrome, as well as turnover mechanism of mucosal immunocytes.

## REFERENCES

- 1 Brandtzaeg P, Nilssen DE, Rognum TO, Thrane PS. Ontogeny of the mucosal immune system and IgA deficiency. *Gastroenterol Clin North Am*, 1991;203(3):397 - 439
- 2 Brandtzaeg P, Halstensen TS, Kepp K, Krajci P, Kvale D, Rognum TO *et al.* Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. *Gastroenterology*, 1989;97(6):1562-1584
- 3 Van Der Heijden PJ, Stok W, Bianchi TJ. Contribution of immunoglobulin-secreting cells in the murine small intestine to the 'background' immunoglobulin production. *Immunology*, 1987;62(2):551-555
- 4 Schreiber S, Raedler A, Stenson W, MacDermott RP. The role of the mucosal immune system in inflammatory bowel disease. *Gastroenterol Clin North Am*, 1992;21(2):451-502
- 5 Hansen DU, Ericsson UB, Henricsson V, Larsson A, Manthorpe R, Warfving G. Autoimmune thyroiditis and primary Sjögren's syndrome: clinical and laboratory evidence of coexistence of the two disease. *Clin Exp Rheumatol*, 1991;9(2):137-141
- 6 Gerschenson LE, Rotello RJ. Apoptosis: a different type of cell death. *FASEB J*, 1992;6(4):2450-2455
- 7 Kroemer G, Martinez-A C. Pharmacological inhibition of programmed lymphocyte death. *Immunol Today*, 1994;15(5):235-242
- 8 Chen XQ, Zhang YL, Song YG, Tan XH, Zhang WD, Zhou DY. A method for rapid induction of apoptosis in rat spleen by cycloheximide. *Chin J Cell Biol*, 1996;18(3):144-145
- 9 Celis JE, Celis A. Cell cycle dependent variations in the distribution of the proliferating cell nuclear antigen in cultured cells: subdivision of S phase. *Proc Natl Acad Sci USA*, 1985;82(4):3262-3266
- 10 Guy-Grand D, Griscelli C, Vassalli P. The gut-associated lymphoid system: nature and properties of the large dividing cells. *Eur J Immunol*, 1974;4(2):435-443
- 11 Willian O Dobbins III. Human intestinal intraepithelial lymphocytes. *Gut*, 1986;27(3):972-985
- 12 Terui Y, Furukawa Y, Kikuchi J, Saito M. Apoptosis during HL-60 cell differentiation is closely related to a G0/G1 cell cycle arrest. *J Cellular Physiol*, 1995;164(1):74-84
- 13 Martin SJ, Lennon SV, Bonham AM, Cotter TG. Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J Immunol*, 1990;145(3):1859-1867
- 14 Evans DL, Dive C. Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. *Cancer Res*, 1993;53(9):2133-2139
- 15 Borner MM, Myers CE, Sartor O, Sei Y, Toko T, Trepel JB, Schneider E. Drug-induced apoptosis is not necessarily dependent on macromolecular synthesis or proliferation in the p53 negative human prostate cancer cell line PC-3. *Cancer Res*, 1995;55(10):2122-2128
- 16 Hockenbery D, Nunez G, Milman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, 1990;348(6299):334
- 17 Ijiri K, Potten S. Further studies on the response of intestinal crypt cells of different hierarchical status to eighteen different cytotoxic drugs. *Br J Cancer*, 1987;55(1):113-123
- 18 Karam SM, Leblond CP. Dynamics of epithelial cells in the corpus of the mouse stomach: I identification of proliferative cell types and pinpointing of the stem cell. *Anat Rec*, 1993;236(3):259 - 279
- 19 Crabbe PA, Heremans JF. Selective IgA deficiency with steatorrhea. A new syndrome. *Am J Med*, 1967;42(2):319 - 326

# Current concept of Spleen-Stomach theory and Spleen deficiency syndrome in TCM

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**Subject headings** spleen deficiency syndrome; spleen-stomach theory

## INTRODUCTION

Spleen-Stomach theory is an important constituent of the theoretical basis of Traditional Chinese Medicine (TCM), the Spleen here is not synonymous with the Spleen in western medicine anatomically, physiologically or pathophysiologically. Conceptually, Spleen-Stomach theory is a comprehensive one, it mainly involves the digestive system, its vegetative nervous system, immunologic function, hemopoiesis, muscle metabolism, endocrine function (thyroid, adrenal cortex and medulla), hepatic metabolic function, protein, nucleotide, energy, water and salt metabolism. Experimental researches on animal models and clinical studies on Spleen deficiency syndrome have yielded fruitful results in this field which lead to a better understanding of its mechanism and help open a new avenue for treatment of diseases relevant to Spleen deficiency.

### *Physiology of Spleen-Stomach*<sup>[1]</sup>

The Spleen-Stomach has various physiologic functions as follows: Spleen governs transport and transformation. Spleen-Stomach transforms food into nutrients which are the sources of Qi and blood. The nutrients include glucose, amino acids, lipid, cations-anions and trace elements. Stomach is considered a reservoir which empties into the intestine. Water and salt absorption depends also on the transportating function of Spleen-Stomach. Qi and blood are both vital to life, Qi means energy, blood points to circulating blood and its formed elements, blood and nutrients furnish nourishment to all organs and tissues of the body.

### *The up- and down-bearing function of Spleen-Stomach*

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**Received** 1997-04-21 **Revised** 1997-05-10

Stomach governs down-bearing function and Spleen governs up-bearing which signify the motility, secretory, assimilative, absorptive and dispersing functions of upper digestive tract, among which, gut hormones and functional activities of vegetative nervous system of the gut are involved. Dysfunction of up- and down-bearing function of Spleen-Stomach can cause gastrointestinal disturbances and various Spleen deficiency syndromes; furthermore, they may also affect the functions of other organs.

### *Spleen governs the flesh or muscles*

Transport and transformation function of Spleen-Stomach provided nutrients for muscle and energy metabolism. When Spleen-Stomach is diseased, muscles will be atrophied and become asthenic.

### *Spleen governs thought*

Brain activities depend normally on furnishing the nutrients, Qi (energy), blood, Yin and Yang. With adequate Qi and blood and normal function of Spleen-Stomach, brain activities can proceed normally.

### *Spleen manages blood*

Spleen has hemopoietic functions; the blood encased within the vessels of all solid and hollow organs is managed by Spleen.

### *Relationship between Spleen-Stomach and other solid and hollow organs*

**Spleen and Heart** The heart governs blood vessels and blood circulation. The lung is related to oxygen uptake. Dispersion of nutrients over the whole body depends on heart, lung and brain function which can also affect Spleen-Stomach motility and function.

**Spleen and Kidney** In TCM, the Kidney stores the body's essence, and its function also depends on the nutrients transformed by Spleen-Stomach. The transport and transformation function of Spleen-Stomach is also influenced by warming and nourishing functions of Kidney. The two are interrelated and mutually potentiating. The Kidney in TCM involves the genital-urinary system, sexual glands and hormones, immunologic function, nervous system and the heart in terms of western medicine. Spleen and Kidney are both

involved in water and salt metabolism. The Kidney-Yang denotes the hypothalamic-pituitary-adrenal, thyroid and gonadal axes, secretion of the corresponding hormones, sodium pump activity of red blood cells, caloric energy production and immunomodulating functions. The Kidney-Yin involves cAMP/cGMP activity.

### ***Diagnostic approach and preventive treatment of Spleen-Stomach diseases<sup>[2]</sup>***

Spleen-Stomach theory forms the basis of diagnostic approach and treatment of Spleen-Stomach disease, according to the presence or absence of Stomach-Qi. Poor appetite signifies grave prognosis and absence of Stomach-Qi; good appetite signifies good prognosis and presence of Stomach-Qi. By looking at the complexion and feeling the strength of pulse, one can predict the prognosis of the patient. Sallow face is the usual manifestation of Spleen disease. Color of skin over thenar muscles reflects Cold or Heat. Cyanotic hue of vein over thenar region indicates Cold in the stomach, whereas redness shows Heat in the stomach. During convalescence from postoperative state or infectious diseases, doctors should prescribe those herbal medicine for replenishing and modulating the Spleen-Stomach function.

Spleen deficiency syndrome is a multi-system and multi-organal functional impairment, but mainly manifests as digestive tract disturbance. It can be classified into Spleen-Qi deficiency, Spleen-Yang deficiency and Spleen-Yin deficiency. The diagnostic criteria in common are poor appetite, abdominal fullness after meal, loose bowel movements, pale or sallow complexion. The characteristic features of Spleen-Qi deficiency are fatigue, asthenia, atrophied muscle, pale tongue with thin white coating and moderate, weak pulse. Spleen-Yang deficiency is characterized by cold limbs, fear of cold, puffy pale tongue with slippery coating and slow fine pulse. Spleen-Stomach-Yin deficiency has dry mouth, low urine output, dry stool, shrunken smooth bare, red tongue and rapid fine pulse.

### ***Pathophysiology of Spleen deficiency<sup>[1]</sup>***

Clinical and animal studies were conducted with controls and statistical analysis made in the past years.

### ***Changes in secretory and absorption functions of gastrointestinal tract***

Salivary flow and salivary amylase activity. Salivary gland activity is related to Spleen function. In patients with chronic gastritis, gastric or duodenal ulcer or chronic colitis with Spleen deficiency syndrome, the salivary flow of parotid glands on citric acid stimulation decreased together with decrease in amylase activity. After Spleen-fortifying treatment, the condition was improved and normalized.

Gastrin and acid secretion. Serum gastrin level was found significantly lowered in Spleen deficiency syndrome but elevated after treatment. In peptic ulcer patients with Spleen deficiency, BAO and MAO were both significantly higher than normal. In chronic gastritis patients with Spleen deficiency, the day and night and 24h uropepsin activities were much decreased.

Pancreatic exocrine function, BT-PABA test. In peptic ulcer patients with Spleen deficiency, urine excretion of BT-PABA diminished. On treatment with Spleen-fortifying drugs, urine BT-PABA and amylase activity in chronic atrophic gastritis patients were increased.

Xylose excretion test. Decrease in xylose excretion in chronic atrophic gastritis patients with Spleen-deficiency syndrome indicated impaired absorption function of small intestine. After treatment, the xylose excretion rate was increased.

Motilin and gastrointestinal motility. In patients after major surgery on digestive tract with Spleen deficiency, serum motilin level was significantly higher than normal. The gastric tone was decreased with retention of fasting gastric juice but transit time of small and large intestine was reduced, resulting in rapid emptying, these motility disturbance led to loss of appetite and epigastric fullness after meal.

Structural and biochemical changes in gastric mucosa. In Spleen deficiency syndrome patients, the turnover rate of epithelial cells was hastened, showing a short life span. In the mucosal lamina propria of superficial gastritis with Spleen deficiency, the glandular atrophy was more severe, intestinal metaplasia more frequent, whereas in disharmonic Liver and Stomach type, the metaplasia was modest. Ultrastructural studies revealed reduced microvilli of epithelial cells, increased junctional width, membrane damage, swollen mitochondria with disrupted cristae and dilated endoplasm in parietal cells<sup>[4]</sup> decreased pepsinogen granules within chief cells; increased plasma cell infiltration in lamina propria. These changes were not seen in disharmonic Liver and Stomach type. Substance P and VIP were found increased in sigmoid colon, correlated with loose bowel movement. Elevated cAMP levels in gastric mucosa and plasma of Spleen deficiency syndrome were found, and plasma cAMP/cGMP ratio was decreased markedly in those patients with intestinal metaplasia. Likewise, gastric mucosal SOD content and plasma LPO also decreased significantly; these might correlate with metaplasia.

### ***Dysfunction of vegetative nervous system of GI tract***

Cerebral cortical function is extensively suppressed, presented with unstable somatic evoked potential, diminished amplitude and poor reproducibility. The hypofunction of sympathetic nervous activity

manifested a decrease in skin electric potential activity, reactivity of peripheral vessels to cold, urine VMA contents as well as plasma dopamine hydroxylase level; all of them increased after adequate treatment. In these patients, blood acetylcholine level was elevated, usually accompanied by bradycardia and lower systolic and diastolic blood pressure; these indicated presence of relative hyperfunction of parasympathetic nervous activity.

Motility and secretion modulating functions of GI tract are relevant to gut hormones. Besides, they are also related to modulating function of the vegetative nervous system of GI tract. Deficiency of Spleen in chronic diarrhea and peptic ulcer patients have overactive parasympathetic nervous system in the majority and hyperactivity of both parasympathetic and sympathetic nervous system in a minority of the patients.

### Immunologic functional changes

In Spleen deficiency patients, peripheral blood lymphocyte count is lower than normal. In patients with chronic hepatitis B with liver depression and Spleen deficiency, it is also lower than normal, but can be restored to near normal after Spleen-fortifying therapy. T cell subset study revealed significantly decreased total T cell and TH lymphocytes. Among cancer patients with Spleen deficiency, CD4 was lower than normal, whereas CD8 had no change. On treatment with astragalus and atractylodes, CD4 was elevated significantly.

Immunoglobulin G changed very little but the content of secretory IgA in GI disease with Spleen deficiency decreased significantly. After treatment with the Si Jun Zi decoction, these can be restored to the level of controls. Some Spleen-fortifying prescriptions can enhance proliferation of mice splenic cells and increase significantly the mice specific antibody secretory cell number, antigen-induced delayed allergic reaction and mixed lymphocytic reaction; they also enhance cytotoxic action of lymphocytes and promotes ConA-stimulated mice splenic cells to secrete IL-2. Phagocytic function of monocyte-macrophages also increase as seen by the clearance of carbon particles. In cancer-bearing patients, NK activity and TK activity are lowered but can be restored by Spleen-fortifying therapy. With Hp infection, these patients have a weaker lymphocyte and plasma cell infiltration and local SIgA response. It was believed that red blood cells also have the function of clearing circulatory immune complex and phagocytosis. Astragalus and Si Jun Zi decoction can restore the immunologic function of red blood cells, probably through their promoting effect on C<sub>3</sub>b receptor expression and the activity on the red cells surface.

Recently, it was found that Spleen-deficiency

patients had a high frequency of HLA-B<sub>12</sub>-whereas disharmony of Liver and Stomach patients had a high frequency of HLA-B15. These showed that immune response is closely related with vulnerability to disease.

### Endocrine changes

Urine 17-ketosteroid was found significantly lower than normal but there was no significant change in 17-hydroxysteroids as compared with normals. In patients with Spleen deficiency the level of catecholamine was also low. There was thyroid hypofunction in Spleen deficiency patients, with total T<sub>3</sub> and fT<sub>3</sub> significantly lower than normals, whereas rT<sub>3</sub> was significantly higher; low metabolic rate, low skin temperature, poor tolerance to cold and lack of adaptation to environmental changes were also present. This poor tolerance to cold is a special feature of Spleen-Yang deficiency. Asthenia and loss of weight might also be due to hypofunction of thyroid.

### Changes of fecal bacteria flora and *Helicobacter pylori*

In Spleen deficiency mice *Lactobacillus bifidus* and other lactobacilli were decreased but could be restored to normal after the Si Jun Zi decoction treatment. The enterobacteria pathogens were increased but could be reduced after treatment and *Helicobacter pylori* was also decreased in amount. Spleen-fortifying therapy lowers the rate of detection and amount of Hp<sup>[3]</sup>.

### Changes of trace elements in blood

In chronic hepatic disease with Spleen-deficiency the blood Zn was significantly lower than normal, but Cu was on the reverse. Zn is important in enzymatic action, nucleic acid synthesis, membranous function of red blood cells, hemopoiesis and cell respiration. In Spleen-Yang deficiency serum Mg was increased, whereas in Spleen-Yin deficiency it was extremely low. Fe was elevated in Spleen Qi deficiency, and Spleen-Yang deficiency patient at the age of 50 to 60 years, and ten years after it was elevated. In Spleen-Qi deficiency Mn and Cr were both increased significantly<sup>[4]</sup>.

### Muscle metabolism

The muscle glycogen and CPK activity in the quadriceps and plasma were all decreased significantly. The resulting asthenia, and muscular weakness were primarily due to energy depletion from lowered hepatic and muscle glycogen content. Besides, ATP, ADP and AMP contents were also much lowered, and LDH and succinyl dehydrogenase activities significantly elevated because of anaerobic glycolysis; those ions relevant to muscle contraction were decreased<sup>[5]</sup>.

Serum total free amino acids and essential

amino acids including branch chained amino acids were all decreased; lysine, valine, glycine, threonine, tryptophan, isoleucine, serine, alanine and histone were all lower than normal. These may all contribute to muscle emaciation.

#### **Treatment of Spleen-deficiency syndrome<sup>[1,6]</sup>**

Many GI diseases, such as chronic atrophic gastritis, chronic pancreatitis with diarrhea, inflammatory bowel disease, coeliac disease may present Spleen-deficiency at certain stage of the disease. Because they have similar pathophysiology, they can be treated by the same principle but with emphasis on different aspects.

The Si Jun Zi decoction is the major Spleen-fortifying therapy for Spleen-deficiency syndrome, including Ginsen or Codonopsis, Atractylodes, Poria, Glycyrrhiza. It promotes absorption and dispersion of nutrients to the whole body, increases physical strength and mental activity, and has multiple effects on the digestive organs, immunity, hemopoiesis, blood circulation, hepatic synthetic function, muscle metabolism, etc. By adding *Astragalus*, the immunologic function and small intestinal muscular tone can also be increased.

#### **Motility and absorptive function of GI tract**

The Si Jun Zi decoction inhibits the small intestinal activity, presented with decrease of amplitude and spasmodic contraction of small intestine. It possesses anticholinergic effect as well as antihistaminic response. The inhibitory effect on intestinal tract is both neurogenic and myogenic. It also has modulating effect on the function of vegetative nervous system. In Spleen deficiency and Qi deficiency, there are inhibition of transport and sodium pump on the epithelial cells which can be restored by the Si Jun Zi decoction. Codonopsis-Poria-Atractylodes powder can promote absorption of water and chloride ion so as to improve the diarrhea of Spleen deficiency.

#### **Immunologic function**

The Si Jun Zi decoction promotes cellular immunity, enhances peritoneal macrophagic phagocytosis, corrects the immunosuppressive effect of cyclophosphamide and corticosteroids, and reduces the inhibitory effect on bone marrow and atrophy of thymus, Spleen by cyclophosphamide. Experimental study revealed that abundant extramedullary erythroblasts and active proliferation of lymphoblast control the atrophic effect of cyclophosphamide in adrenal cortex and testes, also decreases the toxic adverse effect of chemotherapeutic drugs. It also increases the humoral immunity by increasing antibody production for infected patients with Spleen deficiency, it ameliorates and restores the weight

loss of thymus in undernourished mice as well as the structure within the thymus such as the thickness of cortex/medulla ratio and the diameter of thymus cell nucleus to normal.

#### **Hemopoietic action**

Ginsen promotes marrow cell DNA and protein synthesis, and codonopsis promotes red blood cell formation. If ferrous sulfate is given concomitantly, restoration can be hastened.

#### **Hepatic synthetic function**

It can increase plasma albumin, hepatic RNA and glycogen contents in favor of tissue repair and hepatic detoxification.

#### **Effect on skeletal muscle**

In Spleen-Qi deficiency, the mitochondria decreases in number with decrease of oxidase, and increase of anaerobic glycolysis. The Si Jun Zi decoction can restore muscle glycogen, lipid, ATP synthesis and the ultrastructures of mitochondria to near normal; food consumed, body weight, physical strength and mental spirit all improved.

#### **Blood circulation of gastric wall and mesentery**

On the basis of the Si Jun Zi decoction, the addition of *Astragalus*, *Cinnamon Twig*, *Saussure*, *Tangerine Peel*, may dilate arterioles, increase capillary blood flow and tissue perfusion, and promote tissue repair.

Action of individual component of the Si Jun Zi decoction:

① Ginsen, small dose stimulates central nervous system and large dose inhibits it. Ginsen increases mental reactivity and the contents of dopamine, nor-epinephrine in the brain, promotes brain RNA and protein synthesis, increases blood supply, and blood oxygen to the brain; all these are the pharmacologic basis for increasing brain activity. Besides, it has anti-fatigue action and increases tolerance to cold. Ginsen enhances both cellular and humoral immunity, also activates the phagocytic function of macrophages and activity of NK cells, and in addition, it also induces interferon formation.

In the endocrine system, it enhances the hypothalamic-pituitary-adrenal cortical, thyroid and gonadal axis function. It increases cardiac contractility by inhibiting the sodium pump on cardiac muscle cell membrane. It promotes release of insulin and elevates blood insulin level, enhances the synthesis of protein, RNA and DNA, lowers blood lipid, and improves hepatocytic function. As a therapeutic drug for Spleen deficiency, emphasis should be laid on its systemic effect.

② Codonopsis, it also enhances immunologic function, both cellular and humoral immunity, and increases response to stress and

tolerability to hypoxemia. Codonopsis is important for Spleen-fortifying. It can antagonize the decrease in gastric mucosal content of prostaglandin E<sub>2</sub> and aminohe-xose induced by indomethacin and aspirin, and inhibits acid secretion, hence being useful for combating ulceration and mucosal damage. It can also modulate GI motility, and has bidirectional effect on intestinal motility. It can decrease blood viscosity by inhibiting platelet aggregation, and also has some vasodilating action. Furthermore, it has varying degrees of inhibition on the cyclo-oxygenase pathway, TXA<sub>2</sub> synthetase; and these are dose dependent.

③Atractylodes, being a representative of Spleen-fortifying drug, it modulates intestinal motility bidirectionally, increases smooth muscle tone of GI tract, improves the white slippery coating of tongue, and cellular and humoral immunity and protein synthesis. Moreover, it acts on the hypothalamic-putuitary-adrenal cortical axis.

④Poria, relaxes smooth muscle of GI tract, diminishes the contraction amplitude, reduces acid secretion, and has anti-necrosis and anti-degenerative actions on hepatocytes in hepatitis. Its polysaccharide enhances cellular immunity, increases the weight of thymus and lymph node, and hence is used in treatment of cancer of GI tract.

⑤ Glycyrrhiza, having acidity lowering and antispasmodic actions, it diminishes the smooth muscle tone of the intestine, amplitude of contraction, and inhibits ileal contraction induced by acetylcholine. It also has anti-inflammatory and anti-allergic effects. It can increase the cytochrome P-450 content of hepatocytes, and detoxification effect. Besides, it has corticosteroidal effect.

⑥Astragalus, when added to the Si Jun Zi decoction, it can potentiate the immunoenhancing effect, including induction of interferon production and increase in antibody formation. Astragalus also increases cardiac contractility, in particular, the ejection fraction, promotes blood cells formation and maturation, restores the reticulocytes and megakaryocytes to normal. Through activation of phosphorylase mediated by cAMP, it can promote mitosis, differentiation and growth of marrow cells. Furthermore, it increases the RNA, DNA and protein synthesis of the liver. It has an antioxidant action. Moreover, it may increase corticoid secretion and elevates the plasma level of cortisol. It has long been used in treatment of atrophic gastritis, peptic ulcer and other gastrointestinal diseases with Spleen deficiency by increasing cAMP content for gastric mucosal repair. It is also used in chronic hepatitis with Spleen deficiency to increase hepatocytic RNA, DNA and protein synthesis.

#### REFERENCES

- 1 Wu XZ, Gao JL (eds). Current studies on Spleen deficiency syndrome. Tianjing: Science and Technology Translation Publisher Co, 1992
- 2 Wei BH (eds). Application of Spleen-Stomach theory of traditional Chinese medicine. Beijing: Beijing Publisher, 1993
- 3 Shen Y, Liu ZM, Wei S, Lo ZQ, Zhou MW. Histopathology of gastric mucosa by *Helicobacter pylori* and observation on SigA of gastric juice in Spleen deficiency patients. *Chin J Integrated Trad Western Med*, 95;15(11):664-665
- 4 Yin GY, He XF, Yin YF, Zhang WN, Tu YQ, Chen ZF *et al*. The ultrastructural changes of gastric mucosal mitochondria, trace element and the associated factors in patients with Spleen deficiency syndrome. *Ibid*, 1995;15 (12):719-723
- 5 Sun UT, Xie JY, Li LH, Zhang WN, Tu YQ, Chen ZF *et al*. Some trace elements, energy and ionic changes in skeletal muscles of Spleen deficiency rats. *Ibid*, 1993;13(12):736-738
- 6 Li YQ, Jiang MY (eds). Pharmacology of Chinese herbal medicine. Beijing: Beijing National Chinese Medicine and Herbal Medicine Publisher, 1992

# Clinical and experimental study of therapeutic effect of Weixibaonizhuan pills on gastric precancerous lesions \*

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**Subject headings** Weixibaonizhuan pills; stomach neoplasm/TCM therapy; precancerous conditions; gastric mucosa; Chinese herbal

## Abstract

**AIMS** To observe the therapeutic effect of Weixibaonizhuan pills (胃细胞逆转丸) on gastric precancerous lesions.

**METHODS** Thirty patients with gastric precancerous lesions were treated with Weixibaonizhuan pills for 3 months. Of the 36 cases, 13 (36.1%) were mild atrophic gastritis, 14 (38.9%) moderate atrophic gastritis and 9 (25.0%) severe atrophic gastritis; among them 22 (61.1%) and 27 cases (75.0%) were accompanied with intestinal metaplasia (IM) and dysplasia (DYS) respectively. Of the 36 patients, 20 were men and 16 women, aged from 30-60 years and those aged 30-59 years accounted for 61.1%. The course of disease ranged from 3 months to 21 years, and 20 (55.6%) of them had a course of 5 - 10 years. The clinical manifestations were fullness of the abdomen (31 cases), abdominalgia (27 cases), anorexia (30 cases), gas eructation (26 cases), acid regurgitation (6 cases) and loose stool (9 cases). When treatment ended, the improvement of patients' clinical symptoms, atrophy of gastric mucosa, IM and DYS were analysed.

**RESULTS** After 3 months' treatment with Weixibaonizhuan pills, 7 cases recovered, 11 cases were much improved, 13 cases showed some improvement, and 5 cases were ineffective; the total rate of symptomatic improvement was 86.1%. Of the 13 cases with mild atrophic gastritis, 11 cases changed into superficial gastritis, and 2 cases had no changes. Of the 14 cases of moderate atrophic gastritis, 4

cases changed into superficial gastritis, 7 cases changed into mild atrophic gastritis, and 3 cases had no changes. Five of 9 cases of severe atrophic gastritis were reduced to moderate atrophic gastritis, and 4 cases had no changes. The total effective rate was 77.8% in chronic atrophic gastritis. Of the 9 cases with mild IM, IM disappeared in 6 cases and 3 showed no change. Of the 10 cases with moderate IM, it disappeared in 2 cases, 5 cases changed to mild IM, and 3 cases had no change. One of the 4 cases of severe IM changed to moderate IM and 3 had no change. The total effective rate was 63.6% in IM. Of the 16 cases of mild DYS, 11 cases showed disappearance of DYS and 5 had no change. In 9 cases of moderate DYS, 2 showed disappearance, 5 changed to mild DYS and 2 had no change. Two cases of severe DYS, both showed no change. The total effective rate was 66.7% in DYS. Before treatment, the I, II, III and IV degree positive expressions of CEA were present in 13, 12, 9 and 2 cases, respectively, whereas after treatment, the positive expressions were present in 25, 7, 3 and 1, respectively. Before treatment, the I, II, III and IV degree positive expressions of PCNA were present in 16, 11, 10 and 4 respectively, but after treatment, they were present in 21, 9, 5 and 1 respectively. In short, the positive expressions of CEA and PCNA of gastric mucosa were significantly decreased after treatment ( $P < 0.01$ ).

**CONCLUSION** Weixibaonizhuan pill has a therapeutic effect in gastric precancerous lesions.

## INTRODUCTION

Gastric carcinogenesis often arises from some precancerous lesion for cancer development. Effective treatment of such lesions would be helpful in decreasing the risk of gastric cancer. We have used Weixibaonizhuan pills (WXBNZ pills 胃细胞逆转丸) to treat them and have obtained a good result.

## PATIENTS AND METHODS

### Patients

Thirty-six patients with gastric precancerous lesions were diagnosed through gastroscopy and mucosal biopsy<sup>[1]</sup>. Among them, 13 (36.1%) cases were mild chronic atrophic gastritis (CAG), 14 (25.0%) cases moderate CAG, and 9 severe CAG. Twenty-

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\*Supported by grants from the Shanxi Commission of Science and Technology, No.930001.

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Received 1997-08-02

two (61.1%) and 27 (75.0%) cases were accompanied intestinal metaplasia (IM) and dysplasia (DYS), respectively. Of the 36 patients 20 were men and 16 women; their age ranged from 30 - 60 years, and those between 40 - 59 years of age accounted for 61.1%. The course of disease ranged from 3 months to 21 years, and 20 of them (55.6%) had a course of 5 - 10 years. The clinical manifestations were as follows: ① abdominal fullness or feeling of obstruction in the stomach (31 cases), worse in afternoon or after eating and abated after eructation or passing wind from bowels; ② abdominalgia (27 cases) in middle abdomen and epigastrium, more often vague, or distending, burning, stabbing, with tenderness; ③ anorexia (30 cases); ④eructation after eating or of frequent occurrence (26 cases); ⑤acid regurgitation (6 cases) became severe when fasting; and ⑥loose stool (9 cases), watery or unformed.

### Methods

All patients took WXBNZ pill (composed of 15 kinds of herbs including *Radix Codonopsis pilosulae*, *Radix Salviae Miltiorhizae*, *Herba Solani Nigri*, *Radix et Rhizoma Rhei*, *Rhizoma coptidis*, etc, prepared by the Research and Treatment Center of Gastroenterology of Taiyuan) 6 grams three times a day for a course of 3 months. Gastroscopic and pathologic studies of biopsy specimens were performed before and within 2 weeks after treatment. During gastroscopy, 1 biopsy specimen was taken each from the antral lesser curvature, greater curvature, anterior wall and antrum-body transitional zone, respectively, and additional 1-2 biopsy specimens were taken from the grossly identifiable lesions. The examinations were made by fixed doctors. In addition to routine HE staining, we also used immunohistochemical technique to detect the expression of CEA and PCNA of gastric mucosa. CEA antibody (rabbit against human) and PCNA antibody (mouse against human) were provided by Zymed Co. and universe type biotinylated secondary antibody was made in our lab. The staining procedure was performed according to the literature<sup>[2]</sup>.

## RESULT

### Improvement of clinical symptoms after treatment

Of the 36 cases, 7 recovered with a disappearance of clinical symptoms, 11 had significant improvement of symptoms, 13 showed some improvement, and 5 had no change. The total effective rate was 86.1%.

### Improvement of gastric mucosal atrophy

In 13 cases of mild CAG, 11 cases changed to superficial gastritis, and 2 had no change, the effective rate being 84.6%. Among 14 cases of

moderate CAG, 4 changed to superficial gastritis, 7 changed to mild CAG (Figures 1, 2), 3 cases had no change, the effective rate being 78.6%. Five of the 9 cases of severe CAG changed to moderate CAG (Figures 3, 4), and 4 cases had no change, the effective rate being 55.6%. The total effective rate for CAG was 77.8%, and the therapeutic effectiveness seemed to be better in mild and moderate CAG.

### Improvement of IM after treatment

In the 9 cases of mild IM, it disappeared in 6 cases, and 3 cases had no change, the effective rate being 66.7%. In the 10 cases of moderate IM, 2 cases showed a disappearance of IM, 5 cases changed to mild IM and 3 cases had no change (Figures 5, 6), the effective rate being 70.0%. Only one of the 4 cases of severe IM changed to moderate IM and 3 had no change, the effective rate being 25.0%. The total effective rate for IM was 63.6% and the therapeutic effectiveness was better in mild and moderate IM.

### Improvement of DYS after treatment

In the 16 cases of mild DYS, 11 cases showed its disappearance and 5 had no change, the effective rate being 68.8%. In the 9 cases of moderate DYS, 2 had a disappearance of DYS, 5 changed to mild DYS and 2 had no change, the efficacy rate was 77.8%. Both of the 2 cases of severe DYS showed no change after treatment, the efficacy rate was 0.0%. The total effective rate was 66.7% in DYS, and the therapeutic effectiveness was better in mild and moderate DYS.

### Improvement of gastric CEA

A semiquantitative method was used to judge the brown-stained positive cells<sup>[3]</sup>. The positive degree was scored according to the number of positive cells. Grade I: 0% - 25%, grade II: 26% - 50%, grade III: 51% - 75%, and grade IV: 76% - 100%. The expression of CEA decreased significantly after treatment ( $P < 0.01$ ), (Table 1).

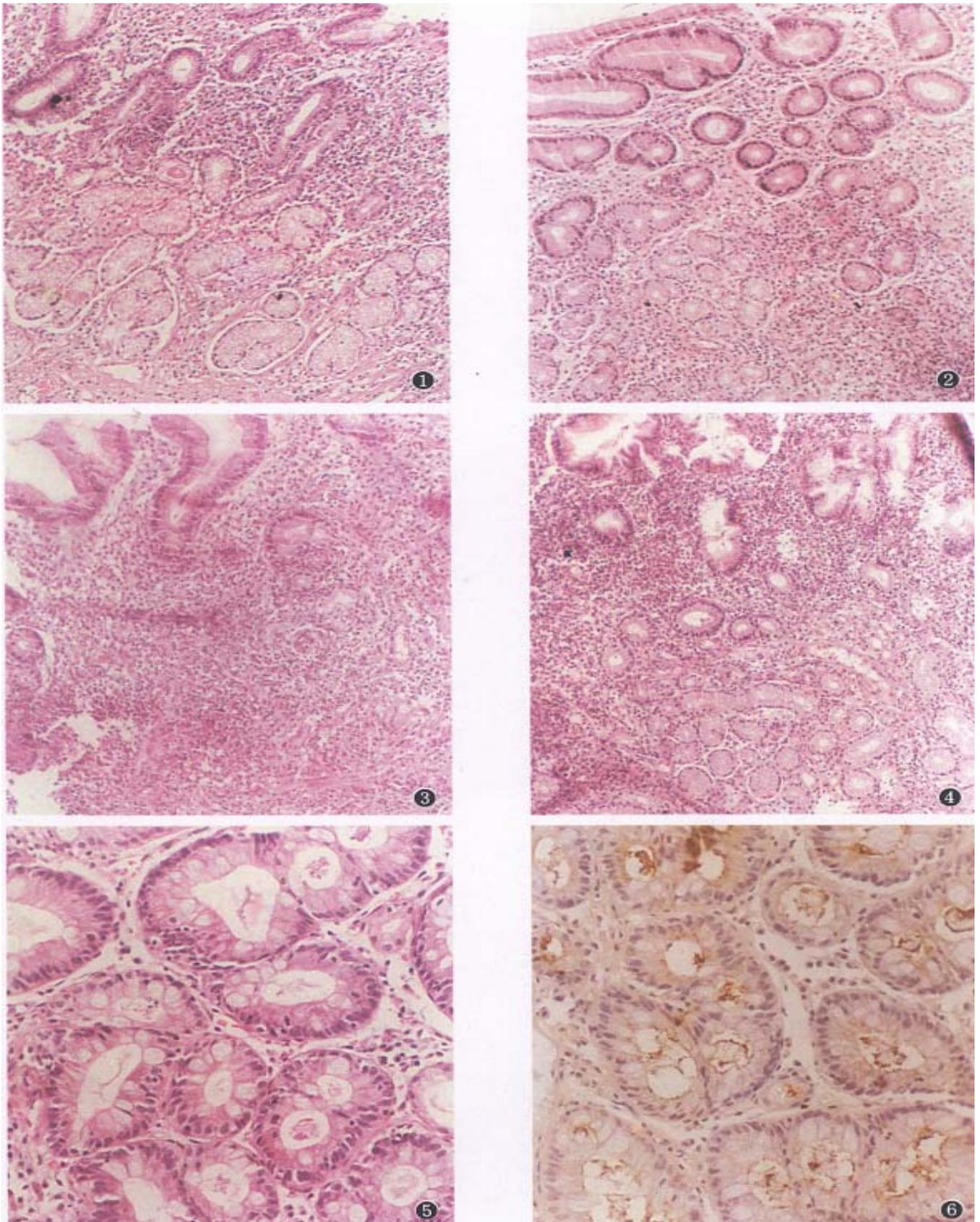
**Table 1** Positive expression of CEA in gastric mucosa before and after treatment with WXBNZ pills

Treatment	Grades			
	I	II	III	IV
Before	13	12	9	2
After	25	7	3	1

$u = 27.68$ ,  $P < 0.01$ .

### Improvement of gastric PCNA

The positive criteria of PCNA was similar to CEA. The PCNA expression decreased significantly after treatment ( $P < 0.01$ ) (Table 2).



**Figure 1** Moderate CAG before treatment. (HE×100)  
**Figure 2** Mild CAG after treatment. (HE×100)  
**Figure 3** Severe CAG before treatment. (HE×100)  
**Figure 4** Moderate CAG after treatment. (HE×100)

**Figure 5** Severe CAG accompanied with severe IM, there is no significantly difference between before and after treatment (HE×200)  
**Figure 6** Severe CAG accompanied with severe IM, there is nosignificantly difference of CEA expression between before

and after treatment (LSAB×200)

**Table 2 Positive expression of PCNA in gastric mucosa before and after treatment with WXBZ pills**

Treatment	Grades			
	I	II	III	IV
Before	26	11	10	4
After	21	9	5	1

$u = 13.85, P < 0.01.$

## DISCUSSION

The rate of cancer transformation in patients with CAG was reported to be 0.2%<sup>[4]</sup>; those with mild DYS was 2.5% - 11%<sup>[5]</sup>; moderate DYS, 4% - 35%<sup>[5,6]</sup>; severe DYS, 10% - 83%<sup>[7]</sup>; and IM, 1.9%<sup>[4]</sup>. It can be suggested that treatment of precancerous lesions may decrease the incidence of gastric cancer effectively. The long-standing chronic gastritis may result in gastric mucosal atrophy, dysplasia and IM of glandular epithelium. As a consequence of such pathologic changes, the patient may have hyosecretion of gastric acid and pepsin, along with symptoms of dyspepsia, such as anorexia and abdominal distention after eating, loose stool, malnutrition, etc. According to the theory of traditional Chinese medicine (TCM), such manifestations belong to an “insufficiency of the Spleen and Stomach *qi*”. Another group of patients presenting symptoms of epigastric pain, eructation, acid regurgitation, nausea, etc belong to “disharmony between the Liver and the Stomach” with stagnation and reversed flow of *qi*, together with the production of Heat. So chronic gastritis with precancerous lesions is a complicated ailment in TCM with regard to its cause and pathogenesis. The rationale of its management is to strengthen the Spleen and Stomach, to enhance the body resistance, to consolidate the constitution, to promote flow of *qi* and blood, and to disperse Heat. Only by this way can the symptoms and pathology be improved effectively. We used WXBZ pills, which was composed of 15 kinds of herbs and had the above-mentioned effects of strengthening the Spleen and Stomach, strengthening the body resistance, to consolidate the constitution, promoting flow of *qi* and blood and dispersing Heat, in treating the patients with gastric precancerous lesions. The total effective rates for clinical symptoms, CAG, DYS, IM were 86.1%, 77.8%, 66.7%, 63.6%, respectively. These results indicated that WXBZ pills had a

good therapeutic effect in gastric precancerous lesions.

From the results in this study we can infer that WXBZ pills has a better therapeutic effects in mild and moderate CAG, DYS and IM, but gives poor results in severe CAG, DYS and IM. Therefore, early diagnosis and early treatment of such lesions are recommended. In normal gastric mucosa, CEA staining is negative or restricted to the secretory margin of cells. In precancerous lesions, the CEA staining showed a positive expression not only at the secretory margin of cells but also in the apical cytoplasm or further dispersed in the whole cell membrane and cytoplasm. We found in our previous study that positive expression of CEA increased progressively as the precancerous lesions progressed<sup>[2]</sup>. The positive expression of CEA may reflect the beginning of a deviation or transition of normal state of cell differentiation toward malignancy. In this study, the positive expression of CEA decreased and disappeared gradually after WXBZ pills treatment, indicating that WXBZ pill might have some effect of inhibiting the CEA expression and accelerating the recovery of gastric mucosal function. Recent studies have shown that PCNA is related intimately to the DNA synthetic phase (S phase), and is a good marker reflecting the activity of cell proliferation<sup>[3]</sup>. In this study, the high positive expression of PCNA in the gastric mucosa with precancerous lesions decreased distinctly after WXBZ pills treatment. These results suggested that WXBZ pills could inhibit the cell proliferative activities and abnormal hyperplasia of gastric mucosa in patients with precancerous lesions.

## REFERENCES

- 1 Bureau of Medical Administration of People's Republic of China. Diagnostic and therapeutic standardization of common tumors in China, First ed. Beijing: Press of Beijing Medical University and Peking Union Medical College, 1991:37 - 41
- 2 Li BQ, Zhang XC, Mei LX, Liu FQ. Immunohistological study of CEA and PCNA in gastric epithelial dysplasia. *Chin J New Gastroenterol*, 1996;4(Suppl 5):29 - 30
- 3 Garcia RL, Coltrera MD, Gown AM. Analysis of proliferating grade using anti-PCNA/cyclin monoclonal antibodies in fixed embedded tissue. *Am J Pathol*, 1989;134(4):733 - 736
- 4 Xu CP, Liu WW. Follow-up study of chronic gastritis, intestinal metaplasia and atypical hyperplasia. *Chin J Digestion*, 1984;4(1):10 - 11
- 5 Rugge M, Farinati F, Mario FD, Baffa R, Valiante F. Gastric epithelial dysplasia: a prospective multicenter follow-up study from the interdisciplinary group on gastric epithelial dysplasia. *Hum Pathol*, 1991;22(10):1002 - 1008
- 6 Del-Corral MIC, Pardi-Mindan FJ, Razquin S, Ojeda C. Risk of cancer in patients with gastric dysplasia, follow-up study of 67 patients. *Cancer*, 1990;65(9):2078 - 2085
- 7 Farinatio F, Cardin F, Di-Mario F, Valiante F, Naccarato R. Follow-up in gastric dysplasia patients. *Am J Surg Pathol*, 1989;13(2):173 - 174

# Aflatoxin sufferer and p53 gene mutation in hepatocellular carcinoma \*

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**Subject headings** Aflatoxin B1; genes, p53; mutation; carcinoma, hepatocellular; liver neoplasms

## Abstract

**AIM** To study the p53 gene mutation and its relationship to aflatoxin B1 exposure in hepatocellular carcinoma (HCC).

**METHODS** Restriction fragment length polymorphism analysis method was used in 62 HCC samples, and DNA direct sequencing in another 45 HCC samples.

**RESULTS** In HCC and AFB1 high and low-risk areas, 36/52 (69%) and 2/10 (20%) cases were found losing the HaeIII allele respectively, suggesting one of the base G mutation at the p53 gene codon 249. Similar results appeared in DNA direct sequencing, 20/35 (57%) and 1/10 (10%) respectively mutated at the codon 249 third base G to C transversion.

**CONCLUSION** In HCC after AFB1 exposure, mutation of p53 gene is fixed at codon 249 third base and take the form of G to T transversion. This is a definite marker of mutation which is induced by AFB1 mutagen. It is applicable for molecular epidemiologic survey of the sufferers of AFB1 among HCC cases and for discovering more unknown natural AFB1 contaminated areas.

## INTRODUCTION

Previous reports from Qidong and southern Africa discovered that the p53 gene of hepatocellular carcinoma (HCC) had a mutational hot-spot at codon 249<sup>[1-3]</sup>. Later there were repeated reports from Qidong. However, reports from worldwide other than those two areas showed no such special mutational hot-spot in HCC<sup>[4-6]</sup>. It was supposed that codon 249 mutation might be caused by exposure to aflatoxin B1 (AFB1) which was a common environmental factor prevalent in the two mentioned areas. *Aspergillus flavus* is widely distributed throughout the world. However, well-known natural high AFB1 contaminated areas are uncommon. It is of significance to study one more known natural high AFB1 risk area Fusui and its neighboring counties where HCC mortality rate was high up to 40/100 000 annually. AFB1 exposure and hepatitis B virus (HBV) were both suspected to be the principal aetiological factors.

## MATERIALS AND METHODS

Surgical specimens of HCC were fixed in formalin and embedded in paraffin. The pathological diagnosis was made by standard histologic criteria. The DNA of 62 HCC samples were amplified focus on the p53 gene exon 7 by polymerase chain reaction (PCR). Primers were introduced by Murakami *et al.* PCR products containing exon 7 were digested with the restriction enzyme Hae III, then electrophoresed for restriction fragment length polymorphism (RFLP) analysis. Another 45 HCC samples were sent for DNA direct sequencing. In the meantime, all samples were stained for HBsAg by ABC method of immunohistochemistry.

## RESULTS

A lot of HCC samples came from high HCC prevalent and high AFB1 exposed area in Guangxi lost the Hae III restriction site GG/CC by method of RFLP analysis. It suggested a mutation occurring at the sequence AGG, CCC. Undoubtedly, the mutational point occurred at the hot-spot codon 249 nucleotides G rather than non-hot-spot C of codon 250. Surprisingly, this mutation was found in 36/52 samples (69%). Compared to the samples from a low prevalent area in Guangxi, the frequency was 2/10 (20%). The difference was significant ( $P < 0.01$ ). The results by direct DNA sequencing were similar. The samples from high prevalent area were 20/35 (57%) mutation at codon 249 third

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\*Project supported by the National Natural Science Foundation of China, No. 39560030. and Natural Science Foundation of Guangxi

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Received 1997-09-01 Revised 1997-10-13

nucleotide G to T transversion, while low prevalent area showed this transversion only in 1/10(10%). The difference was also significant ( $P < 0.01$ ). The total positive rate of HBsAg was 94.4%, with no difference between high and low prevalent areas.

## DISCUSSION

The p53 tumor suppressor gene is one of the genes with great interest, because it is commonly mutated in human cancer, and the spectra of p53 mutations in these cancers provide clues to the etiology and molecular pathogenesis of tumors. In HCC, p53 gene mutation is related to AFB1 and HBx protein of HBV. The present data is a research on the spot on great number of cases. The conclusions are as follows: the principal etiology and molecular pathogenesis of HCC at the part of high prevalent area in Guangxi are caused by AFB1 without doubt, and HBV chronic infection is also high. AFB1 induces p53 gene mutational hot-spot in the high prevalent local area up to 57% - 69%. This hot-spot is located at exon 7 codon 249 third nucleotide G to T transversion. It is a special and stable mutational point. It is useful for HCC molecular epidemiologic study for general survey whether the residents are at the risk of aflatoxin exposure, and distinguish the

AFB1 sufferers of HCC cases individually. Until now, there has been no report from high AFB1 contaminated area where HCC samples escape this p53 gene mutational hot-spot. More and more human materials from high AFB1 areas show the mutation point clustering at the codon 249 third nucleotide rather than the first or second nucleotide, and only G to T single form but not G to A or C multiple forms mutation. Therefore it would be confirmed as a mutational marker of a AFB1 sufferer in human HCC.

## REFERENCES

- 1 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hot-spot in the p53 gene in human hepatocellular carcinomas. *Nature*, 1991;350(6317):427-428
- 2 Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. *Nature*, 1991;350(6317):429-431
- 3 Ozturk M and Collaborators. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet*, 1991;338(8779):1356-1359
- 4 Li D, Cao Y, He L, Wang NJ, Gu JR. Aberrations of p53 gene in human hepatocellular carcinoma from China. *Carcinogenesis*, 1993;14(1):169-173
- 5 Fujimoto Y, Hampoton LL, Wirth PJ, Wang JP, Xie JP, Thorgeirsson SS. Alterations of tumor suppressor genes and allele losses in human hepatocellular carcinomas in China. *Cancer Res*, 1994;54(2):281 - 285
- 6 Scorsone KA, Zhou YZ, Butel JS, Slagle BL. p53 mutations cluster at codon 249 in hepatocellular carcinomas from China. *Cancer Res*, 1992;52(7):1635-1638
- 7 Yet FS, Yu MC, Mo CC. Hepatitis B virus, aflatoxin, and hepatocellular carcinoma in southern Guangxi, China. *Cancer Res*, 1989;49(10):2506 - 2509
- 8 Murakami Y, Hayashi K, Hirohashi S, Sckiya T. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res*, 1991;51(10):5520 - 5525

ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(1):29

## Effect of garlic and garlic-green tea mixture on serum lipids in MNNG-induced experimental gastric carcinoma and precancerous lesion\*

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**Subject headings** garlic tea; stomach neoplasms/prevention & control; precancerous conditions/blood; lipids/blood

### INTRODUCTION

To study effect of garlic and garlic-green tea mixture on serum contents of Tch, LDL and HDL in MNNG-induced gastric carcinoma (GC) and precancerous lesion (PL) in Wistar rats.

### METHODS

Serum contents of Tch, LDL and HDL in normal control group ( $n = 10$ , NG), MNNG group ( $n=30$ , MG), prevention group ( $n = 30$ , PG), treatment group I ( $n = 20$ , TG I) and treatment group II ( $n = 20$ , TGII) were detected by PGE 6000/COD.

### RESULTS

Serum Tch and LDL of rats of MG ( $6.86 \pm 1.39$ ,  $3.72 \pm 1.10$ ) and its GC ( $6.95 \pm 1.37$ ,  $3.77 \pm 1.08$ ) and PL ( $6.42 \pm 1.04$ ,  $3.56 \pm 0.74$ ) were lower than that of NG ( $8.74 \pm 1.89$ ,  $5.89 \pm 1.61$ ), PG ( $7.73 \pm 3.18$ ,  $4.96 \pm 2.89$ ) and its GC ( $8.36 \pm 3.41$ ,  $5.93 \pm 3.31$ ) and PL ( $7.45 \pm 3.16$ ,  $4.55 \pm 2.71$ ), TGI ( $8.86 \pm 1.75$ ,  $5.38 \pm 1.76$ ) and its GC ( $9.10 \pm 2.27$ ,  $5.55 \pm 2.51$ ) and PL ( $8.61 \pm 1.17$ ,  $5.22 \pm 0.55$ ) and TGII ( $8.16 \pm 0.76$ ,  $5.32 \pm 0.72$ ) and its GC ( $8.52 \pm 0.67$ ,  $5.96 \pm 0.48$ ) and PL ( $8.02 \pm 0.79$ ,  $5.09 \pm 0.65$ ), respectively ( $P < 0.01-0.05$ ). Serum HDL of MG rats ( $2.76 \pm 0.48$ ) and its GC ( $2.79 \pm 0.48$ ) were remarkably higher than that of MG ( $2.20 \pm 0.85$ ) and GC of PG ( $2.24 \pm 0.38$ ) ( $P < 0.05$ ).

### CONCLUSION

Experimental gastric carcinoma and precancerous lesion were associated with hypocholesterolaemia, LDL and HDL. Garlic and garlic-green tea mixture can inhibit and reverse MNNG-induced gastric carcinoma and precancerous lesion in Wistar rats.

nucleotide G to T transversion, while low prevalent area showed this transversion only in 1/10(10%). The difference was also significant ( $P<0.01$ ). The total positive rate of HBsAg was 94.4%, with no difference between high and low prevalent areas.

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## REFERENCES

- 1 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hot-spot in the p53 gene in human hepatocellular carcinomas. *Nature*, 1991;350(6317):427-428
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- 4 Li D, Cao Y, He L, Wang NJ, Gu JR. Aberrations of p53 gene in human hepatocellular carcinoma from China. *Carcinogenesis*, 1993;14(1):169-173
- 5 Fujimoto Y, Hampoton LL, Wirth PJ, Wang JP, Xie JP, Thorgeirsson SS. Alterations of tumor suppressor genes and allele losses in human hepatocellular carcinomas in China. *Cancer Res*, 1994;54(2):281 - 285
- 6 Scorsone KA, Zhou YZ, Butel JS, Slagle BL. p53 mutations cluster at codon 249 in hepatocellular carcinomas from China. *Cancer Res*, 1992;52(7):1635-1638
- 7 Yet FS, Yu MC, Mo CC. Hepatitis B virus, aflatoxin, and hepatocellular carcinoma in southern Guangxi, China. *Cancer Res*, 1989;49(10):2506 - 2509
- 8 Murakami Y, Hayashi K, Hirohashi S, Sckiya T. Aberrations of the tumor suppressor p53 and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res*, 1991;51(10):5520 - 5525

ISSN 1007-9327 CN 14-1219/R WJG, 1998;4(1):29

## Effect of garlic and garlic-green tea mixture on serum lipids in MNNG-induced experimental gastric carcinoma and precancerous lesion\*

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**Subject headings** garlic tea; stomach neoplasms/prevention & control; precancerous conditions/blood; lipids/blood

### INTRODUCTION

To study effect of garlic and garlic-green tea mixture on serum contents of Tch, LDL and HDL in MNNG-induced gastric carcinoma (GC) and precancerous lesion (PL) in Wistar rats.

### METHODS

Serum contents of Tch, LDL and HDL in normal control group ( $n = 10$ , NG), MNNG group ( $n=30$ , MG), prevention group ( $n = 30$ , PG), treatment group I ( $n = 20$ , TG I) and treatment group II ( $n = 20$ , TGII) were detected by PGE 6000/COD.

### RESULTS

Serum Tch and LDL of rats of MG ( $6.86 \pm 1.39$ ,  $3.72 \pm 1.10$ ) and its GC ( $6.95 \pm 1.37$ ,  $3.77 \pm 1.08$ ) and PL ( $6.42 \pm 1.04$ ,  $3.56 \pm 0.74$ ) were lower than that of NG ( $8.74 \pm 1.89$ ,  $5.89 \pm 1.61$ ), PG ( $7.73 \pm 3.18$ ,  $4.96 \pm 2.89$ ) and its GC ( $8.36 \pm 3.41$ ,  $5.93 \pm 3.31$ ) and PL ( $7.45 \pm 3.16$ ,  $4.55 \pm 2.71$ ), TGI ( $8.86 \pm 1.75$ ,  $5.38 \pm 1.76$ ) and its GC ( $9.10 \pm 2.27$ ,  $5.55 \pm 2.51$ ) and PL ( $8.61 \pm 1.17$ ,  $5.22 \pm 0.55$ ) and TGII ( $8.16 \pm 0.76$ ,  $5.32 \pm 0.72$ ) and its GC ( $8.52 \pm 0.67$ ,  $5.96 \pm 0.48$ ) and PL ( $8.02 \pm 0.79$ ,  $5.09 \pm 0.65$ ), respectively ( $P<0.01-0.05$ ). Serum HDL of MG rats ( $2.76 \pm 0.48$ ) and its GC ( $2.79 \pm 0.48$ ) were remarkably higher than that of MG ( $2.20 \pm 0.85$ ) and GC of PG ( $2.24 \pm 0.38$ ) ( $P<0.05$ ).

### CONCLUSION

Experimental gastric carcinoma and precancerous lesion were associated with hypocholesterolaemia, LDL and HDL. Garlic and garlic-green tea mixture can inhibit and reverse MNNG-induced gastric carcinoma and precancerous lesion in Wistar rats.

# CEA and AFP expression in human hepatoma cells transfected with antisense IGF-I gene \*

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**Subject headings** insulin-like growth factor-I; carcinoembryonic antigen;  $\alpha$ -fetoprotein; carcinoma, hepatocellular; tumor cells, cultured; radioimmunoassay

## Abstract

**AIM** To determine whether antisense insulin-like growth factor-I (IGF-I) gene can modulate CEA and AFP expression in human hepatoma cells (HepG2).

**METHODS** Transfection of HepG2 cells was accomplished using Lipofectin reagent. Northern blot analysis confirmed the antisense IGF-I RNA of the transfected cells. CEA and AFP levels were measured using radioimmunoassay.

**RESULTS** Human hepatoma cell lines (HepG2) were transfected with antisense IGF-I gene. Northern blot analysis confirmed that antisense IGF-I RNA was expressed in the transfected cells. The effect of antisense IGF-I gene on CEA and AFP expression was demonstrated by the fact that the CEA and AFP levels in the supernatant of transfected cell culture were significantly lower as compared with the parent cells, [CEA  $7.0 \mu\text{g/L} \pm 0.76 \mu\text{g/L}$  and  $3.29 \mu\text{g/L} \pm 1.80 \mu\text{g/L}$  ( $P < 0.05$ ) and AFP  $53.63 \mu\text{g/L} \pm 6.02 \mu\text{g/L}$  and  $9.0 \mu\text{g/L} \pm 5.26 \mu\text{g/L}$  ( $P < 0.01$ ), respectively].

**CONCLUSION** The malignant potentiality of the transfected cells was partially suppressed. Antisense IGF-I gene can modulate the expression of CEA and AFP in human hepatoma cell lines (HepG2)

## INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a cytokine with multiple biological functions, which can stimulate cell differentiation and DNA synthesis and transcription<sup>[1]</sup>. In recent years, it has been found that many kinds of tumors expressed abnormal IGF-I, e.g., hepatoma, astrocytomas, etc. IGF-I might be important mitogens in the growth of many tumors<sup>[2]</sup>. Tumor cells may be able to enhance their own growth by synthesis of endogenous IGF-I. This process of autocrine secretion contributes to the partial autonomy and rapid growth in tumor cells<sup>[2-4]</sup>. We have applied the antisense strategy to block the function (e.g., by binding to a splice junction) of IGF-I in tumor cells so as to promote its degradation, and alter the structure of the target sequence. In a recent report, the stable transfectants of rat glioblastoma cells transfected with antisense IGF-I gene lost its tumorigenicity, and the inhibition of IGF-I expression elicited a highly immunogenic phenotype in glioma cells<sup>[3,4]</sup>. On the other hand, tumor markers related with the malignant hepatocarcinoma are CEA and AFP. IGF-I expression was increased significantly in the cell proliferation and high malignancy of human hepatoma. It can be used in the subsidiary diagnosis of the relapse and the metastasis of cancer, and in the research of neoplastic transformation and primary tumor diagnoses. To observe the effect of antisense IGF-I gene on the tumor markers (CEA and AFP), antisense IGF-I gene was transfected into human hepatoma cells (HepG2) to observe whether antisense IGF-I gene can regulate the changes of CEA and AFP, and the effect of neoplastic transformation.

## MATERIAL AND METHODS

### *Cell lines and materials*

Human hepatoma cells were purchased from the Institute of Shanghai Cell Biology. Antisense IGF-I expression plasmid was obtained from the University of Case Western Reserve. Hygromycin was purchased from Sigma. Lipofectin and RPMI-1640 were purchased from GIBCO. The DIG system for hybridization was purchased from Boehringer Mannheim Biochemica. The CEA and AFP kit were the products of Institute of Shanghai Biologic-Products.

*The antisense IGF-I plasmid preparation and its DNA separation, appraisal and purification*  
Detailed methodology followed Kliieg Ler M<sup>[5]</sup> and Trojan *et al*<sup>[3]</sup>'s.

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\*Project supported by the Natural Science Foundation of Guangdong Province, No. 930305.

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Received 1997-09-02 Revised 1997-09-30

### Antisense IGF-1 gene transfer into tumor cells mediated by Lipofectin

Transfection of HepG2 cells was accomplished using Lipofectin reagent (GIBCO) according to the instructions. Solution A: 100  $\mu$ l RPMI-1640 containing 10  $\mu$ g/L antisense IGF-I gene. Solution B: 90  $\mu$ l RPMI-1640 containing 10  $\mu$ l Lipofectin. Both solutions were mixed gently, and placed at room temperature for 15min. Two serum-free RPMI-1640 was added to each tube containing the Lipofectin reagent-DNA complexes, and mixed gently and overlay out cells. The cells were incubated for 5h - 12h at 37°C in a CO<sub>2</sub> incubator. Two ml of RPMI-1640 was supplemented with 10% FCS and the cells were incubated at 37°C, 5% CO<sub>2</sub> incubator for another 48h. The cells grow in the presence of hygromycin until positive clones were selected.

### Northern blotting hybridization

Total RNA in cells was extracted according to Kliegler M<sup>[5]</sup>. Transfer blot hybridization was carried out as described in manual of DIG system kit. A dilution series of the total RNA was transferred to Nylon membrane and baked for 30min at 120°C. The membranes were put in a sealed plastic bag and used for Northern hybridization

### Measurement of CEA

CEA kit contained CEA marker, 125I-CEA, CEA antibody and immune separation reagent. The operation and analysis of the results were carried out according to the manual.

### Measurement of AFP

AFP kit contained marker, 125I-AFP, first antibody of AFP, analysis reagent of AFP and second antibody of AFP. The operation and analysis of the results were carried out according to the manual.

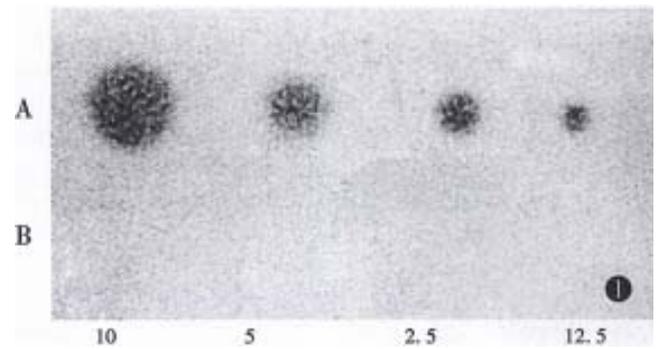
### Statistical methods

Data were analyzed using the Student's *t* test.

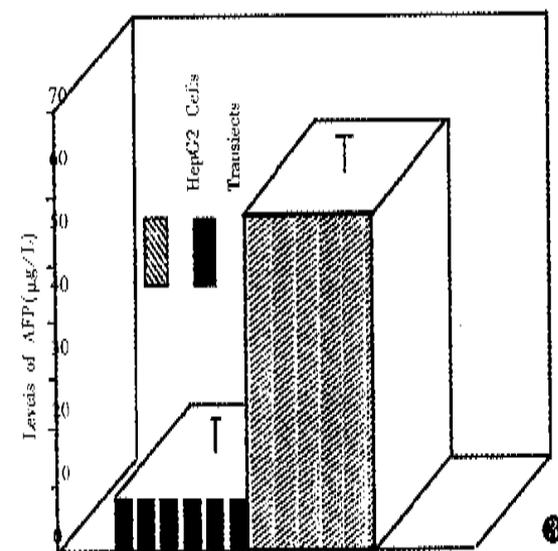
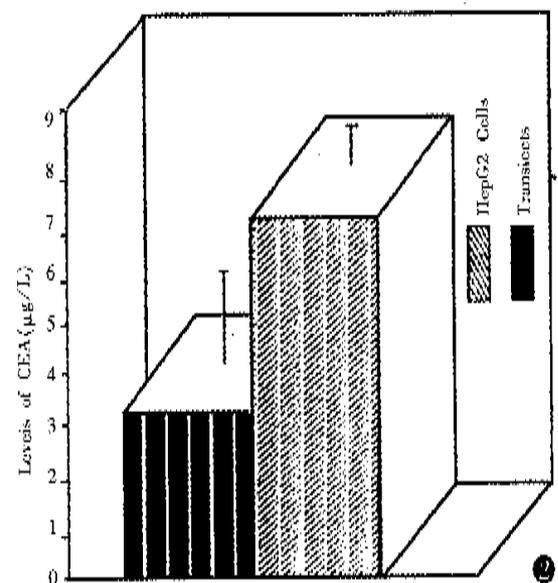
## RESULTS

### The analysis of Northern Blot

The antisense IGF-I plasmid was purified by abstraction and electrophoresis. It was wrapped up by Lipofectin and conducted into human hepatoma cells (HepG2). IGF-I antisense transcripts were selected in the presence of hygromycin. The total RNA in positive clone was extracted and its RNA transcription levels were analyzed by Northern Blot hybridization (Figure 1). The results showed that strong expression of the antisense transcript and the IGF-I transcripts of parent HepG2 cells was not apparent.



**Figure 1** Northern blot analysis of antisense IGF-I transcripts in human HepG2 cells. Lane A, RNA of transfectant cells, Lane B, RNA of parent cells.



**Figure 2** Expression of CEA in human HepG2 cells transfected with antisense IGF-I gene and parent HepG2 cells.

**Figure 3** Expression of AFP in human HepG2 cells transfected with antisense IGF-I gene parent HepG2 cells.

### **CEA levels of HepG2 cells transfected with antisense IGF-I**

The transfectant cells were kept in the presence of hygromycin for 8 - 12 days. After the transfectants and parent cells were cultured for 24 hours, the supernatants were collected and CEA levels were determined (Figure 2). The CEA levels of positive clones were markedly lower than that of the parent cells ( $7.0 \mu\text{g/L} \pm 0.76 \mu\text{g/L}$  and  $3.29 \mu\text{g/L} \pm 1.80 \mu\text{g/L}$ , respectively) ( $P < 0.05$ ).

### **The levels of AFP of antisense IGF-I transfectants of HepG2**

The supernatants were collected by the same method for CEA. AFP levels are shown in Figure 3. The AFP levels of positive clones were lowered markedly as compared with parent cells ( $53.63 \mu\text{g/L} \pm 6.02 \mu\text{g/L}$  and  $9.0 \mu\text{g/L} \pm 5.26 \mu\text{g/L}$ , respectively) ( $P < 0.01$ ).

## **DISCUSSION**

It has been known for a long time, abnormal materials related with the diseases exist in the sera of cancer patients. The tumor markers are used for the diagnosis, prognosis and treatment of tumor. Although, the tumor markers were expressed nonspecifically by tumors and produced differently, they are still important indexes to predict carcinogenesis. CEA and AFP are carcinoembryonic protein. They appear in the embryonic term and disappear in adult. When they appear in the serum of adult, it shows that the growth of tumor was active so that these proteins were secreted into the blood. The half-life of CEA and AFP in plasma were 6 - 8 and 6.5 days. Recent reports indicated that CEA was a member of immunoglobulin supergene family<sup>[6]</sup>. Both were important tumor markers in the course of the carcinogenesis and development. As both were regarded as the index of the tumor biological changes and of the malignant degree in vivo, in this study, CEA and AFP were used as the index of positive clone biological changes after tumor was transfected by antisense IGF-I gene. It showed that antisense IGF-I gene affect the changes of tumor markers related with malignancy of the tumors. Because many gastrointestinal tumors were produced by CEA and AFP, human hepatoma cells (HepG2) were selected as the target tumors. Antisense IGF-I gene was wrapped up and

transfected into the HepG2 cells, and cells grew in the presence of hygromycin for the positive clone. Northern Blots proved that transfectants of HepG2 cells showed detectable antisense IGF-I transcripts. The positive clone grew in the presence of hygromycin for 8 - 12 days. After the transfectants and parent cells were adjusted to  $2 \times 10^6$ /well for 24 hours the supernatants were collected. Figures 1-2 show the results of radioactive immune technique. The CEA and AFP levels of positive clones were markedly lower than that of the parent cells [CEA,  $7.0 \mu\text{g/L} \pm 0.76 \mu\text{g/L}$  and  $3.29 \mu\text{g/L} \pm 1.80 \mu\text{g/L}$  ( $P < 0.05$ ) and AFP,  $53.63 \mu\text{g/L} \pm 6.02 \mu\text{g/L}$  and  $9.0 \mu\text{g/L} \pm 5.26 \mu\text{g/L}$  ( $P < 0.01$ ), respectively]. The results indicated that transfectants could affect the levels of CEA and AFP. When the secretive levels of both decreased, it suggested that malignancy of positive clone was lower than that of the parent cells and original biological courses were changed. At present, a lot of papers show that many kinds of tumors expressed abnormal IGF-I and suggest the involvement of the continuous proliferation and tumorigenic phenotype<sup>[2]</sup>. After antisense IGF-I gene was transfected into target cells, a specially nucleotide sequence was expressed in cells that was complementary to a portion of IGF-I gene in target cells. The process and transcript of IGF-I mRNA were inhibited. As the secretion of IGF-I in target cells were changed, it could affect the role of biology in cells, the event could inhibit the growth and the tumorigenicity of rat glioblastoma cells and murine teratocarcinoma in vivo<sup>[3,4]</sup>. That antisense IGF-I gene blocks the corresponding IGF sequences may be the reason for the decrease of CEA and AFP in target cells. The exact mechanisms await further researches.

## **REFERENCES**

- 1 Froesch ER, Schmid Chr, Schwander J, Zapf J. Actions of insulin-like growth factors. *Ann Rev Physiol*, 1985;47:443 - 467
- 2 Macaulay VM. Insulin-like growth factors and cancer. *Br J Cancer*, 1992;65 (3):311-320
- 3 Trojan J, Johnson TR, Rudin SD, Ilan J, Tykocinski ML, Ilan J. Treatment and prevention of rat glioblastoma by C6 cells expressing antisense IGF-I RNA. *Science*, 1993;259(5091):94 - 97
- 4 Trojan J, Johnson TR, Rudin SD, Blossey BK, Kelley KM, Shevelev A *et al*. Gene therapy of murine teratocarcinoma: separate functions for insulin-like growth factors I and II in immunogenicity and differentiation. *PNAS*, 1994;91(13): 6088 - 6092
- 5 Kliegler M. Gene transfer and expression. New York: M Stockton Press, 1990:47 - 81
- 6 Zimmerman W. Recent development concerning the carcinoembryonic gene family and their clinical implications. *Tumor Biol*, 1990;11(1-2):1 - 4

# Arterial chemoembolization for hepatocellular carcinoma \*

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**Subject headings** Liver neoplasm/therapy; Lipiodol; sinobufagin microsphere; gelatin sponge; chemoembolization, therapeutic

## Abstract

**AIM** To study the therapeutic effects of transcatheter arterial three-segment chemoembolization for hepatocellular carcinoma (HCC).

**METHODS** According to the anatomy of vessels, the tumor capillary networks, muscular arterioles and feeding arteries were successively occluded using lipiodol ultra-fluid (LUF), sinobufagin microsphere (SBMs) and particles of gelatin sponge (PGS). In this series of 80 cases, therapeutic effects were evaluated in 76 cases.

**RESULTS** There were 22 cases (28.9%) with partial response and 41 (53.9%) with minor response in the 76 evaluated patients. The 6-month, 1-year, 2-year and 3-year survival rates were 97.4%, 86.8%, 46.1% and 27.6% respectively.

**CONCLUSION** This regimen was a rational chemoembolization method for HCC patients.

## INTRODUCTION

Transcatheter arterial embolization was recommended for the treatment of unresectable HCC in the 70s. Recently, various embolic materials and anticancer agents have been developed. But these methods still have some unsolved problems such as the level of tumor vessels embolization, selection of embolic agents and anticancer drugs, etc. Therefore, we devised the three-segment chemoembolization of the tumor vessels (TSCTV) using lipiodol ultra-fluid, sinobufagin microsphere and particles of gelatin sponge for the treatment of HCC patients. The results of the clinical trials are reported below.

## MATERIALS AND METHODS

### Patients

Eighty consecutive patients with HCC were observed from March 1990 to March 1992. There were 72 males and 8 females, aged from 24 to 69 years (average 49 years). None of them underwent chemotherapy before. The diagnosis of HCC was established by various imaging techniques (including ultrasonography, US; computed tomography, CT; magnetic resonance imaging, MIR; and hepatic angiography, HAG;) and serum alpha-fetoprotein (AFP). The histologic diagnosis was made in 10 cases.

The cases were classified into four stages according to the Manual for Staging of Cancer<sup>[1]</sup>, 2 cases as stage I (2.5%), 6 cases as Stage II (7.5%), 62 as Stage III (77.5%), and 10 as Stage IV (12.5%). The serum AFP levels were greater than 400 $\mu$ g/L in 42 cases (52.5%). Hepatitis B surface antigen was positive in 62 cases (77.5%). The characteristics of these patients are shown in Table 1.

### Response criteria

The clinical response to the treatment was assessed objectively by the change in tumor size, which was estimated by US, CT and MRI before and after the therapy. The reduction in tumor size was measured at the same image level, presenting the maximum diameter. In cases of the multiple tumors, the largest mass was measured in the same way<sup>[2]</sup>. Response criteria were defined based on the reduction of the perpendicular diameter in the tumor as follows: ① A complete response was total disappearance of tumor; ② A partial response was a reduction in tumor size of more than 50%; ③ A minor response was a reduction of 25% to 50%; ④

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Received 1997-4-27

No change was a change in tumor size less than  $\pm 25\%$ ; ⑤ Progressive disease was an enlargement of more than 25%. Response criteria were determined once a month for 6 months after TSCTV. Response criteria had to be maintained for at least a month.

#### Preparation of chemoembolization agents

SBMs were prepared in the department of pharmacy of our college. The drug microsphere, with a mean diameter of 200 $\mu\text{m}$ , was composed of approximately 6% (w/w) of sinobufagin in gelatin<sup>[3]</sup>. The doses of embolic and anticancer agents were determined by the tumor size and degree of liver dysfunction.

Usually, 10 ml - 20 ml LUF and 50 mg - 100 mg SBMs were used when the tumor diameter was less than 10 cm, and 20 ml - 30 ml and 100 mg - 200 mg respectively when the tumor size was more than 15cm. The doses of the anticancer drugs were aclarubicin (ACR) 50 mg and cis-diaminedichloroplatin (CDDP) 80 mg. For patients with poor liver function anti-cancer drug doses should be reduced. The anticancer drugs were divided into two portions of same dose. A portion was suspended in LUF to make emulsion, the other portion was mixed in 10 ml of radiopaque contrast medium and SBMs to form mixture. The PGS, with a mean diameter of 1mm, was prepared in our laboratory.

#### TSCTV

TSCTV was performed through the femoral artery using the technique of Seldinger. A 6.5-French (Cook Co., USA) angiographic catheter was inserted superselectively into the hepatic artery feeding the target tumor.

Initially, the emulsion of the LUF and anticancer drugs were slowly infused till the vessels within tumors were filled. Subsequently, the microsphere mixture was gently infused till the tumor arteries completely disappeared. Finally, the PGS, approximately 1 g - 2 g, were infused until the feeding artery of the tumor was occluded. All of these procedures were performed under fluoroscopic guidance to avoid the reflux or spill-over of the embolic agents and anticancer drugs. The second therapy was performed about a month after the first TSCTV. TSCTV was repeated at 3 - 8 months depending on the patients' condition. This therapy should be carried out immediately if any of the followings appeared: increase in tumor size, occurrence of new focus within the liver, inadequate accumulation of LUF in the tumor, reelevation of serum AFP ( $>200\mu\text{g/L}$ ) or GGT (r-Glutamyltranspeptidase $>350\mu\text{g/L}$ ).

## RESULTS

#### TSCTV courses and drugs doses

A total of 240 courses of TSCTV were performed, averaging 3 courses per patient, with a range of 1 to 6 courses. Fifty-six cases (70%) underwent more than 3 courses. The mean dose of embolic agents and

anticancer drugs in TSCTV are shown in Table 2.

#### Response

Of 80 patients, 4 was excluded from this study because surgical operations were performed about a month after TSCTV. The remaining 76 patients (95%) were evaluated for responses. According to the response criteria, 22 (28.9%) had partial response, 41 (53.9%) minor responses, while no change in 10 cases (13.1%) and progressive diseases were observed in 3 (3.9%) cases (Table 3). The state of the tumor reduction maintained 4.5 to 49 months. The liquefaction and necrosis within tumors occurred in 60 (79%) cases after the first TSCTV. The necrosis area ranged from 2.5 cm to 6.5 cm (mean 3.8 cm  $\times$  3.2 cm). The second hepatectomy was performed in 6 cases within 3 - 6 months after 1 - 3 courses of TSCTV. The focuses were resected in 5 (6.6%) of 6 cases. The histological examination demonstrated that there were massive coagulation necrosis and fibrosis of tumor tissue in all cases. No living tumor cells could be seen in the specimens in 2 cases (Figure 1).

#### Survival rate

The starting point was defined as the day 0 initial TSCTV therapy. The 6-month, one-year, 2-year and 3-year survival rates were 97.4% , 86.8%, 46.1% and 27.6% (Table 4). The longest survival time was up to 49 months. During the disease palliation phase, the life quality of the patients with tumors was satisfactory.

**Table 1 Clinical characteristics of patients with hepatocellular carcinoma**

Average age in year (range)	49(24-69)
Sex (male: female)	72:8
Serum AFP level ( $\mu\text{mol/L}$ )	
<50	18(22.5%)
50-399	20(25%)
>400	42(52.5%)
HBsAg+	62(77.5%)
TBILT(mg/L)	16.9 $\pm$ 6.0
ALT(u/l)	68.9 $\pm$ 36.6
ALB(g/L)	38.4 $\pm$ 4.4
Child's grade (a:b:c)	30:47:3
Stage (I:II:III:IV)	2:6:62:10

TBILT: bilirubin, ALT: glutamic-pyruvic transaminase, ALB: albumin.

**Table 2 Mean doses of embolic agents and anticancer drugs**

TSCTV courses	No of patients	Embolic agents		Anticancer drugs		
		SBMs (mg)	LUF (ml)	SB (mg)	ACR (mg)	CDDP (mg)
1	80	145.0	22.3	8.7	41.8	80.0
2	72	114.3	13.7	6.9	37.9	75.6
3	56	107.8	15.4	6.5	40.8	76.4
4	24	91.7	11.7	5.5	40.0	80.0
5-6	6	100.0	11.2	6.0	40.0	80.0
Mean	240	111.8	14.9	6.7	40.1	78.4

SB: Sinobufagin

**Table 3 Response to TSCTV in evaluated patients**

Stage	CR	PR	MR	NC	PD	Total
I	0	0	0	0	0	0
II	0	0	2	2	0	4
III	0	22	36	4	0	62
IV	0	0	3	4	3	19
Response	0	22	41	10	3	76
Percentage	(0%)	(28.9%)	(53.9%)	(13.1%)	(3.9%)	

**Table 4 Survival of 76 patients undergoing TSCTV**

Stage	No of patients	No of survival (year)			
		0.5	1	2	3
I	0	0	0	0	0
II	4	4	4	3	2
III	62	62	60	32	19
IV	10	8	2	0	0
Total	76	74	66	35	21
Percentage		(97.4%)	(86.8%)	(46.1%)	(27.6%)

**Laboratory examinations**

The liver function, peripheral white blood cell counts, platlet counts and AFP level were examined before and after TSCTV (1, 2 and 4 weeks). The serum AFP, which was more than 200 μg/L before treatment in 62 patients, apparently fell in 58 patients (94%) about 2 weeks after TSVCTV. Serum AFP was lowered to normal (AFP < 200 μg/L) after 2 - 4 courses of TSCTV in 28 (48.3%) cases. The changes of serum AFP level in 42 cases (AFP>400 μg/L) are shown in Figure 2. The ALT level showed dropping tendency a week after TSCTV and then, elevated again in some patients after 2 - 3 weeks. The peripheral WBC of all patients increased after first TSCTV, <3.5×10<sup>9</sup>/L in 2 cases after the third TSCTV. The results of laboratory examinations are shown in Figure 3.

**Side effects and complications**

The postembolization syndrome (including fever, right hypochondrium pain, and nausea and vomiting) was seen in all patients after TSCTV. The serious degree of the syndrome was strongly correlated with the area of the liquefaction and necrosis within tumor and the doses of the embolic agents and anticancer drugs. After the first TSCTV, the syndrome was severe in some patients and generally maintained 1 to 3 weeks. The side effects and complications of TSCTV are shown in Table 5.

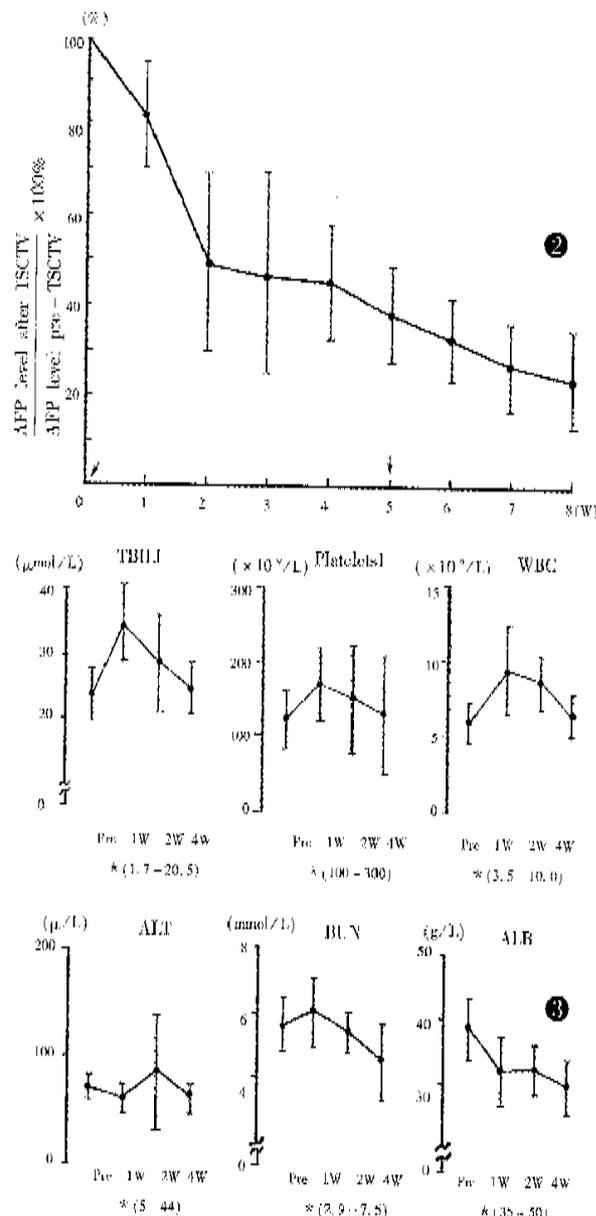
**Table 5 Side effects and complications**

Side effects and complications	Incidence No.(%)
Fever 38-39°C	71(88.7)
>39°C	18(22.5)
Nausea and vomiting	60(75.0)
Right hypochondrium pain	72(90.0)
Cholecystitis	5(6.2)
Liver abscess	1(1.2)
Haptic failure	4(5.0)
Ascites	6(7.5)

\*The floccule was found in gall bladder by US

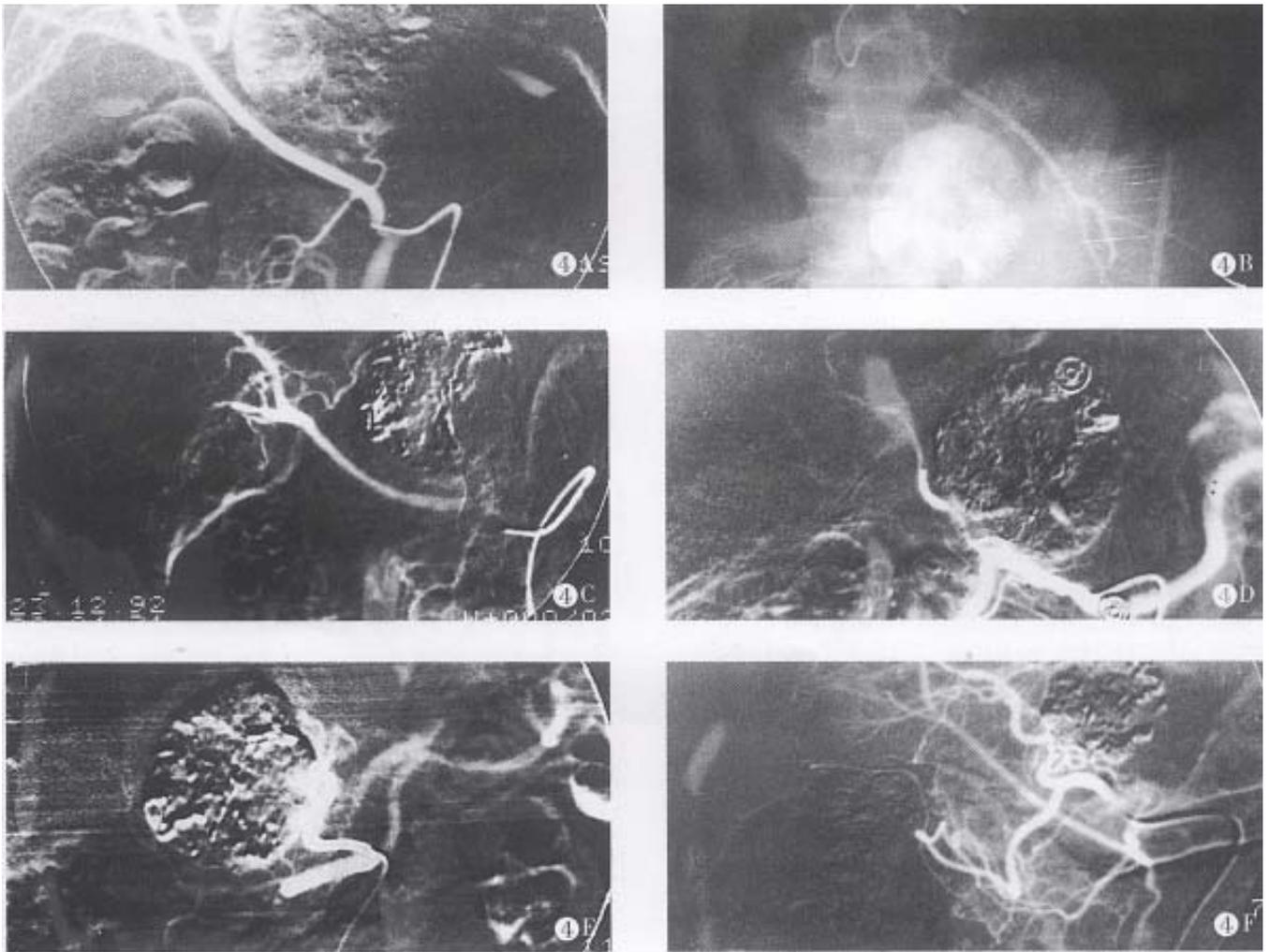


**Figure 1** The resected specimen showing massive coagulation necrosis and fibrosis in tumor.



**Figure 2** Serum AFP levels in 42 cases (AFP > 400μmol/L) after TSCTV, assuming that pre-embolization value was 100% (↓ TSCTV treatment).

**Figure 3** Changes in blood chemical data after first TSCTV (pre: level before TSCTV; W: week; \*: normal range)



**Figure 4 A.** The hepatic right artery derived from superior mesenteric artery after TSCTV was performed. The left hepatic artery derived from gastroduodenal artery and supplying left and lower 1/3 of the tumor, to prevent embolic agents reflux, lipiodol chemoembolization (LCE) was performed.

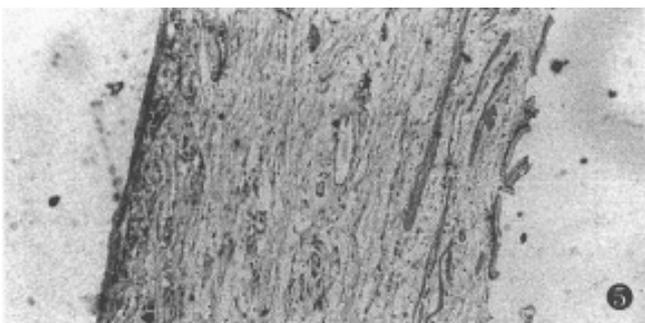
**B.** The tumor vessels disappeared completely and the lipiodol accumulated within tumor after embolization.

**C.** The lipiodol was obviously washed out in the LCE area after 4 months, TSCTV and LCE were repeated.

**D.** The lipiodol was washed out fourth in the LCE area after 6 months.

**E.** LUF 5ml and SBMs 50mg were infused through hepatic left artery, the tumor vessels completely disappeared, the LUF accumulated in the LCE area.

**F.** The tumor obviously shrank (PR) and lipiodol accumulated within tumor after 19 months.



**Figure 5** The necrosis of the gallbladder mucosa.

## DISCUSSION

According to the electronic microscopic observation, the arterial system of the HCC is composed of three kinds of successive vessels. There are massive neonate capillary networks within tumor consisting of single layer endothelium cells. Its angiographic imaging is revealed as vascular mass. The successive vessels are muscular arterioles with diameters of about  $100\ \mu\text{m}$  -  $250\ \mu\text{m}$ . There are extensive arterio-portal vein shunts in these massive neonate vessels. The blood flow of the portal vein might reflux into the empty arterioles through these shunts as soon as the feeding arteries are occluded. The blood supply of the tumor renews in short time so that the inhibiting effect in tumors disappear soon. The successive feeding arterioles usually become wide and tortuous and are accompanied by "stealing blood"

phenomenon<sup>[4,5]</sup>. In light of the vessels anatomy, we designed the TSCTV method. There are several possible mechanisms of TSCTV that can prolong the survival rates: ①The capillary networks, muscular arterioles and feeding arteries of the tumor are successively occluded using three embolic agents of various diameters and properties so that direct ischemic changes within tumors are caused by liquid embolic agent, LUF, and solid embolic agent, SBMs; while proper occlusion of feeding arteries surrounding the tumor is also encountered by expansive embolic agent, PGS. Thus, the occlusion of tumor vessels is more rational and complete. ②The less viscosity and better compatibility of LUF are favourable to occlusion of capillary networks in tumor and dispersion of anticancer drugs into oil phase<sup>[6]</sup>. ③By complex chemical cross linking, the degradation of the gelatin microsphere is delayed *in vivo*. After infusion, SBMs can block blood flow, delay the “washout” of LUF (Figure 4), prevent reflux of the portal vein blood and, simultaneously, slowly release the drug locally. ④While occluding feeding and collateral arteries of the tumor, the PGS can avoid the refluxing of embolic agents and anticancer drugs into gallbladder and gastroduodenal arteries, resulting in severe side effects<sup>[7]</sup>. ⑤Because anticancer drugs suspended in LUF and SBMs, forming the structure of “three-ply-board” after infusion introduced as regional chemotherapy, anticancer drugs seem to be delivered to and accumulated selectively within the tumor<sup>[8,9]</sup>. ⑥Sinobufacin, as an anticancer drug of Chinese traditional materials, has its own efficiency on HCC treatment, which is superior to fluorouracil. The drug can cause vessels inflammation and the remarkable endovasculitis and leading secondary embolization in the embolized vessels, enhancing the effect of arterial occlusion. In addition, the drug is very effective for cardiogenic, diuresis, antiseptic, analgesia and increased peripheral white blood cells, enhancing body immunofunction, etc<sup>[3]</sup>. These complementary pharmacologic effects can augment the therapeutic effects and reduce systemic side effects. ⑦ACR and CDDP are, at present, a better combination of chemotherapy, and have synergistic anticancer effects<sup>[7]</sup>. ⑧This method caused relatively mild, damage to liver and renal functions so that it is acceptable to most patients.

The outcomes of our clinical trial demonstrate that TSCTV can significantly improve the completeness of the tumor vessels occlusion, the area of liquefaction and necrosis of the tumor, stay time of embolic agents within tumor vessels, partial response rate and survival rate, as compared with the treatment using microsphere or lipiodol alone.

It was found that after occlusion, the collateral circulation occurred sooner or later in most tumors. Where there was vessels occlusion, there was the formation of collateral circulation. Therefore, occlusion should be performed until the tumor vessel

completely disappeared in each course of treatment so that the extensive and fast necrosis of the tumor cells can be induced. The necrosis of tumor cells might result in high response rates, survival rates and prevent metastasis within the liver<sup>[3,7]</sup>. The doses of embolic agents should be modest, as excessive embolic agents might cause reflux and side effects.

Tumor progression occurred in some patients with time. Timely re-TSCTV could usually inhibit or prevent the tumor progression. Therefore, repeated TSCTV was very important in ensuring the therapeutic effects. We found that more than 3 courses of TSCTV was essential.

However, the effect of TSCTV was not so satisfactory in stage IV patients, especially in those with tumor thrombus of the major portal branch. In our study, there were 4 cases of hepatic failure. Therefore, this method should be carefully adopted in those patients.

The post-embolization syndrome was significant by this method. The causes might be as follows: ①Complete occlusion of the tumor vessels resulted in remarkable necrosis of the tumor; ②Sinobufacin caused the vasculitis of the embolized vessels; and ③The embolic agents and anticancer drugs refluxed into gallbladder artery, leading to necrosis of the gallbladder mucosa (Figure 5). To prevent the occurrence of the second liver abscess and cholecystitis, it was essential to administer sufficient antibiotics after TSCTV.

In conclusion, based on anatomy of the vessels, TSCTV combines with the advantages of the three embolic agents so that occlusion of the tumor vessels was more complete and rational. The embolization effect was strengthened by SBMs causing endovasculitis and second occlusion. The structure of the “three-ply-board” can form a high concentration region of anticancer drugs. Three anticancer drugs complement with each other in pharmacologic action, thus augmenting the anticancer effect and reducing systemic side effects. From these results, it is concluded that the TSCTV is a ideal therapeutic regimen for HCC patients.

## REFERENCES

- 1 American Joint Committee on Cancer. Liver. In: Hermanek P, ed. manual for staging of cancer. Ed 3. Philadelphia: LB Lippincott, 1988:87-94
- 2 Yamashita Y, Takahashi M, Koga Y. Prognostic factors in the treatment of hepatocellular carcinoma with transcatheter arterial embolization and arterial infusion. *Cancer*, 1991;67(2):385-387
- 3 Fan J, Huang MK, Wu GY. An experimental study of regional chemotherapy with adriamycin and gelatin microsphere. *TIEDAO YIXUE*, 1995;14(6):434
- 4 Akerman N. Experimental studies of the circulatory dynamics of intrahepatic tumor blood supply. *Cancer*, 1972;29(2):435-437
- 5 Li CL, Wong CE, Shoa XH. Vascular cast of primary liver cancer and its scanning electron microscopic observations. *Chin J Surg*, 1986;34(10):620
- 6 Shibata J, Fujiyama S, Sato T *et al*. Hepatic arterial injection chemotherapy with cisplatin suspended in an oily lymphographic agent for hepatocellular carcinoma. *Cancer*, 1989;64(12):1586-1589
- 7 Toru B, Chitoshi O, Yasuo Y *et al*. A new approach to chemoembolization for unresectable hepatocellular carcinoma using aclarubicin microspheres in combination with cisplatin suspended in iodized oil. *Cancer*, 1991;68(12):1555-1558
- 8 Fan J, Huang MK, Wu GY. An experimental study on hepatic arterial chemoembolization with sinobufagin microspheres. *Chin J Cancer*, 1995;14(6):434-436
- 9 Xue KX. Experimental embolization in patients with unresectable hepatocellular carcinoma: a randomized controlled trial. *Gastroenterology*, 1988;94(3):453-456

# Expression of glutathione S-transferase placental mRNA in hepatic preneoplastic lesions in rats \*

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**Subject headings** liver neoplasms; glutathione S-transferase; in situ hybridization; RNA, messenger; precancerous conditions

## Abstract

**AIM** To detect glutathione S-transferase placental (GST-P) mRNA expression in hepatic preneoplastic lesions in rats.

**METHODS** Using Solit-Farber model, the GST-P mRNA expression was observed in hepatic preneoplastic lesions induced by diethylnitrosamine (DEN) in rats and normal and regenerated hepatic tissues in the control group by in situ hybridization.

**RESULTS** GST-P mRNA was mainly expressed in altered hepatic foci (AHF) and some of the oval cells in hepatic preneoplastic lesions and the extent of its expression was different among various foci or/and positive cells in the same focus whereas no expression was observed in normal and regenerated hepatic tissues.

**CONCLUSION** Cells in AHF and oval cells may be the preneoplastic cells in the experimental hepatocellular carcinoma at the molecular level and heterogeneity exists in GST-P transcription levels.

## INTRODUCTION

Glutathione S-transferases (GSTs, EC2,5,1,18) are a family of dimeric proteins that may play important roles in both the intracellular transport of hydrophobic molecules and the metabolism of toxic compounds. The cytosolic GSTs can be divided into at least four different families, i. e.  $\alpha$ ,  $\mu$ ,  $\pi$  and  $\theta$  based on N-terminal sequences, substrate specificities and affinity for non-substrate ligands in human. GST-P (the rat enzyme equivalent to human GST- $\pi$ ) was first found in placenta, but later also in kidney, lung and testis<sup>[1]</sup>. Recently, it has been shown by Northern blot and Dot blot analysis that GST-P mRNA is present abundantly in preneoplastic lesions and hepatocellular carcinoma induced by genotoxic carcinogen<sup>[2]</sup>. However, in hepatic preneoplastic lesions, which cell type has GST-P mRNA expression has not been well elucidated. This study was to investigate the expression of GST-P mRNA in hepatic preneoplastic lesions in rats by in situ hybridization and reveal the significance and regulation mechanism of its expression.

## MATERIALS AND METHODS

### *Animal models and samples processing*

Seventy male Wistar rats, weighing 100 g - 150 g, were randomly divided into experimental group (A) and control groups (B,C). Group A (30 rats), according to Solt-Farbar model<sup>[3]</sup>, was initially given a single intraperitoneal injection of DEN at 200 mg/kg (Sigma product) dissolved in 0.9% NaCl to initiate hepatocarcinogenesis. After 2 weeks on basal diet, they were fed with 0.02% 2-acetylaminofluorene (AAF, Sigma product) for 2 weeks. All the rats were subjected to partial hepatectomy (PH) at the 3rd week. Group B (20 rats) were injected with 0.9% NaCl instead of DEN solution and then underwent PH at the 3rd week. Group C (20 rats) were injected with 0.9% NaCl only (Figure 1).

Rats of each group were killed at the 1st, 2nd, 3rd, 4th and 5th week. The livers were removed. A portion of lesion tissues were stored at -80 °C. Frozen serial sections were made for in situ hybridization and histochemical demonstration. The remainders were fixed in 4% paraformaldehyde for routine staining with hematoxylin and eosin.

### *Morphological observation and $\gamma$ -GT histochemical staining*

Pathomorphological observation and histochemical assay were made according to the National<sup>[4]</sup> and Rutenberg method<sup>[5]</sup>.

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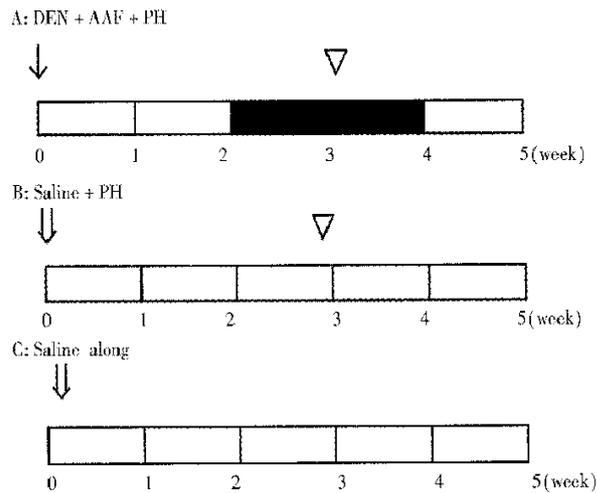
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\*Project supported by the National Natural Science Foundation of China, No. 39270303.

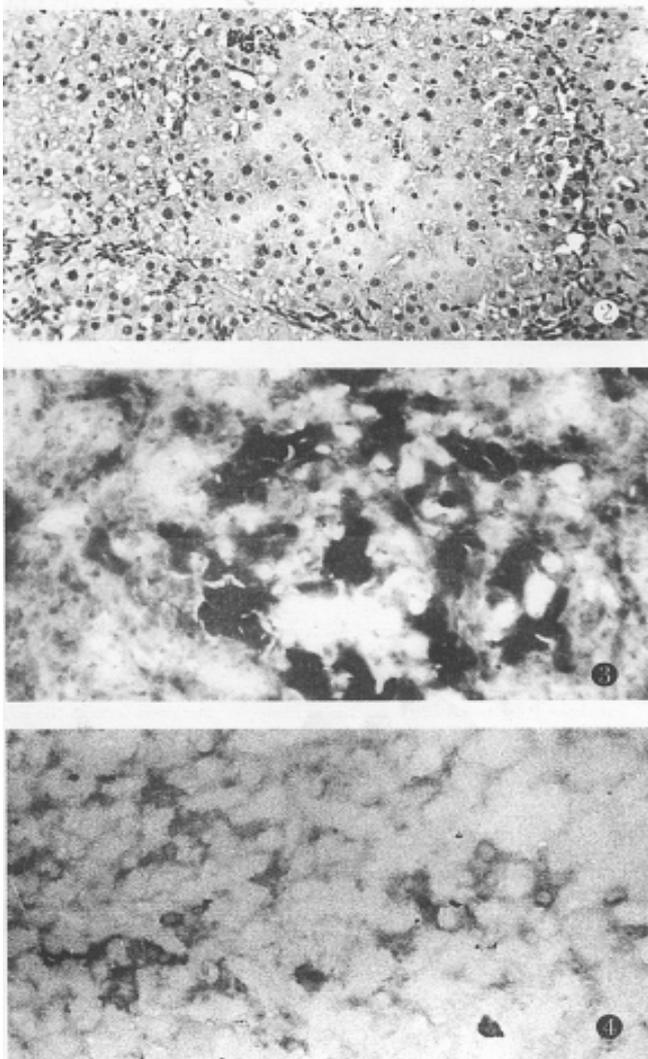
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Received 1997-03-26 Revised 1997-05-28



**Figure 1** Experimental protocol

↓ DEN 200 ng/kg ip      ▽ 0.9% NaCl 0.5 ml/kg ip  
 ▽ PH: 2/3 partial hepatectomy    ■ 0.02% 2-AAF in diet



**Figure 2** Altered hepatic- foci at the 5th week (HE×150)

**Figure 3**  $\gamma$ -GT positive foci of liver cells at the 5th week (Enzymo histochemistry) ×200

**Figure 4** GST-P mRNA positive foci of liver cells at the 5th week (ISH×400)

### *In situ hybridization*

The bacteria carrying the pUC18 plasmid containing full length 0.75kb GST-P cDNA, PGP5 were gifts from Dr. Sugioka. The special restricted endonucleases cutting sites were EcoRI-SaI. Probes were prepared according to J. Sambrook's method<sup>[6]</sup> and labeled by digoxigenin (DIG) using DIG-labelling and detection kit (Boehringer Mannheim Inc).

In situ hybridization was done according to SU HC *et al*<sup>[7]</sup>. Frozen sections were fixed in 4% paraformaldehyde/PBS and then rinsed with PBS, glycine/PBS and Triton-100/PBS in turn. Hybridization buffer contained 110  $\mu$ g/L DIG-GST cDNA probes, 5  $\times$  SSC, 50% formamide, 5  $\times$  Denhardt's solution, 5% dextran sulfate and predenatured 0.5 g/L ssDNA. Optimal hybridization temperature was about 42°C in humid chamber. As a negative control, hybridization buffer did not contain DIG-GST-P cDNA probes, and sections were digested with RNase at 37°C before hybridization.

## RESULTS

### *Pathomorphological and histochemical changes*

One week after PH, there was a grey granular appearance on gross inspection in experimental group rat liver. Histological examination of the liver tissues showed many AHF. These foci were mainly composed of basophilic cells. Proliferation of oval cells was found in portal areas (Figure 2). AHF was  $\gamma$ -GT positive (Figure 3).

### *In situ hybridization*

At the 3rd week, GST-P mRNA was expressed in a few clusters of oval and liver cells in preneoplastic lesions. At the 4th and the 5th week, GST-P mRNA was mainly detected in hyperplastic AHF and some of the oval cells. Hybridization signal positive granules were mainly distributed in the cytoplasm of altered hepatic cells (Figure 4). The extent of the GST-P mRNA expression was different among the foci or/and positive cells in the same focus. Results of negative control showed only background staining, indicating the specificity of hybridization. No positive signals were found in control groups.

## DISCUSSION

The binding of electrophilia metabolites of carcinogens to macromolecules, especially the DNA, is a critical event in chemical carcinogenesis. GST can detoxify a number of carcinogenic electrophiles, including diol-epoxide metabolites of polycyclic aromatic hydrocarbons by catalysis of the conjugation with reduced glutathione. Four multigene families of GSTs were characterized in human and rats, i. e.,  $\alpha$ ,  $\mu$ ,  $\pi$  and  $\theta$ <sup>[1]</sup>. The expression of xenobiotic metabolizing enzymes in

human hepatocellular carcinoma is complicated and a few findings support the proposal that GST- $\pi$  could be used as a marker of hepatocellular carcinoma<sup>[8]</sup>. However, GST-P has been demonstrated to be a very useful marker enzyme, its mRNA is hardly detectable in normal rat livers, but is very strongly expressed in preneoplastic livers by induced genotoxic carcinogen by Northern blot and Dot blot hybridization methods<sup>[2]</sup>. There are many cell types in the liver tissues. Northern blot and Dot blot hybridization only showed an average level of the whole cell gene expression, not single cell gene expression and its characteristics. It provides the most accurate identification of RNA transcripts at single cell level by in situ hybridization technique. Our findings showed that GST-P mRNA was mainly expressed in AHF and some of the oval cells in hepatic preneoplastic lesions and the extent of its expression was different among foci or/and positive cells in the same focus whereas no expression was observed in normal and regenerated hepatic tissues. This study indicates that cells in AHF and oval cells may be the preneoplastic cells in experimental hepatocellular carcinoma at the molecular level and heterogeneity in GST-P transcript levels.

GST-P is known as a reliable tumor marker for chemical carcinogen-induced and spontaneously occurring preneoplastic lesions and hepatomas in rats. Thus, GST-P expression may be closely related to the process of hepatocarcinogenesis in rats. In multistep carcinogenesis, changes in the pattern of gene expression might be an important step in oncogene activation and/or inactivation of antioncogenes. To investigate possible alterations in gene expression pattern during neoplastic transformation, it may be useful to explore tumor marker gene expression. Why and how is the GST-P gene activated inevitably during the course of liver cell transformation? Two possible mechanisms are considered: One is that the GST-P gene is located in close proximity to a gene that is directly involved in liver cell transformation and local chromosomal changes activate both genes simultaneously. The other is that those genes are not physically linked, but their expression is regulated by some common transcription factors. The GST-P gene has a strong enhancer element, GPE1, located at 2.5kb upstream from the cap site. The enhancer consists

of the TRE-like sequence with palindromical orientation. GPE1 is a major control element responsible for GST-P expression in preneoplastic lesions. The regulatory region of the GST-P gene has another TRE-like sequence located at 61bp upstream from the cap site. These TRE-like sequences are active as AP-1 (Jun/Fos) binding sites and TREs, at least in vitro DNA binding and transfection analyses. From these results, it is inferred that the GST-P gene is controlled, at least in part by AP-1. However, AP-1 is not the only factor activating GPE1, because the expression of Jun and Fos do not always correlate with GST-P expression. Recently, the consense Maf recognition element (MARE) sequence was identified and is similar to the TRE sequence. Since the TRE sequence of the proximal region of the GST-P gene is internal to a MARE, Maf too might regulate GST-P expression. Sakai found that peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ ) can interact the Jun, not Maf, and inhibit the activation of GST-P expression<sup>[9-13]</sup>.

## REFERENCES

- 1 Rushmore TH, Pickertt CB. Glutathione S-transferases, structure, regulation and therapeutic implications. *J Biol Chem*, 1993;268(16):11475 - 11478
- 2 Satio K. Glutathione S-transferases and hepatocarcinogenesis. *Jpn J Cancer Res*, 1988;79(5):556 - 572
- 3 Solt DB, Farber E. New principle for the analysis of chemical carcinogenesis. *Nature*, 1976;263(6):702 - 703
- 4 Inst Lab Animal Resources. Histologic typing of liver tumors of the rat. *JNCL*, 1980;64(1):179 - 190
- 5 Rutenberg AM. Histochemical and ultrastructural demonstration of  $\gamma$ -GT activity. *J Histochem Cytochem*, 1969;17(3):517 - 519
- 6 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989:16 - 34
- 7 Su HC. In situ hybridization. Beijing: China Science and Technology Press, 1994:59-90
- 8 Ewen SW, Melvin WT, Burke MD. The expression of cytochrome P-450, epoxide hydrolase, and glutathione S-transferase in hepatocellular carcinoma. *Cancer*, 1993;71(1):36 - 43
- 9 Morimura S, Suzuki T, Hoshi S, Yuki A, Nomura K, Kitegawa T *et al*. Transactivation of glutathione transferase P gene during chemical hepatocarcinogenesis of the rat. *Proc Natl Acad Sci USA*, 1993;90(3):2065-2068
- 10 Suzuki T, Imagawa M, Hirabayashi M, Yuki A, Histatake K, Nomura K *et al*. Identification of an enhancer responsible for tumor marker gene expression by means of transgenic rats. *Cancer Res*, 1995;55(6):2651 - 2655
- 11 Sakai M, Hibiya YM, Nishizawa M, Nishi S. Suppression of rat glutathione transferase P expression by peroxisome proliferators: interaction between Jun and peroxisome proliferator-activated receptor  $\alpha$ . *Cancer Res*, 1995; 55 (11): 5370-5376
- 12 Sakai M, Muramatsu M, Nishi S. Suppression of glutathione transferase P expression by glucocorticoid. *Biochem Biophys Res Commun*, 1992; 187 (9): 976-983
- 13 Kataoka K, Noda M, Nishizawa M. Maf nuclear oncoprotein recognizes sequences related to an AP-1 site forms heterodimers with both Fos and Jun. *Mol Cell Biol*, 1994;14(4):700-712

# Cloning of 3H11 mAb variable region gene and expression of 3H11 human-mouse chimeric light chain \*

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**Subject headings** gene, expression; stomach neoplasms; antibodies, monoclonal; chimeric antibody

## Abstract

**AIM** To clone mouse anti-human gastric cancer mAb (3H11) variable genes and to construct 3H11 human-mouse chimeric antibody.

**METHODS** The entire VH and VL genes of anti-gastric cancer mAb 3H11 were cloned by RT-PCR method from 3H11 hybridoma cells, using 5' primers for leader sequences. The 3H11 VL gene was then inserted into human-mouse chimeric light chain expression vector and transfected into murine Sp2/0 myeloma cells.

**RESULTS** DNA sequence analysis indicated that the cloned genes included the whole leader sequences and the mature Ig variable region encoding sequences. After gene transfection, transient expression of chimeric light chain protein was detected.

**CONCLUSION** DNA sequences and transient expression indicated that the cloned gene was functional. This work laid basis for constructing 3H11 human-mouse chimeric antibody in the future.

## INTRODUCTION

The mouse mAb 3H11 was raised against human gastric cancer cells<sup>[1,2]</sup>. But 3H11 mAbs were derived from mouse hybridoma and their inherent immunogenicity in patients precluded its long-term use. In an attempt to circumvent this problem, chimeric antibodies in which the antigen-specific variable (V) regions of the mouse antibodies were joined to the constant (C) regions of human antibodies. These molecules should be much less immunogenic and hence be more suitable for application. In order to obtain the correct VL and VH genes, we designed 5' primers according to the leader sequences and cloned the entire VL and VH genes by RT-PCR method. The transient expression of chimeric light chain protein indicated that the cloned gene was functional in expression.

## MATERIALS AND METHODS

### Materials

Mouse 3H11 mAb hybridoma was provided by Biochemistry Department of Beijing Institute of Cancer Research. Chimeric light chain expression vector pAG4622 was a gift from S.L. Morrison (University of California, Los Angeles);  $\gamma$ -<sup>32</sup>P-ATP was from Beijing Ya Hui Ltd. Fmol DNA sequencing system, pGEM-T vector system, Tag enzyme and restriction enzymes were purchased from Promega. Lopofectin was from Gibco BRL.

### Design of PCR primers

The 5' primers for amplification of mouse V genes were synthesized according to Coloma *et al*<sup>[3]</sup>. An extra VH5' primer was added according to newly published sequences data<sup>[4]</sup> (VHL4 in Table 1). The 5' primers were designed to hybridize to partially conserved sequences in the leader regions of VH and VL. The 3' primer for VL genes was located at V-C junction of VL. The 3' primer for VH genes was located at the boundary of VH and CH1, which was effective for IgG, IgG2a, IgG2b and IgG3 subclass. Restriction sites were incorporated into the primers as shown in Table 1 (R = A/G S = C/G Y = C/T M = C/A K = T/G H = A/C/T D = G/T/A).

### Cloning of VL and VH genes

Total RNA was isolated from 107 cells of 3H11 hybridoma cells using Trizol reagent from Gibco BRL. The VL and VH genes were amplified by RT-

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\*Supported by the National 863 project of China, No. 863-102-09-01.

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Received 1997-04-14 Revised 1997-09-20

PCR method, purified by agarose gel electrophoresis and cloned with pGEM-1 system.

### DNA sequencing

The VL and VH genes were sequenced by dideoxy-mediated chain-termination method, using fmol DNA sequencing system (Promega).

**Table 1 PCR primers for cloning variable genes of rearranged immunoglobulin light and heavy chains**

5'Primers for VL Signal peptide	
LL1:	GGGGATATCCACCATGGAGACAGACACTCCTGCTAT
LL2:	GGGGATATCCACCATGGATTTTCAAGTGCAGATTTTCAG
LL3:	GGGGATATCCACCATGGAGWCACAKWCTCAGGTCTTTRTA
LL4:	GGGGATATCCACCATGKCCCCCWRCTCAGTYCTKGT
LL5:	GGGGATATCCACCATGAAGTTGCCTGTTAGGCTGTTG
3'Primer for VK spanning CK and JK1,2&4	
MVK:	GGATACAGTTGGTGCAGTCCGACTTACGTTTKATTTCCARCTT
5'Primers for VH signal peptide	
VHL1:	GGGGATATCCACCATGGRATGSAGCTGKGTMATSTCTT
VHL2:	GGGGATATCCACCATGRACCTCGGGYTGAGCTKGGTTTT
VHL3:	GGGGATATCCACCATGGCTGTCTTGGGGCTGCTCTTCT
VHL4:	GGGGATATCCACCATGATRGTTTTRAGTCTTYTGTRCCTG
3'Primer for VH spanning CH1 and JH	
MVH:	GACHGATGGGGSTGYGTGCTAGCTGNRGAGACDGTGA

### Construction of chimeric light chain expression vector

The pGEM-T vector containing 3H11 VL gene was digested with EcoR V and SalI. The 3H11 VL gene was purified by electrophoresis and electroelution and cloned into expression vector pAG4622. Transfection of DNA into Sp2/0 cells was accomplished by two methods: Lipofectin procedure: 20µg plasmid DNA was mixed with 40µl lipofectin and left at room temperature for 15 minutes. The mixture was added into  $1.6 \times 10^7$  Sp2/0 cells in 2ml serum-free culture medium drop by drop. The cells were then cultured at 37°C in a CO<sub>2</sub> incubator for 20 hours and 2ml RPMI1640 containing 20% fetal bovin serum was added. After further 72 hours incubation, the supernatant was collected.

Electroporation<sup>[3]</sup>: 20µg PvuII linearized plasmid DNA was added into  $1.6 \times 10^7$  Sp2/0 cells. After 10 minutes in ice bath, the cells were electroporated at 960µF, 2kV/cm by GENE PULSER (Bio-RAD). After another 10 minutes in ice bath, the cells were transferred into culture flask that contained 10 ml RPMI1640 containing 20% fetal bovin serum. The cells were cultured at 37°C in a CO<sub>2</sub> incubator for 72 hours and the supernatant was collected.

### Detection of human-mouse chimeric light chain

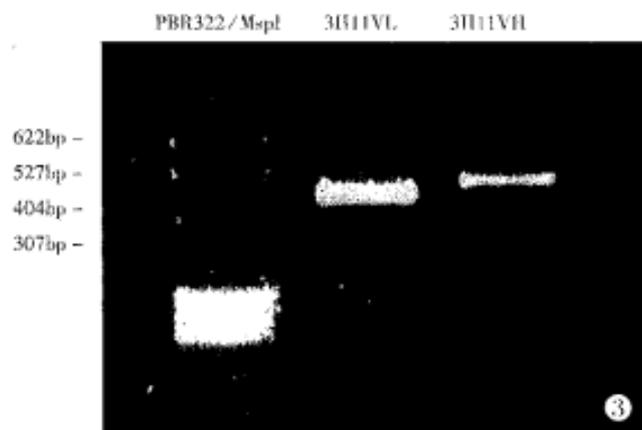
The expression of chimeric light chain was determined by ELISA. Goat anti-human IgG Fab (Sigma) at 2.3 mg/L in 0.05M borate buffer, was absorbed overnight in a 96-well plate. Either a

control or supernatant samples was added and incubated for 1h. This was followed by a secondary antibody coupled to horseradish peroxidase (HRP) (Sigma) and developed with OPD (0.5 g/L) (Sigma) and absorbance at 492nm. Between each addition, the plate was washed with phosphate-buffered saline with 0.05% Tween. The positive control was normal human blood serum, and the negative control was the supernatant of Sp2/0.

## RESULTS

### Cloning of 3H11 VL and VH genes

Total RNA was isolated from 3H11 cells and covered to cDNA. PCR amplification of VL and VH genes were done separately with sets of primer pairs. Agarose gelelectrophoresis showed that 3H11 VL gene was amplified with LL2 and VH gene with VHL1 (Figure 1)



**Figure 1** Amplification of variable genes of immunoglobulin light and heavy chains with PCR. The PCR products were resolved on 1.2% agarose gel.

### Sequencing of 3H11 VL and VH genes

The purified VL and VH PCR products were cloned into pGEM-T vector and sequenced with fmol DNA sequencing system. Comparison of the sequences with published antibody variable region data<sup>[4]</sup> indicated that the 3H11 VL gene contained a leader sequence of 57 nucleotides encoding leader peptide of 19 amino acid residues, while the 3H11 VH gene had a 66 bases long leader sequences that encoded a 22 residues leader peptide (Figure 2). The sequences of the mature VL and VH regions were the same as what we cloned previously using primers for framework region<sup>[5]</sup>.

### Construction of chimeric light chain expression vector

The 3H11 VL fragments in pGEM-T were digested with EcoR V and SalI and ligated into pAG4622 vector (Figure 3). The resulting expression vector pAG4622-3H11 was verified by restriction enzyme analysis.

A.  
-66-

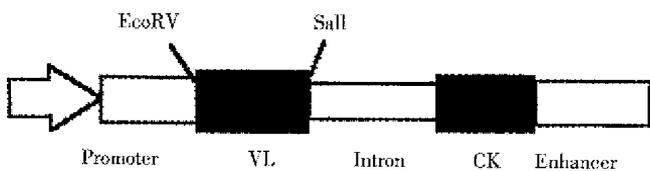
A T G G A T  
M D

-60-TTT CAA GTG CAG ATT TTC AGC TTG CTG CTA ATC AGT GTC ACA GTC ATA GTG TCT AAT GGA  
F Q V Q I F S L L L I S V T V I V S N G  
1-CAA ATT GTA CTC ACC CAG TCT CCA ACC ACC ATG GCT GCA TCT CCC GGG GAG AAG ATC ACT  
Q I V L T Q S P T<sub>CDR1</sub> T M A A S P G E K I T  
61-ATC ACC TGC AGT GCC AGC TCA AGT ATA ACT TCC AAT TAC TTG CAT TGG TAT CAA CAG AGG  
I T C S A S S S I T S N Y<sub>CDR2</sub> L H W Y Q Q R  
121-CCA GGA TTC TCC CCT AAA CTC TTG ATT TAT AGG ACA TCC AAT CTG GCT TCT GGA GTC CCA  
P G F S P K L L I Y R T S N L A S G V P  
181-GTT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATT GGC ACC ATG GAG  
V R F S G S G S G T S Y S L<sub>CDR3</sub> T I G T M E  
241-GCT GAA GAT GTT GCC ACT TAC TAC TGC CAG CAG GGT AAT TAT TTA TCA CGC ACG TTC GGA  
A E D V A T Y Y C Q Q G N Y L S R T F G  
301-GGG GGG ACC AAG CTG GAA ATA AAA  
G G T K L E I K

B.

-57- A T G G A A T G G A G C T G T G T C A T G C C C T T C A T C C T C T C A G G A A C T G C A G G T G T C A C T C C  
M E W S C V M P F I L S G T A T V H S  
1-CAG GTT CAG CTG TGG CAG TCT GGA GCT GAG CTG GCG AGG CCC GGG GCT TCA GTG AAG CTG  
Q V Q L W Q S G A E L A R P G A S V K L  
61-TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT GAC TAC TAT ATA AAC TGG GTG AAA CAG AGG  
S C K A S G Y T F T D Y Y I N W V K Q R  
121-ACT GGA CAG GGC CTT GAG TGG ATT GGA GAG ATT TAT CCT GGA AGT GGT AAT ACT TAC TAC  
T G Q G L E W I G E I Y P G S G N T Y Y  
181-1 AAT GAG AAT TTT AAG GGC AAG GCT ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC  
N E N F K G K A T L T A D K S S S T A Y  
241-ATG CAG CTC ATT AGC CTG ACA TCT GAG GAC TCT GCA GTC TGT TTC TGT GCA AGA TAC TCT  
M Q L I S L T S E D S A V C F C A R Y S  
301-GGT TAC GAC GGA TAT TAC TAT GCT ATG GAC TAT TGG GGT CAA GGA ACC TCA GTC ACC GTC  
G Y D G Y Y Y A M D Y W G Q G T S V T V  
361-TCC TCA  
S S

**Figure 2** The nucleotide and amino acid sequences of 3H11 mAb VL(a) and VH(b) genes. Underlined sequences are CDR regions. Numbering according to mature protein sequences.



**Figure 3** Human-mouse chimeric light chain expression vector pAG4622.

**Table 2** Expression of chimeric light chain in transfectants determined by ELISA

ELISA system	Supernatant			Human Ig	Mouse Ig
	3H11	Sp2/0	Transfectant		
Anti-human	0.169	0.112	1.214	1.660	0.122
Anti-mouse	1.564	0.147	0.128	0.102	1.538

- a. Anti-human Fab and HRP-anti-human Fab Ab were used as catcher and second Ab.
- b. Anti-mouse Fab and HRP-anti-mouse Fab Ab were used as catcher and second Ab.
- c. ABS492

**Expression of chimeric 3H11 light chain**

The expression vector pAG4622-3H11 was transfected into murine myeloma Sp2/0 cells by lipofectin or electroporation. Culture supernatant was harvested after the transfected cells were incubated at 37°C for 72 hours. Expression of chimeric 3H11 K chain was tested by ELISA. As shown in Table 2, human Ck was expressed in the culture supernatant of transfected cells indicated that the cloned gene was functional.

**DISCUSSION**

Monocloned antibodies (mAbs) are well-characterized highly specific reagents, and widely applied *in vitro* into immunochemical characterization and quantitation of antigens. They are being used clinically for both diagnosis and therapy increasingly. Their *in vivo* application is limited because most available mAbs are derived from mouse hybridomas, and their inherent immunogenicity in patients precludes their long-term administration. In an attempt to circumvent this problem, chimeric antibodies have been produced. Immunoglobulin V gene used to be

obtained by genomic library which was tedious and time-consuming. Recent developments in PCR technology have greatly facilitated the cloning of variable region genes<sup>[6]</sup>. Two approaches have been used to design the 5' primers. In one approach, degenerated primers for the 5' end are designed to recognize relatively conserved sequences within the 5' end of framework 1. Because the primers are within the coding sequences of the mature antibody and the introduction of restriction sites, amino acid substitutions that change the Ag-binding specificity or the affinity of the cloned antibody may result<sup>[7]</sup>. An alternative approach takes advantage of the conservation of the leader sequences. Since the leader is not present in the mature antibody molecules, any sequence alternations introduced by the degenerated primers will not influence antibody function. The phenomenon occurred in our work, the deduced N-terminal sequence of mature 3H11 VH protein had 4 amino acid residue discrepancies between "leader primer" amplified VH gene in this work (QUQLWQS) and previous "framework 1 primer" amplified VH gene (LLELVQS). While in 3H11 VL gene there were 2 discrepant residues, QIVLT of authentic VL versus DIVMT from "framework 1 primer" amplified VL<sup>[8]</sup>. Indeed the changes had profound influence on the binding of engineered 3H11 Ab molecules to gastric cancer cells (manuscript in preparation). In order to maintain the original sequences of the C terminal of the V regions, Coloma *et al*<sup>[3]</sup> suggested that the V genes be first amplified with 3' primer located at constant regions. Then a second set of 3' primers were designed according to the authentic sequences, and the V genes were PCR amplified again. Since there were only 4J segments for murine VL and VH genes respectively, we designed 3' primers for VL and VH

genes that straddled the V-C junction and might at most cause one residue substitution in VL (Leu<sup>106</sup>→Ile<sup>106</sup> in JK5) or VH (Ser<sup>113</sup>→Ala<sup>113</sup>) while the labour of cloning was greatly reduced.

Due to the diversity of Ab leader sequences, it would be more difficult to amplify V genes from leader by PCR<sup>[3]</sup>. There were some reports about RT-PCR method by primers for leader sequences abroad, but there is no report yet in our country. The facts that we had successfully amplified the variable genes not only of 3H11 but also of CD3 (manuscript in preparation) indicated that the primers were effective.

The amplified 3H11 VL gene was cloned into chimeric light chain expression vector and transfected into SP2/0 cells. Transient expression analysis demonstrated that the VL gene was functional, thus laid the basis for constructing 3H11 human-mouse chimeric antibody.

## REFERENCES

- 1 Dong ZW, Wei SM, Zhang MY, Li ZF, Wan WH, Mou ZY *et al*. Application of the monoclonal antibody 3H11 against gastric cancer. *Chin J Cancer Biother*, 1995 Jun; 2(2):84 - 87
- 2 Xu GW, Zhang MY, Dong ZW. Radioimmunoguided surgery in gastric cancer using 131I labelled monoclonal antibody 3H11. *Seminars in Surgical Oncology*, 1994;10(2):88 - 92
- 3 Coloma MJ, Hastings A, Wims LA, Morrison SL. Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J Immunol Methods*, 1992;152(1):89-104
- 4 Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. *Sequences of proteins of immunological interest*, 5th edition. U.S. Department of Health and Human Service, Bethesda, 1991
- 5 Padlan EA. Anatomy of the antibody molecule. *Mole Immunol*, 1994;31(3): 169 - 174
- 6 Oi VT, Morrison SL. Chimeric antibodies. *Biotechniques*, 1986;4(3):214-219
- 7 Orlandi K, Gussow DH, Jones PT, Winter G. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc Natl Acad Sci USA*, 1989;86(10):3833 - 3837
- 8 Fu YX, Ma SL, Wang Y, Yuan YH, Dong ZW. Cloning and sequence analysis of variable genes of anti-gastric cancer mAb 3H11. *J Beijing Medical University*, 1994;26(5):345 - 347

# Relationship between different sources of drinking water, water quality improvement and gastric cancer mortality in Changle County<sup>\*</sup>

## -A retrospective-cohort study in high incidence area

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**Subject headings** stomach neoplasms/mortality; water supply; risk factors; cohort studies; incidence; retrospective studies

### Abstract

**AIM** To investigate the relationship between different sources of drinking water supply, water quality improvement and gastric cancer mortality rate in a high risk area.

**METHODS** A retrospective-cohort survey was carried out in all towns of this county to study the effect of different sources of drinking water supply and water quality improvement on gastric cancer mortality rate.

**RESULTS** The gastric cancer mortality rate among the population 124.05/105 drinking river water was obviously higher than that of drinking shallow well water (74.85/105) ( $P < 0.01$ ) according to the Zhanggang Town 16 years accumulated data. The same pattern was presented in 7 towns after balancing the confounders. The gastric cancer mortality rate of population drinking river water was 86.03/105, which was higher than those drinking shallow well water (62.03/105) and tap water (29.78/105) ( $P < 0.01$ ). When the drinking water switched from river and well water to tap water, the gastric cancer incidence decreased to 30.33/105 and 26.10/105, and the gastric cancer mortality decreased by 59% and 57% respectively.

**CONCLUSION** The quality of drinking water is one of the important factors of increased incidence of gastric cancer in Changle County, and water quality improvement has a beneficial effect, but the cause of high gastric cancer incidence may be multi-factorial in this area.

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\*Supported by "8-5" national major project, No. 95-914-01-10.  
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Received 1997-07-10

### INTRODUCTION

Changle County is located in the south-east beach area of Fujian Province with a population of over 600 thousand and has 17 towns. The standardized gastric cancer mortality rate ranged from 60.18/10<sup>5</sup> - 98.68/10<sup>5</sup> between 1971-1990. The highest standardized mortality rate in male was 161.20/10<sup>5</sup>, remaining at a high level for a long time<sup>[1]</sup>. It is one of the highest gastric cancer incidence area in China. The result of a retrospective cohort study of six towns in this county had been reported<sup>[2]</sup>, which indicated that the gastric cancer mortality rate is related to drinking water supply. The drinking water was surveyed between 1991 - 1992 throughout the county. The stratifying analysis results of the seven towns investigated are presented as follows.

### MATERIALS AND METHODS

#### *Reliability of data*

A resident retrospective survey of deaths from all causes has been conducted four times since 1973. Diagnosis of gastric cancer death made by hospitals at county level or above was 77.99%, 81.64%, 71.46%, and 81.79% respectively. Diagnosis of grades I and II were 75.60%, 78.58%, 88.58% and 97.48% (grade I: postmortem; grade II: pathology). The reliability and integrity of the survey and data all met the quality requirements set by the National Cancer Prevention and Treatment Bureau "Death Cause Survey Handbook" and the Ministry of Public Health "Resident Death Cause Survey".

#### *Type of drinking water sources*

Type of drinking water sources was divided into river water (partly including pond, stream), shallow well (partly including deep well and spring water) and tap water. For calculating the person-year observed, only the people who must use one type water source for over four years can get into the cohort. It had been defined before the survey was begun because the drinking water source may be changed during the resident's lifetime. The types of drinking water drunk by people with gastric cancer before death were checked at the same time.

**Data analysis and calculation**

SAS software was used to analyze the data of this survey. Chisquare test was done by Mentel-Haneszel method. Gathering analysis for equilibrium of food, smoking and drink factors was proceeded by Systat software.

**RESULT**

**Water type and gastric cancer mortality rate**

Gastric cancer mortality rate in Zhanggang Town was the highest in this county from 1973 - 1990. Sixteen years accumulated data were analyzed, which indicated that gastric cancer mortality rate of population drinking river water was significantly higher than that of the shallow well source ( $P < 0.01$ ) (Table 1).

**Table 1 Gastric cancer mortality of Zhanggang Town according to the type of water supply (1/10<sup>5</sup>) (1973 - 1990)**

Type of water source	Male	Female	Total
River Water	176.91 (141/79 699)*	57.29 (40/69 822)	121.05 (181/149 521)
Shallow well water	112.70 (320/267 979)	31.58 (74/234 356)	74.85 (276/502 335)
RR	1.57	1.81	1.62
$\chi^2$	19.88	9.49	28.79
P	<0.01	<0.01	<0.01

\*Number of gastric cancer death cases/person-year observed.

In view of the fact that there were different factors among the towns in this county, such as geographical environment and living habits. According to the recent information<sup>[3]</sup>, after controlling confounders of diet (mouldy grain, fresh vegetable, fish-sauce intake amount), smoking, drinking, etc., the different drinking water supply

and gastric cancer mortality rates for the seven towns are presented in Table 2. The results showed that gastric cancer mortality rate of population with river water supply is higher than that of the shallow well water and tap water supply ( $P < 0.01$ ).

**Table 2 Types of the drinking water supply and gastric cancer mortality rates for seven towns, Zhanggang (1/10<sup>5</sup>)**

Types of drinking water supply	Male	Female	Total
River water (1)	122.83 (107/87 113)	46.10 (37/80 263)	86.03 (144/167 376)
Shallow well (2)	85.02 (650/764 555)	36.06 (244/676 618)	62.03 (894/1 441 173)
Tap water (3)	51.31 (16/31181)	3.86 (1/25906)	29.78 (17/57087)
RR	2.39	11.94	2.89
$\chi^2$ (1:3)	10.617	8.618	18.005
P	<0.01	<0.01	<0.01
RR	1.66	9.34	2.08
$\chi^2$ (2:3)	3.673	6.524	8.872
P	>0.01	>0.05	>0.01
RR	1.44	1.28	1.39
$\chi^2$ (1:2)	12.158	1.686	13.017
P	<0.01	<0.05	<0.01

**Water quality improved and gastric cancer mortality rate**

Using population with persistent river and shallow well water supply as control group, the effect of the improved water quality on gastric cancer mortality rate was studied. The results showed that after switching river water or shallow water to tap water, the gastric cancer mortality rate presented a decreasing trend, especially in population that the river water was changed as summarized in Table 3.

**Table 3 Improved water and gastric cancer mortality rates for seven towns in Changle County (1/10<sup>5</sup>)**

	Male			Female			Total		
	Person-year observed	No. of deaths	Death rate	Person-year observed	No. of deaths	Death rate	Person-year observed	No. of deaths	Death rate
Persistent river water supply	37 484	43	114.72	36 856	12	32.56	74 340	55	73.98
River water changed to tap water	35 088	14	39.90	24 262	4	16.49	59 350	18	30.33
RR		0.35			0.51			0.41	
$\chi^2$		12.93			1.44			11.52	
P		0.0003 <sup>b</sup>			0.2295			0.0007 <sup>b</sup>	
Persistent well water supply	411 547	355	86.26	362 687	118	35.23	774 234	473	61.10
Well water changed to tap water	10 449	3	28.71	8 711	2	22.96	19 160	5	26.10
RR		0.33			0.71			0.43	
$\chi^2$		3.98			0.24			3.80	
P		0.0460 <sup>a</sup>			1.0000			0.0512 <sup>a</sup>	

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

## DISCUSSION

The relationship between different sources of drinking water and gastric cancer has been reported by several authors and comparatively consistent results were observed<sup>[4-7]</sup>. In the population with raw river water supply, the gastric cancer incidence was significantly higher than those with underground water supply. In our study, a retrospective-cohort of sixteen years accumulated data was analyzed. The result indicated that the type of drinking water was related to gastric cancer mortality. Even though the survey was extended to seven towns and under the condition that the confounders related to gastric cancer were balanced, the result showed the same pattern. In view of these results, the drinking water was thought to be one of important factors in the increased incidence rate of gastric cancer in this area.

According to the result of this investigation, altering drinking water has presented a significantly beneficial effect in decreasing gastric cancer rates. When the river water was changed to tap water, the rate decreased by 59% and 57% in case the well water was changed to tap water. Mutagenicity of different drinking water source has been compared in an earlier study which showed that the raw water had more mutagenic effects than the water purified in this area<sup>[8,9]</sup>. This result was identical with the results of epidemiological survey in this study. It is suggested that improving the drinking water quality

should be a preventive method for decreasing the gastric cancer incidence rate in this high risk area. This study was only referred to one aspect of this problem, that is 'drinking water and gastric cancer'. Even if that drinking water had been improved in this district, the gastric cancer mortality rate was still on the median level. So the etiology of gastric cancer may be attributed to multi-factors in this high incidence area<sup>[10-12]</sup>.

## REFERENCES

- 1 Chen JS. Analysis of gastric cancer death and prevalence trend in Changle County in 1973-1990. *J Fujian Med Coll*, 1992;26(5):116-118
- 2 Wang ZQ, Zhou TS, Chen W. Investigation of the drinking water and cancer mortality in Changle County. *J Fujian Med Coll*, 1991;25(4):354-356
- 3 Ye WM, Yi YN, Lin RT. Case-control study of fish sauce and gastric cancer. *Cancer*, 1995;15(2):157
- 4 Yang KY, Tu JT, Li HM. Exploring to factors of high gastric cancer incidence in geographic fault zone. *Cancer*, 1986;6(3):107-110
- 5 Yeh BF, Xu YC, Yu H. Retrospective-cohort study of the drinking water and cancer. *Chin J Epidemiol*, 1986;7(4):219-221
- 6 Chen K, Shen YZ, Shen GF. Study on incidence rate of some cancer in areas with difference in drinking water sources. *Chin J Public Health*, 1994; 12 (3): 146 -148
- 7 Miao YD. A study on the relation between the type of drinking water and cancer. *Cancer*, 1989;9(1):8 - 10
- 8 Wang ZQ, Ling YX, Lin RT. Study on drinking water, cancer mortality and mutagenicity of water organic extract substances. *China Comprehensive Med*, 1995;7:796-798
- 9 Chen H, Lin YC. A study on mutagenicity of source water for drinking in Changle. *Carcinogenesis. Teratogenesis. Mutagenesis*, 1995;7(2):87-90
- 10 Zhu HG. Health assessment of organic chemical contaminants in water. *China Environ*, 1987;7(4):67 - 73
- 11 Deng DJ, Zhang RF, Jin S. The salmonella mutagenicity of cancer high risk area. *Cancer*, 1989;9(6):251 - 252
- 12 Yi YN, Ye WM, Rou NX. Ecologic study of fish sauce and gastric cancer. *Cancer*, 1995;15(2):158

# Expression of somatostatin mRNA in various differentiated types of gastric carcinoma \*

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**Subject headings** stomach neoplasms; somatostatin; RNA, messenger; in situ hybridization; immunohistochemistry

## Abstract

**AIM** To investigate the expression of somatostatin mRNA in various differentiated types of gastric carcinoma.

**METHODS** By using in situ hybridization and immunohistochemical techniques, the expression of somatostatin mRNA and somatostatin immunoreactivity in the normal gastric mucosa, the poorly, moderately and well-differentiated gastric carcinomas, and various clinical stages of carcinoma were observed.

**RESULTS** In comparison with the normal gastric mucosa, the significantly increased expression of somatostatin mRNA positive cells was displayed in gastric carcinoma ( $t = 2.681$ ,  $P < 0.01$ ). The positive signal cells were distributed in a scattered form or aggregated as a mass or a cord, and the positive cells were more significantly enhanced in poorly differentiated carcinomas than those in well and moderately differentiated carcinomas ( $t = 2.962$ ,  $P < 0.01$ ). The somatostatin mRNA hybridization signals in stages III and IV of gastric carcinoma were significantly higher than those in stages I and II. The results of somatostatin immunoreactivity were consistent with those of in situ hybridization.

**CONCLUSION** The alteration of the expression of somatostatin mRNA was associated with the development of gastric carcinoma and may play an important role in the process of tumor differentiation.

## INTRODUCTION

The study of the effect of endocrine hormone on the tumor development and cellular localization of the hormone DNA, and mRNA at molecular level using in situ hybridization techniques has been considered as the target of the tumor endocrinal investigation. It has been known that somatostatin involves the cell division and differentiation. However, there has been no report on somatostatin mRNA expression and its effect on normal and gastric carcinoma tissues with in situ hybridization at molecular level. In this study, in situ hybridization with digoxigenin-labelled antisense RNA probe and immunohistochemical techniques were used to investigate the alteration of somatostatin gene transcription and the expression of the above-mentioned tissues in the normal and the various differentiated types of gastric carcinoma tissues.

## MATERIALS AND METHODS

### Materials

Fifteen normal gastric mucosa and 32 gastric carcinoma samples were obtained from the surgical operations in Henan Tumor Hospital. Before operation no patients had been treated with anti-tumor drugs. After surgery, the specimens were rinsed immediately in saline and fixed in freshly prepared 4% polyformaldehyde (PFA) for 1-4 hours at 4°C. Washed with 0.01 mol/L PBS, the specimens were immersed in autoclaved 30% sucrose overnight at 4°C. The frozen sections and paraffin-embedded sections were prepared and used for in situ hybridization and immunohistochemistry respectively. The frozen sections were incubated for 12 - 16 hours at 43°C, and then preserved at -20°C.

### Slide treatment

Washed with water, the slides were immersed in clean solution overnight. Washed with water and distilled water, the slides were roasted for 2h at 180°C. Coated with DEPC created gelatin, the slides were dried overnight at 37°C.

### Histopathological diagnosis and classification

The gastric carcinoma specimens were classified into poorly, moderately and well differentiated types, and four clinical stages (stages I - IV) according to the standard of the clinical classification for gastric carcinoma set up by the National Gastric Carcinoma Association.

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\*Project supported by the National Natural Science Foundation of China, No. 39170440 and Natural Science Foundation of Henan Province.

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Received 1997-06-10

### ***In situ hybridization***

The frozen sections of normal and gastric carcinoma tissues were treated with 0.1 mol/L PBS for 5 - 10 minutes, 0.1 mol/L glycine/PBS for 5 minutes, 0.3% Triton X-100/PBS for 15 minutes. Digested with 1 mg/L proteinase K (England) in 0.1 mol/L Tris-HCl, pH 8.0, 50 mmol/L EDTA buffer for 20 minutes at 37°C, the specimens were postfixed with 4% PFA for 5 minutes. Washed with 0.1 mol/L PBS, the specimens were immersed in freshly prepared 0.25% acetic anhydride in 0.1 mol/L triethanolamine for 10 minutes to reduce the background. Washed with 2×SSC for 10 minutes, each slide was covered with 10 µl - 30 µl hybridization solution (prehybridization procedure was omitted) containing 5% formamide, and 10% dextran sulfate (Fluka), Digoxigenin-labelled SOM antisense RNA probe, 10 mmol/L Tris-HCl (pH 8.0), 0.03 mol/L NaCl, 1 mmol/L EDTA, 250 mg/L salmon sperm DNA, 10 mmol/L DTT, 0.25% PVP, 0.35% BSA, 0.24% Ficoll 400. The sections were covered with 20 mm × 20 mm Parafilm (Greenwich CT), and hybridization was performed at 43°C for 16-20 hours in a moisture chamber. After hybridization, the specimens were washed with 2×SSC for 30 minutes. Following the removal of parafilm, the specimens were digested with 20mg/L RNase A for 30 minutes at 37°C to eliminate the unhybridized probe. Washed 3 times with 0.05mol/L PBS for 5 minutes, the specimens were incubated with Anti-Dig-AP (Boehringer Mannheim) 1:1000 diluted in 1% BSA, 0.4% Triton X-100/0.05mol/L PBS for 12-16 hours at 4°C. Washed 4 times with 0.01mol/L PBS for 10 minutes, the specimens were stained with freshly prepared 400mg/L NBT/20mg/L BCIP for 3-16hours. The reaction was stopped by washing with 20mmol/L EDTA for 30 minutes.

### ***Control experiment***

The sections were pretreated with RNase A (0.05 g/L) for 30 minutes at 37°C. All procedures were the same as above except for no probe in hybridizationsolution.

### ***Immunohistochemistry***

Immunohistochemistry was used according to the PAP method. The samples were incubated with normal goat serum 1:10 diluted with 0.3% Triton X-100 for 30 minutes. Then, the somatostatin antibody (DAKO) (diluted 1 : 100 in PBS) was added and incubated for 24 hours at 37°C. Goat anti-rabbit IgG (diluted 1:25 in PBS) was added and incubated for 1 hour at 37°C. Then, PAP complex (diluted 1:100 in PBS) was added to the sections and incubated for 1 hour at 37°C. After that, the samples were colored in freshly prepared 0.5 g/L DAB for 10 - 15 minutes at room temperature.

### ***Control experiment***

Normal rabbit serum was substituted for goat anti-

rabbit IgG and PBS was substituted for PAP complex.

### ***Statistical treatment***

The data were statistically analysed with *t* test.

## **RESULTS**

### ***Intracellular distribution of in situ hybridization positive signal***

Distinct hybridization signal was detected with digoxigenin-labelled antisense RNA probe in both the normal tissues and in the gastric carcinoma tissues. The hybridization signal was blue-violet, and located in cytoplasm; and there was no signal in the nucleus. The background of the hybridization specimen was colorless.

### ***Results of in situ hybridization in normal gastric mucosa***

The positive signal cells were distributed in a scattered form. The hybridization signal could be seen in 13 of the 15 normal gastric mucosa specimens. However, the hybridization positive cells were less (Table 1), mainly located in the middle and the lower portion of the mucosa, and appeared in round or irregular shape (Figure 1), and some cells had a slender process.

**Table 1 In situ hybridization results in normal and various differentiated types of gastric carcinoma (x±s)**

Histological types	Cases	Positive cases	Positive cells/mm <sup>2</sup>
Normal gastric mucosa	15	13	7.3±5.2 <sup>b</sup>
Poorly differentiated carcinoma	9	9	201.3±41.3 <sup>d</sup>
Ring cell carcinoma	3	3	212.6±58.3
Moderately differentiated carcinoma	9	7	40.0±18.4 <sup>f</sup>
Well-differentiated carcinoma	11	9	32.4±15.4 <sup>b</sup>

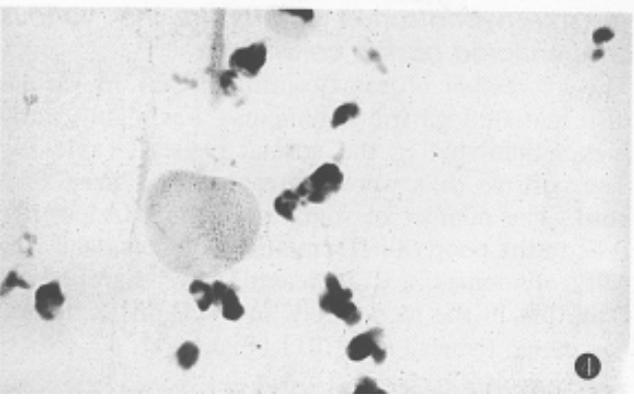
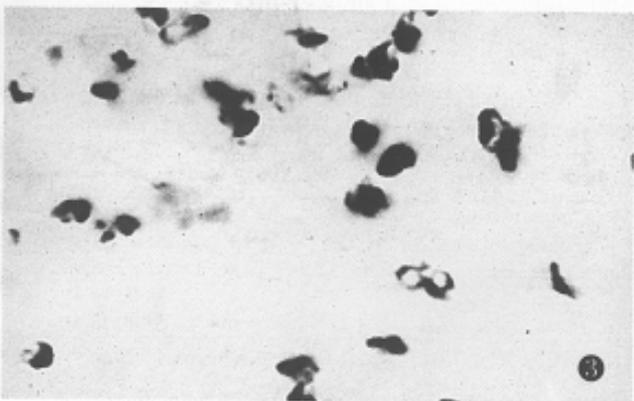
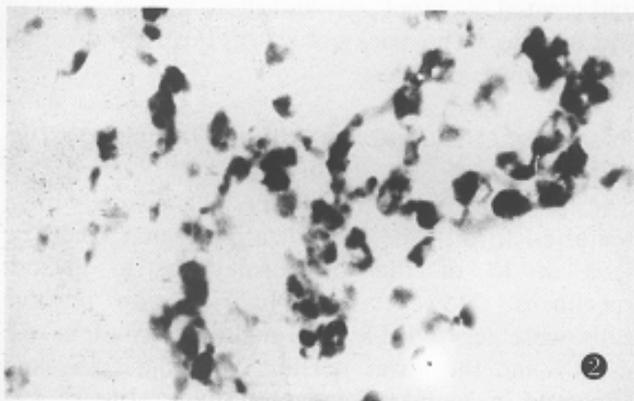
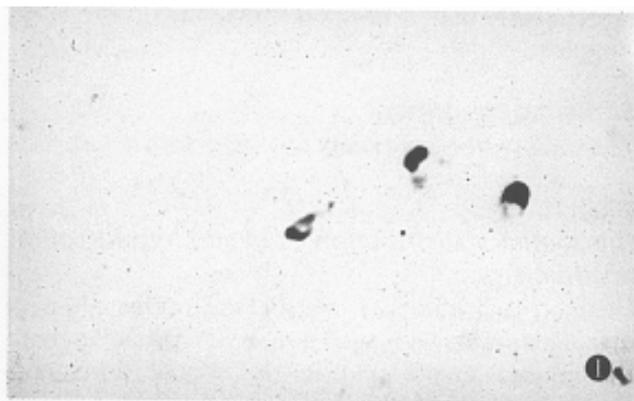
<sup>b</sup>*P*<0.01, <sup>d</sup>*P*<0.01, <sup>f</sup>*P*<0.01 vs carcinoma.

### ***In situ hybridization results in the various differentiated gastric carcinoma***

The expression of somatostatin mRNA in various differentiated gastric carcinomas was significantly higher than that in the normal tissues (Table 1). The positive cells were aggregated as a mass or a cord. The number of somatostatin mRNA positive cells in the poorly differentiated carcinoma and ring cell carcinomas was increased more significantly than that in the moderately and well differentiated carcinoma tissues (*P*<0.01) (Figures 2-4).

### ***Relationship between the in situ hybridization signals of somatostatin mRNA and the clinical stage of gastric carcinoma***

The in situ hybridization signals detected with somatostatin antisense RNA probe in various clinical stages of gastric carcinoma tissues are displayed in Table 2.



**Figure 1** Somatostatin mRNA in normal gastric mucosa.  $\times 400$   
**Figure 2** Somatostatin mRNA in poorly differentiated gastric carcinoma.  $\times 400$   
**Figure 3** Somatostatin mRNA in moderately differentiated gastric carcinoma.  $\times 400$   
**Figure 4** Somatostatin mRNA in well differentiated gastric carcinoma.  $\times 400$

**The results of somatostatin immunohistochemistry**

The somatostatin positive immunoreactivity appeared as brownish colored granules and was located in the cytoplasm. The positive cells in the poorly differentiated carcinoma were significantly higher than those in the moderately and the well differentiated carcinomas. The results of somatostatin immunoreactivity was consistent with those by in situ hybridization (Table 3).

**Table 2 Relationship between the in situ hybridization signals of somatostatin mRNA and clinical stages of gastric carcinoma**

Clinical stage	Cases	Positive cases	Hybridization positive cells/mm <sup>2</sup>
I	5	2	39.2 $\pm$ 14.1
II	10	6	83.7 $\pm$ 36.2 <sup>ab</sup>
III	14	11	130.5 $\pm$ 70.7
IV	3	1	133.5 $\pm$ 64.3 <sup>d</sup>

<sup>a</sup>P<0.05 vs I, <sup>b</sup>P<0.01 vs III; <sup>d</sup>P<0.01 vs II.

**Table 3 The correlation analysis of the expression between somatostatin mRNA and somatostatin immunoreactivity**

	Normal gastric mucosa	Gastric carcinoma		
		Poorly differentiated	Moderately differentiated	Well differentiated
r	0.874	0.725	0.836	0.836
P	<0.001	<0.001	<0.01	<0.01

**Control experiment**

Both in situ hybridization and immunohistochemistry were negative.

**DISCUSSION**

Up to date, there have been many reports about the relationship between gastroenteric hormones and endocrinal tumors in the digestive tract<sup>[1,2]</sup>. However, there have been few reports about the research of endocrinal regulation of gastroenteric non-endocrinal tumor and its mechanism with in situ hybridization. Chen<sup>[3]</sup> and Ooi<sup>[4]</sup> found endocrine granules or hormones in the common gastroenteric carcinoma cells and in the tumor endocrine cells of the digestive tract with ultrastructural and immunohistochemical techniques. However, immunohistochemical techniques could only show the gastroenteric hormone preserved in a certain kind of tumor cells, but could neither reveal whether the hormone came from protein synthesis by itself or was the exogenous substance, nor distinguish if amino acid composition was similar, such as  $\alpha$  or  $\beta$  cGRP<sup>[5]</sup> by proteins or polypeptides. In situ hybridization was not only the unique effective method to identify the result of immunohistochemistry, but also an index to watch

the hormone synthesis process, since the alteration of mRNA transcription rate was much faster than that of the translation production<sup>[6]</sup>. In this study, the in situ hybridization with digoxigenin-labelled somatostatin antisense RNA probe was first used to study the human normal and gastric carcinoma tissues. The results displayed intense signals with clear background and no nonspecific reaction, which indicated the satisfactory results of cellular localization at molecular level for somatostatin mRNA hybridization signal positive cells in normal gastric mucosa and the various differentiated gastric carcinoma tissues.

In the previous studies, it was believed that somatostatin inhibited cell growth<sup>[7]</sup>. However, the recent studies demonstrated that somatostatin could stimulate tumor cell growth<sup>[8,9]</sup>. Although the exact mechanism is still unclear, at least two possible sorts of explanation can be considered. The first is the inhibition of trophic action of gastrin on mucosal cells caused by exogenous and endogenous gastrin, and the second is the effect of somatostatin on cell proliferation. Our study on various clinical stages of gastric carcinoma showed that the somatostatin mRNA hybridization signals in stages III and IV were significantly higher than those in stages IV and II. Because there was a high incidence of migration to lymph nodes and peripheral organs in stage III and IV, it was indicated that the over expression of somatostatin was significantly correlated with the development and prognosis of gastric carcinoma.

Generally, the poorer the tumor cell differentiation, the worse the prognosis. In this study, the hybridization positive cells were more

significantly enhanced in poorly differentiated gastric carcinoma than those in well and moderately differentiated gastric carcinomas ( $P < 0.01$ ). The results indicated that, to some extent, somatostatin mRNA and somatostatin were associated with the differentiation induction of gastric carcinoma at the level of transcription and translation. The abnormal regulation at transcription level may stop the cell differentiation at a certain stage and the differentiated features of carcinoma cells occur. It is worth considering that the alteration of the expression of somatostatin mRNA may play an important role in the process of tumor differentiation.

## REFERENCES

- 1 Rindi G, Luinetti O, Cornaggia M. Three subtypes of gastric argyrophil carcinoma and the gastric neuroendocrine carcinoma: a clinicopathologic study. *Gastroenterology*, 1993;104(4):994 - 1006
- 2 Walker FM, Lehy T, Bernuan DG. Detection of gastrin mRNA in human antral mucosa and digestive endocrine tumors by in situ hybridization. *J Histotechnol*, 1992;40(9):1363-1372
- 3 Chen BF, Yin H. Neuroendocrine type of gastric carcinoma. Immunohistochemical and electron microscopic studies of 100 cases. *Chin Med J (Engl)*, 1990;103(7):561-564
- 4 Ooi A, Mai M, Ogino T. Endocrine differentiation of gastric adenocarcinoma. *Cancer*, 1988;62(6):1096-1104
- 5 Noguchi K, Senba E, Morita Y.  $\alpha$ -CGRP and  $\beta$ -CGRP mRNA are differentially regulated in the rat spinal cord and dorsal root ganglion. *Mol Brain Res*, 1990;7(4):299-304
- 6 Hiroshi K, Piers CE, Masaya T. Recent progress in the use of technique of non-radioactive in situ hybridization histochemistry: new tools for molecular neurobiology. *Neuroscience Res*, 1990;9(1):1-21
- 7 Kraenzlin ME. Long-term treatment of VIPoma with somatostatin analogue resulting in remission of symptoms and possible shrinkage of metastasis. *Gastroenterology*, 1985,88(1):185 - 187
- 8 Koper JW. Somatostatin inhibits the activity of adenylate cyclase in cultured human meningioma cells and stimulates their growth. *J Clin Endocrinol Metab*, 1992;74(3):543-547
- 9 Moyer MP, Armstrong A, Aust JB. Effects of gastrin glutamine and somatostatin on the in vitro growth of normal and malignant human gastric mucosal cells. *Arch Surg*, 1986;121(3):285 - 289

# Arylsulfatase, $\beta$ -galactosidase and lysozyme in gastric cancer cells and its relationship to invasion\*

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**Subject headings** stomach neoplasms; hydrolases; proteoglycans histochemistry; neoplasm invasiveness; arylsulfatase; beta-galactosidase; muramidase

## Abstract

**AIM** To study the distribution of arylsulfatase,  $\beta$ -galactosidase and lysozyme in gastric cancer cells, and its relationship to differentiation and invasion of gastric cancer cells.

**METHODS** Histochemical, immunohistochemical and ruthenium red (RR) electrocytochemical technique for three types of hydrolases and proteoglycans in pericancerous matrix in 33 cases of gastric cancer were observed under light and electron microscopy.

**RESULTS** The expression intensities of arylsulfatase,  $\beta$ -galactosidase and lysozyme in mucinous cell carcinomas were more intensive than those in well-differentiated and poorly-differentiated adenocarcinomas ( $P < 0.05 - 0.01$ ). The fibrous tissues smooth muscle and proteoglycans close to the cancer cells were degraded. They were found in the region far from the cancer cells. Expression of three enzymes mentioned above was low in adenocarcinoma cells, and fibrous tissues and RR granules were present and intact near the well-differentiated and poorly-differentiated adenocarcinoma cells.

**CONCLUSION** Mucinous cell carcinoma may release various hydrolases into extracellular matrix, inducing degradation of pericancerous matrix and facilitating cancer cell invasion and metastasis.

## INTRODUCTION

Tumor invasion and metastasis is a highly complex process. The mechanism of tumor invasion and metastasis is still not clear. In this paper the content and distribution of arylsulfatase  $\beta$ -galactosidase and lysozyme in three histological types of gastric cancer cells were studied by means of histochemical and immunohistochemical stains and the proteoglycans in pericancerous matrix by ruthenium red electrocytochemical stains respectively. The relationship between hydrolases and differentiation as well as invasion of gastric cancer cells were discussed.

## MATERIALS AND METHODS

Surgically resected specimens of 33 cases of gastric cancer were studied. Histologically there were 14 cases of mucinous cell carcinoma, 9 cases of well-differentiated adenocarcinoma, and 10 cases of poorly-differentiated adenocarcinoma. Each specimen was treated as follows: ① The fresh tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5  $\mu$ m section, and were stained by HE and lysozyme PAP immunohistochemical staining<sup>[1]</sup> (lysozyme antibody from Dako). ② Fresh tissue were fixed in 3% glutaraldehyde solution for 18 - 20 hours, and cut in a cryostat into 8  $\mu$ m sections for arylsulfatase and  $\beta$ -galactosidase histochemical staining<sup>[2,3]</sup>. Substrates were p-nitrocatecholsulfate dipotassium salt and 5-bromo-4-chloro-3-indoxyl- $\beta$ -galactoside (Sigma). ③ 0.1 mm<sup>3</sup> tissues from 20 of 33 cases of gastric cancer (including 10 cases of mucinous cell carcinoma, 5 cases of well-differentiated and 5 cases of poorly-differentiated adenocarcinoma) were fixed in 2% ruthenium red (Merck) mixed with 4% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4). The proteoglycans of the extra-cellular matrix and the ultrastructures of cancer cells were observed under a Hitachi-600 electron microscope<sup>[4]</sup>.

## Grading of staining patterns

The staining patterns in tissue sections were scored according to the relative intensity and extent of positive staining. Weak positive (+): the number of positive cells less than 1/3 of the total cells, moderate reaction (++) : 1/3 to 2/3 of the total, strong positive (+++): more than 2/3 of the total.

## Statistics

The same enzyme staining grades from the tissues were compared by rank sum test.

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**Received** 1997-09-10

## RESULTS

### *Hematoxylin and eosin staining*

In 14 cases of mucinous carcinoma, the cancer cells were diffusely distributed, some secreting a lot of mucin into extra-cellular matrix forming the mucin pool. There were no fibrous tissues and smooth muscle and lymphocyte infiltration in the pericancerous tissues. Nine cases of well-differentiated adenocarcinoma (including 2 cases of papillary carcinoma) displayed a tubular structure surrounded by fibrous tissue proliferation or smooth muscle and lymphocyte infiltration. The cancer cells of 10 poorly-differentiated adenocarcinomas arranged in solid trabeculae of incomplete gland pattern accompanied by fibrous tissues and lymphocytes.

### *Histochemical staining of arylsulfatase*

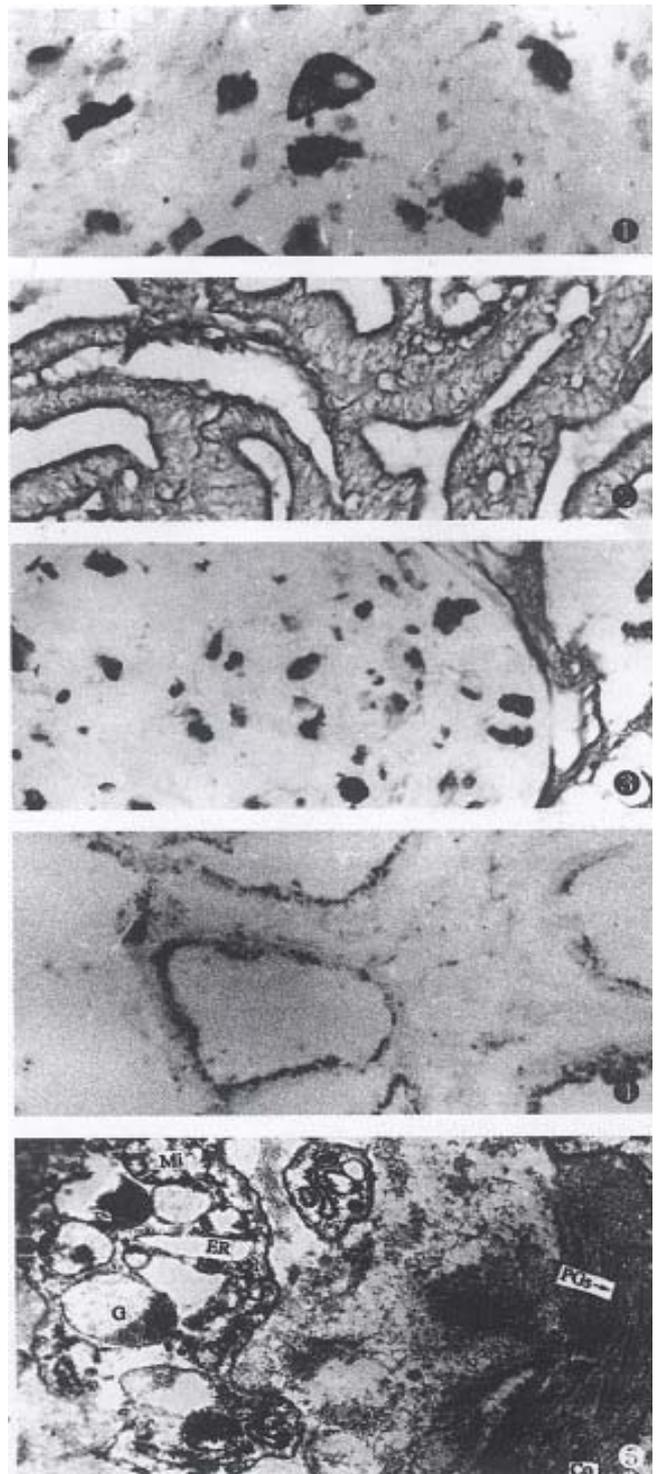
The positive reaction was dark-brown or yellow-brown in color. In cancer cells of mucinous carcinoma, the staining reaction was distributed in the cytoplasm and varied with the amount of cytoplasm, the more cytoplasm, the more staining intensity (Figure 1). There were light yellow-brown fine granules in the mucin pool. In well-differentiated adenocarcinomas the positive reaction was mostly located at the gland luminal border (Figure 2). In poorly-differentiated adenocarcinomas, most of cancer cells were negative, but were positive in the cytoplasm.

### *$\beta$ -galactosidase histochemical staining*

The enzyme-active sites were stained blue or turquoise. The reaction was most intense in mucinous cell carcinomas. The size of indigo particles was dependent on the cytoplasm, the particle was largest in abundant cytoplasm cells or signet-ring cells (Figure 3). Fine light blue granules were seen in the mucin-pool. In well-differentiated adenocarcinoma, the fine blue granules were mostly located in the apical cytoplasm of cancerous gland (Figure 4). In poorly-differentiated adenocarcinoma, some cancer cells were positive, and others were negative in reaction.

### *Lysozyme immunohistochemical staining*

The positive reaction was yellow-brown. In mucinous cell carcinoma the positive product was diffusely distributed in the cytoplasm of most cancer cells, in signet-ring cell, the positive reaction was located on one side of the cytoplasm. In well-differentiated adenocarcinoma, positive product was located at the luminal side or in the cytoplasm in some cells, a portion of cancer cells were negative in reaction. In poorly-differentiated adenocarcinoma, most cancer cells were negative. The content and distribution of three type enzymes in gastric cancer cells are shown in Table 1.



**Figure 1** Mucinous cell carcinoma. The positive product of arylsulphatase is diffusely distributed in the cytoplasm.  $\times 400$

**Figure 2** Well-differentiated adenocarcinoma. The positive product of arylsulphatase is located at gland luminal side.  $\times 200$

**Figure 3** Mucinous cell carcinoma. The positive reaction of  $\beta$ -galactosidase is diffusely distributed in the cytoplasm.  $\times 200$

**Figure 4** Well-differentiated adenocarcinoma. Fine blue granules are mostly located in the apical cytoplasm. The positive product is seen in macrophage at the matrix.  $\times 200$

**Figure 5** Mucinous cell carcinoma. Mitochondria (Mi), endoplasmic reticulum (ER), secretory granules (G) in the cytoplasm were found. PGs (arrow) and collagens (Co) close to cancer cells are degraded. Both are seen in the region far from the cancer cells. RR $\times 10\ 000$

**Table 1 Staining intensity of three hydrolases in gastric cancer**

Histopathological types	No.	Arylsulfatase <sup>b</sup>			β-galactosidase <sup>d</sup>			Lysozyme <sup>c</sup>		
		+	++	+++	+	++	+++	+	++	+++
Well-differentiated	9	7	2	0	5	2	2	8	1	0
Poorly-differentiated	10	8	2	0	7	3	0	7	3	0
Mucinous	14	3	4	7	2	5	7	4	3	7

<sup>b</sup>P<0.01, <sup>d</sup>P<0.05 <sup>c</sup>P<0.05.

These types of hydrolases in different types of gastric cancers were compared by rank sum test. There were significant differences between mucinous carcinoma and well-differentiated and poorly-differentiated adenocarcinoma ( $P < 0.01$ ,  $P < 0.05$ ). There were no significant differences between well-differentiated and poorly-differentiated adenocarcinoma ( $P > 0.05$ ).

### Ultrastructural findings

In mucinous carcinoma, the Golgi complex, endoplasmic reticulum, swollen mitochondria and secretory granules with various electron densities in the cytoplasm were easily found. RR granules and collagens close to the cancer cells of mucinous carcinoma were degraded but were observed in the region far from the cancer cells (Figure 5).

In well-differentiated adenocarcinoma, there were microvillus on the surface of luminal gland, and close connection and desmosomes between cancer cells. There were more swollen mitochondria, and smaller secretory granules with moderate density in the apical cytoplasm in some cancer cells. RR granules and collagens were present and intact near the adenocarcinoma. In poorly-differentiated adenocarcinoma, there were abundant ribosomes, a few secretory granules with moderate density in a few cancer cells. RR granules were found in surrounding cancer cells, which looked like short rods with 20 nm - 50 nm in diameter with high electron density, and attached on the collagens or located between collagens.

## DISCUSSION

### Relationship between hydrolases and differentiation

The results showed that Golgi complex, endoplasmic reticulum, secretory granules, and staining intensity of arylsulfatase, β-galactosidase and lysozyme were more intense in mucinous cell carcinoma and varied with cell differentiation. In well-differentiated adenocarcinoma, three kinds of hydrolases were mostly distributed in apical cytoplasm of luminal glands, electron-microscopically there were moderate electron density secretory granules in apical cytoplasm. In poorly-differentiated adenocarcinoma, there were few secretory granules, and, three types of enzymes were scanty. These results suggested that the content and distribution of these three enzymes are related to the differentiation and secretory function of cancer cells. The more differentiated of the secretory system, the more secretory granules in the cytoplasm and the more intensive enzymes staining reaction. Expression of three

hydrolases in mucinous cell carcinoma was more intensive than those in well-differentiated and poorly-differentiated adenocarcinomas ( $P < 0.05$ ). GERL (Golgi complex, endoplasmic reticulum and lysosome)<sup>[5]</sup> system was mature with a good secretory function, which may secrete various hydrolases.

### Relationship between hydrolases and invasion

Malignant tumors may produce various hydrolases, which degrade pericancerous matrix at different sites in favour of the invasion<sup>[6]</sup>.

Arylsulfatase and β-galactosidase were lysosomal enzymes which degrade proteoglycans (PGs) in the extracellular matrix and basement membrane. PGs are very complex macromolecules containing a protein core, to which one or more glycosaminoglycans (GAGs) chain are covalently bound and the chain radiate out from central core protein<sup>[7]</sup>, owing to the polyanionic on the chains which tend to separate from each other to form the network structure, which can hinder the cancer cell spread. Arylsulfatase is a key enzyme in hydrolysing PGs. Removal of sulfate is a key step. Only after the sulfate group of PG was degraded, PG may be further hydrolyzed by other lysosomal enzymes. β-galactosidase is another important hydrolase which degrades the PGs. β-galactosidase not only cleaves the linkages between β-N-acetylglucosamine and β-galactoside (R-GlcNAc-Gal) on the GAG chains, but also degrade oxosylserine residues of the carbohydrate-protein linkage region on PGs<sup>[8]</sup>, resulting in macromolecular PGs hydrolyzation, cancer cell spreading in matrix. Lysozyme is a representative enzyme of macrophage cells. The functional mechanism of lysozyme is to cleave the linkage between N-acetylmuramic acid and N-acetylglucosamine of mucopeptide in cell wall of bacteria<sup>[8]</sup>, but, whether it may degrade similar structure on PGs and hyaluronide in extracellular matrix of human body to the bacteria has not been reported in literature. In the present study, three hydrolases were abundant in mucinous cell carcinoma, the positive reaction was also found in the extracellular mucous pool. PGs and collagens and smooth muscle in pericancerous matrix close to the cancer cell disappeared, which suggest that the cancer cells may secrete various hydrolases to degrade the extracellular matrix in different sites. So the cancer cells can easily invade and metastasize.

## REFERENCES

- Klockars M, Reitamo S. Tissue distribution of lysozyme in man. *J Histochem Cytochem*, 1975;23(12):932 - 940
- Leznicki A, Bleszymski W. Histochemical localization of the soluble arylsulfatase activities in rat brain. *Histochem*, 1970;24(3):251 - 265
- Lojda. Indigogenic methods glycosidase: an improved methods for β-Galactosidase and its application to localization studies of the enzyme in the intestine and other tissues. *Histochem*, 1970;23(3):266 - 288
- John J. Ruthenium red and violet: fine structural localization in animal tissues. *Anat Rec*, 1971;171(3):369 - 392
- Weiss L. The cells in leon veiss. *Histology (cell and tissue biology)*. 5th ed. The Macmillan Press, 1983:1 - 90
- Yi F, Mia D. Hydrolases in malignant tumor and invasion. (review) *Acta Univ Sci Med Chongqing*, 1991;16(1):83 - 87
- Lozzo RV. Proteoglycans, structure, function and role in neoplasia. *Lab Invest*, 1985;53(4):373 - 396
- Chipman DM, Nathan Sharon. Mechanism of lysozyme action. *Science*, 1969; 165(3892):454 - 465

# A study of the relationship between trace element Mo and gastric cancer \*

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**Subject headings** stomach neoplasms/mortality; molybdenum/analysis; trace element; risk factors

## Abstract

**AIM** To study the relationship between trace element Mo and gastric cancer.

**MATERIALS AND METHODS** Soil samples were collected according to its type in different areas of Jiangxi Province; available molybdenum content in soil was measured by catalytic polarography and rank correlation method was used to analyse correlation between the mean of soil available molybdenum and mortality rate of gastric cancer in each county and city in Jiangxi Province. Gastric cancer cases were selected from the authors' hospital, occiput hair was collected to measure its molybdenum content with an atomic absorption spectrograph and controls were selected from the same hospital for comparison. Gastric cancer cases were selected from three hospitals at the same time, blood samples were taken on an empty stomach and serum molybdenum contents were measured with the atomic absorption spectrograph, and controls were selected from the same hospitals. Blind method was used in the whole course (chemical analysts did not know the source and nature of samples).

**RESULTS** A negative correlation existed between soil available molybdenum content and mortality rate of gastric cancer ( $r = -0.285$ ,  $P < 0.05$ ); hair molybdenum contents of gastric cancer cases were lower than those of healthy controls ( $0.308 \mu\text{g/g} \pm 0.673 \mu\text{g/g}$  and  $0.707 \mu\text{g/g} \pm 0.561 \mu\text{g/g}$  respectively,  $P < 0.01$ ); serum molybdenum contents of patients were also lower than those of healthy controls ( $21.84 \mu\text{g/L} \pm 7.49 \mu\text{g/L}$  and  $25.38 \mu\text{g/L} \pm 8.58 \mu\text{g/L}$  respectively,  $P < 0.05$ ).

**CONCLUSION** Deficiency of molybdenum may be one of the risk factors in gastric cancer.

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\*Project supported by National Science Foundation of China, No. 39360071.

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Received 1997-05-28

## INTRODUCTION

A study of risk factors in gastric cancer in Jiangxi Province was started in 1983. The relationship between the soil trace element molybdenum (Mo) contents and mortality rates of gastric cancer in different areas of the province was investigated, and hair and serum Mo contents of gastric cancer cases and controls were determined.

## MATERIALS AND METHODS

Eighty-five of 91 counties or cities in the whole Jiangxi Province were chosen for soil Mo determination. A total of 1748 soil samples were taken from farming areas. The characteristics of the representative areas were: nonirrigated farmland of 12 thousand mu; paddy field of 25 thousand mu, hilly and mountainous region (reclaimable wasteland) of 105 thousand mu. Samples were separately packed into plastic pockets, filtered through nylon sieves and digested by oxalic acid-ammonium oxalate. Mo in the extract thus obtained was measured by catalytic polarography. Twenty-three gastric cancer cases from the 2nd Affiliated Hospital of Jiangxi Medical College and 152 non-cancer controls of similar age and sex were chosen for the study. Occiput hair samples (3%) were obtained from each case and control, impregnated in detergent, rinsed, desiccated and acidified for the measurement of Mo contents with an atomic absorption spectrometer (HITACHI 8000).

Another group of gastric cancer and non-cancer matched controls were from the First and Second Affiliated Hospitals of Jiangxi Medical College and Jiangxi Tumour Hospital diagnosed from January to March in 1993 and 1994. Blood samples of these subjects were taken on empty stomachs in the morning. Serum Mo contents were determined with an atomic absorption spectrograph (SHIMATSU A-A 680, Japan).

## RESULTS

### *Relationship between available molybdenum and mortality rate of gastric cancer*

Soil samples were collected from different areas of the whole province, including gastric cancer high-incidence county Gui Xi and low-incidence county Hui Chang. About 20 samples were taken from each county or city. The mean of available Mo in soil was calculated. The correlation between the mean of soil available Mo and mortality rate of gastric cancer in each county or city was analysed by rank correlation. The result was  $r = -0.285$ ,  $P < 0.01$ , which indicated a negative correlation between soil available Mo and mortality rate of gastric cancer.

### Comparison of hair molybdenum between gastric cancer cases and healthy controls

Twenty-three gastric cancer cases were all definitely diagnosed by barium meal study, gastrocopy or biopsy. Among them, 18 were males and 5 females, aged from 30 to 70 years, averaging  $52.2 \pm 12.4$ . A total of 152 healthy persons served as controls. The mean of hair molybdenum content of the 23 gastric cancer cases was  $0.308 \mu\text{g/g} \pm 0.673 \mu\text{g/g}$ , being lower than that of the control group ( $0.707 \mu\text{g/g} \pm 0.561 \mu\text{g/g}$ ) ( $P < 0.01$ ). The difference was significant.

### Comparison of serum molybdenum between gastric cancer cases and non-cancer controls

There were 33 gastric cancer cases, with age ranging from 27 to 80 years (average 54.4 years), and a male:female ratio of 1.00:1.06. There were 79 non-cancer controls, aged from 29 to 83 (average 48.1 years) with a male:female ratio of 1.00:1.13.

Serum molybdenum contents were  $21.84 \pm 7.49$  in the gastric cancer group and  $25.38 \pm 8.85$  in the controls, ( $t = 2.18, P < 0.05$ ).

Serum molybdenum contents in gastric cancer cases were lower than those in non-cancer controls, the difference being significant.

## DISCUSSION

Molybdenum has important physiological functions in human body. It is one of the essential trace elements. Low molybdenum content in soil has been thought to be one of the factors involving a high incidence of esophageal cancer and Keshan disease<sup>[1,2]</sup>. But there have been few reports about the relationship between trace element molybdenum and gastric cancer.

Our study showed a negative correlation ( $r = -0.285, P < 0.01$ ) between soil Mo and mortality rate of gastric cancer. Soil available Mo contents were all low in these counties and cities with high mortality rate of gastric cancer such as Gui Xi. Lack of Mo in soil results in inadequate contents in corn and livestock which in turn, affects human body. Molybdenum is a component of nitrite reductase. In the process of the reduction of nitrate to nitrogen which can then be utilized by plants, the participation of molybdenum is indispensable. In the soil deficient of molybdenum, only part of nitrate can be reduced, thus leaving more nitrite in the environment and corn. This makes the human body and animals absorb and store more nitrites which under certain circumstances combine with nitrogenous substances to form nitrosamines, a group of strong carcinogens. Furthermore, molybdenum has some effects on the synthesis of vitamin C in plants, which may inhibit synthesis of nitroso-compounds in the human body<sup>[3]</sup>. To sum up, we can say that lack of molybdenum in the environment may obviously influence nitrogen fixation and transformation of soil nitrates as well as compound formation, and that Mo deficiency is definitely related to a high mortality rate of gastric

cancer in local inhabitants.

Hair and serum molybdenum contents of gastric cancer cases were lower than those of healthy controls. The difference was statistically significant. This indicates that lack of molybdenum in the human body may be one of the causes of gastric cancer. Zhou *et al* found that molybdenum level in the mucosa of patients with gastritis or gastric ulcer was lower than that of normal people ( $P < 0.01$ ), and that its level in mucosa of gastric cancer patients was also decreased<sup>[4]</sup>. This indicated that molybdenum deficiency may be an important factor in the causation of gastric disease. Wei *et al* used *N*-nitroso-sarcosine-ethyl (NSEE) induced mouse foregastric squamous carcinoma model for studying the effect of molybdenum on carcinogenesis and proved that molybdenum had an obvious inhibitory effect on mouse foregastric squamous carcinoma<sup>[5]</sup>. Miller<sup>[6]</sup> reported that most chemical carcinogens were metabolized by cytochrome P-450 in cells, producing "end-stage carcinogen" with electrophilic groups and then brought about cell deterioration. P-450 also had some relationship with its effect on the detoxification of carcinogens in the body. Lu *et al* studied the effect of molybdenum on the activities of P-450 and demethylase in rat liver and found that small dose of molybdenum could speed up detoxification of carcinogens and large dose of molybdenum might slow down that effect relatively<sup>[7]</sup>. Basing on the literature and our results, we can infer that some amount of molybdenum in the human body may lower the intake and thereby the contents of nitrate and nitrite in the body and enhance detoxification of carcinogens so as to give a protective action on the gastric mucosa. The content of molybdenum in human body may be related to molybdenum level in the environment. In a relative molybdenum-deficient environment, is human body also in a relative molybdenum-deficient status Is it useful to give a proper supplement of molybdenum to the people or give molybdenum-containing fertilizer for the prevention of carcinoma (especially against gastric cancer) in Modeficient areas These questions remain to be answered.

In conclusion, we have reasons to think that lack of molybdenum may be one of the risk factors in gastric cancer.

## REFERENCES

- 1 Yang GH. Molybdenum and esophageal carcinoma. *FMS: Medical Geograph*, 1990;(1):12 - 14
- 2 Gai XD. Research of effect of lack of molybdenum and nitrite toxication in Ke Shan disease. *Chin J Control Endemic Dis*, 1989;4(5):267 - 269
- 3 Kong XL. Nutritional, physiological and clinical significance of essential trace elements. Hefei: Anhui Science and Technology Publishing House, 1982:353-355
- 4 Zhou WG, He LZ, Lin WY, Luo JH. Trace element measurement in gastric mucosa of gastric disease patients. *Guangxi Med*, 1990;12(5):298 - 300
- 5 Wei HJ. Effect of molybdenum on *N*-nitroso-sarcosine-ethyl causing mouse foregastric squamous carcinoma. *Trace Element*, 1986;(2):6 - 8
- 6 Miller EC. Some current perspectives on chemical carcinogenesis in human and experimental animals. *Cancer Res*, 1978;38:1479
- 7 Lu SX, Tian Y, Li FM. Effect of molybdenum on P-450 and the activity of demethylase in rat liver. *Chin J Oncology*, 1984;6(4):257 - 259

# Overexpression of P53 and its risk factors in esophageal cancer in urban areas of Xi'an

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**Subject headings** esophageal neoplasms; tumor suppressor gene; smoking; genes, p53; mutation; risk factors; immunohistochemistry

## Abstract

**AIM** To investigate the risk factors of esophageal cancer (EC) in urban areas of Xi'an and to determine the association between overexpression of P53 and these risk factors.

**METHODS** All cases (89) and controls (97) were permanent residents in urban areas of Xi'an, all cases of primary EC had been histologically confirmed, controls were inpatients with non-cancer and nonsmoking-related disease. Cancer tissues and tissues adjacent to the cancer of 65 cases and 24 available normal esophageal tissues of controls were detected for P53 overexpression by the immunohistochemical method.

**RESULTS** The smoking and familial history of cancer were significantly associated with EC in Xi'an inhabitants. The laboratory assay indicated that P53 positive stain in EC was 50.0%(34/65) and 6.1%(4/65) in tissues adjacent to the cancer, but no positive stain was found in normal esophageal tissues of controls. The results showed that P53 overexpression in EC was closely related to smoking and cases with familial history of cancer.

**CONCLUSION** Smoking and familial cancer history were important risk factors for EC, and the alteration of P53 gene may be due to smoking and inheritance factors.

## INTRODUCTION

Esophageal cancer (EC) is the sixth most common cancer in males. In some areas of China, it is the leading cause of deaths. Previous studies suggested that certain local dietary and environmental factors are important risk factors in the high-incidence areas of China. But in Europe, epidemiological studies have shown that tobacco and alcohol consumption are the prevailing risk factors<sup>[1]</sup>.

Recent progress in the molecular biology of cancer has provided a basis for novel strategies in the prevention and treatment of cancer<sup>[2]</sup>. Cumulating evidence indicates that changes in both dominant oncogene and tumor suppressor genes are likely for malignant transformation of normal cells. Among these genetic abnormalities, the p53 tumor suppressor gene appears to be the most frequent target for DNA damage in carcinogenesis. Recent work suggests that proper function of this gene product in controlling growth is compromised by mutations through a significant region of the coding sequence<sup>[3]</sup>, however, the exact cause of p53 gene mutations is still not clear until now. The current study was initiated to find out the risk factors of esophageal cancer in Xi'an inhabitants, and to correlate p53 overexpression with these factors.

Because almost all of previous studies in etiology of esophageal cancer were limited to study in the high-incidence areas of esophageal cancer, and in general, the high-incidence area tended to be rural<sup>[9]</sup>, the results of these studies could not explain completely the etiology of esophageal cancer in Xi'an.

## MATERIALS AND METHODS

### *Study design and inclusion criteria*

The hospital-based case-control study was performed between August 1994 to January 1995. The subjects were selected from one of the three hospitals affiliated to the Fourth Military Medical University in Xi'an. All eligible subjects should be the permanent residents of the urban area of Xi'an. Informed consent was obtained from the subjects, if they agreed with the immunohistochemical and genetic analysis of tissue samples.

Our cases were selected from among consecutive persons who underwent major esophageal surgery and endoscopy, all were histologically confirmed as primary carcinoma of esophagus and were diagnosed for the first time, none of the case had chemotherapy or

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**Received** 1997-01-02 **Revised** 1997-03-26

radiotherapy prior to biopsies. Patients with esophageal cancer were excluded if they had a clinically apparent malignancy of any other origin.

Controls had a variety of diseases excluding cancer and diseases which are epidemiologically unrelated to tobacco smoking and aged from 45 to 65 years. Other malignancies were excluded by medical history and routine examinations.

### Factors investigated

Factors in the questionnaire included demographic information (such as race, age, sex, educational level, and profession), tobacco smoking, alcohol consumption, intake of pickled vegetables, familial history of cancers, etc. All cases and controls were investigated by two trained investigators and interviewed for 20 minutes.

**Tobacco smoking.** Data on smoking history, i.e., duration of smoking, starting age, time of quitting, smoking, and average number of cigarettes consumed per day, were recorded.

**Family history of cancer.** Positive family history of cancer was defined as the presence of esophageal cancer in one or more relatives for both cases and controls. Data of three degree blood relatives on sex, age (age of death), cause of death, and duration of blood relatives living together with the subjects were investigated.

### Samples

The tumor tissues were obtained from surgical or endoscopic resections. Nonpathologic or grossly normal esophageal tissues were obtained from the margins of the specimen or normal esophageal tissues apart from tumors under endoscopy. Tissue blocks of both grossly apparent carcinoma had nonlesional esophagus were fixed in 4% paraformaldehyde in standard phosphate-buffered saline (PBS), pH 7.4. They were irradiated with microwaves for 10-15 seconds and postfixed for 2 hours at 4°C and then embedded in paraffin.

### Immunohistochemistry

Thin 7 µm slices from paraffin-embedded specimens were deparaffinized routinely, then, added 50 µl 0.3% hydrogen peroxide for 10 minutes, followed by wash in PBS (pH 7.4) three times and treated with 50 µl 1% normal rabbit serum for 10 minutes at room temperature. The slices were incubated with primary antibody for 3 hours at room temperature in a moist chamber. The primary antibody used in this study was monoclonal antibody DO-7, purchased from Maxim Biotech Inc, China. The antibody reacted with wild and mutant type of the P53 protein. Immunostaining was performed by the streptavidin peroxidase conjugated method employing DAB as chromogens<sup>[4]</sup>. Specific staining

was identified by the presence of brown or yellow reaction products.

### Validity of laboratory measurement

All immunohistochemical studies were done without knowing the clinical data. To determine whether the positive stain resulted from contamination, two kinds of negative controls were used. Sections were incubated with PBS and non-immune mouse serum instead of the primary antibody. All sections for immunohistochemical analysis were observed by two experienced pathologists and their judgements should be in close agreement.

### Statistical analysis

The odds ratio(OR) and its 95% confidence interval (CI) were calculated for variables that had been proved statistically significant in univariate analysis. Univariate analysis of factors potentially related to esophageal cancer and P53 overexpression were made to investigate the single effect of these factors. Logistic regression analysis was also performed. Univariate and multivariate analyses were conducted with SPLM program provided by the Department of Statistics in our university.

## RESULTS

### Subjects

Eighty-nine cases and 97 controls were collected. Their mean age ( $55.2 \pm 9.7$  years among cases and  $54.4 \pm 8.8$  years among controls), proportion of gender, educational level and profession were similar. Detailed information on smoking and family cancer history was collected from all of our cases and controls.

### The risk factors of esophageal cancer

The results indicated that the proportion of smokers and those with family histories of esophageal cancer was significantly higher than that in controls. It suggested that smoking and family cancer history were risk factors of EC. The OR for smokers was 3.26(95% CI 1.74 - 6.12), and for individuals with positive family cancer histories was 10.48(95% CI 4.81 - 22.48) (Table 1). Multiple logistic regression analysis showed the same results, in the logistic model, the interaction term was not statistically significant (probably due to sample size limitation) (Table 2).

**Table 1 Single variable analysis of risk factors of EC**

Factors	Cases	Controls	$\chi^2$	OR	95% CI*	P
Smoking						
Yes	65	44				
No	24	53	15.53	3.26	1.74-6.12	0.0012
Family history of cancer						
Yes	43	8				
No	46	83	35.46	10.48	4.81-22.48	0.0000

\*CI: confidence interval

**Table 2 Logistic regression analysis of risk factors of EC**

Factors	OR	Standard error	u value	P
Smoking	3.3	0.2939	4.0760	0.0000
Family history of cancer	3.0	0.2922	3.7090	0.0002

**Table 3 Correlation between clinical features and overexpression of p53 in EC**

Clinical features	P53 status		Univariate		Multivariate	
	Overexpression	Normal	$\chi^2$	P	OR	P
Age	55.41±8.83*	55.32±11.07*	1.5836	0.9710	1.0104	0.8447
Sex						
Male	20	22				
Female	14	9	1.1497	0.5628	1.3611	0.7820
Smoking						
Yes	30	20				
No	4	11	3.8898	0.0486	3.1200	0.0041
Family history of cancer						
Yes	24	7				
No	10	24	17.2834	0.0001	15.8100	0.0002

\*mean ± SD.

### Immunohistochemical analysis

All cases, which were immunohistochemically detected, were squamous cell carcinomas. Among the 65 cases of esophageal cancer 52.0% (34/65) were positive for P53, 31 cases were negative for the immunostain. 6.1% (4/65) tissues adjacent to the carcinoma were positive for P53, but no positive products were observed in normal esophageal tissues of 24 controls. In the majority of tumor cells intense P53 immunostaining was observed in the cell nuclein and some tumor cells demonstrated the intense staining in cytoplasm. In normal esophageal mucosa, immunoreactivity of P53 was observed sporadically in basal and parabasal cells of mucosa. As a histological feature, the overexpression of p53 gene had heterogeneity in esophageal carcinoma. Immunoreactivity described earlier was not observed when incubated with PBS or non-immune mouse serum instead of the primary antibody.

### Correlation of overexpression of P53 with clinical data

This study was unblinded after completing detection of overexpression of P53. Statistical analysis for the overexpression of P53 was then performed to correlate with clinical data. We could not find any statistically significant association between the overexpression of P53 and age, gender and occupation. In univariate analysis, the smoking and family histories of cancer were closely related with the overexpression of P53 ( $P < 0.05$ ). Using the logistic regression model, multivariate analysis was performed to investigate the independent risk of smoking or family history of cancer as a predictive factor for overexpression of P53. Smoking and familial history of cancer were the factors shown to be closely related to overexpression of P53 after adjusting for other covariates ( $P < 0.01$ ), e.g., the odds ratio for overexpression of P53 to develop in

smokers can be estimated at 3-fold increase over nonsmokers. The odds ratio for individuals who had family cancer histories was 15.8 (Table 3).

### DISCUSSION

Numerous epidemiologic studies in China have shown that both particular environmental exposure and certain local dietary factors were responsible for the carcinogenesis of esophagus, but almost all previous studies were limited to the high-incidence areas of EC. Generally, the high-incidence areas have tended to be rural, so the results of these studies can not explain completely the rationale of EC in Xi'an which is not a high-incidence area in China. Our findings contradict the previous studies which stated that there was nosignificant association between smoking and esophageal cancer in China, and it was found that smoking and family history of cancer were important risk factors of EC.

More and more researches have shown the tumor is a molecular disease. Carcinogenesis depends on many genetic events, including mutation of oncogene or/and tumor suppressor gene. Carcinogens with significant mutagenic activity are components of various substances associated with tobacco smoking. P53 tumor supressor gene mutation has been observed in esophageal tumor cells induced by *N*-methyl-*N*-benzylnitrosamine treatment which is one of components in tobacco<sup>[5]</sup>.

It is now clear that the p53 gene is the most frequent target gene among the known genetic alterations in EC<sup>[6]</sup>. P53 protein is a 53 KDa nuclear phosphoprotein and is expressed by many normal cells<sup>[7]</sup>. A decade ago, it was found that P53 protein was specifically overexpressed in transformed cells and undetectable in normal cells<sup>[8]</sup>. Numerous studies have confirmed these observations and shown that P53 protein accumulation is a consequence of its stabilization<sup>[9]</sup>. Normal P53 protein is known to be rapidly eliminated due to its short half-life, therefore, in normal cells, intracellular levels of P53 protein are low, and accumulation of P53 protein is usually the result of which modifies the conformation and stability of the protein<sup>[9,10]</sup>. It is easy to analyse the expression of P53 protein with immunohistochemistry on a large panel of tumors, because there appears to be a good correlation between the p53 gene mutation and its protein accumulation. In accordance with previous studies, P53 protein accumulation was found in 50% tumor specimens and 6.1% (4/65) tissues adjacent to cancer. In our study, in order to investigate whether the overexpression of P53 was present in normal esophageal tissues of controls, we immunostained 24 available specimens of controls, but no positive staining was found. The results suggest that the overexpression of p53 gene is a frequent genetic alteration and plays an important role in the carcinogenesis of esophageal carcinoma.

At present, the P53 tumor suppressor gene has come to the forefront of cancer research because it is commonly mutated in human cancer and the spectrum of p53 mutations in these cancers can provide clues to the etiology<sup>[11,12]</sup>. Various studies indicate that the p53 gene is a good target for molecular epidemiological studies of various human cancer. Our epidemiological investigation also showed that major fraction of EC can be attributed to smoking and familial history of cancer, therefore, identifying the association of P53 overexpression with smoking and family history of cancer in this study is of particular interest. The study showed that the alteration of p53 gene was associated with smoking and family history of cancer.

Because p53 genes are inherited in a recessive form, requiring the loss of both copies for the phenotype to be expressed, it can be hypothesized that genetic predisposition to cancer induction may be related to inherited mutations in all of tumor suppressor genes that regulate cell growth and terminal differentiation, and this kind of inherited susceptibility to esophageal cancer can be provoked by the specific carcinogens contained in cigarettes. Inactivation or altered function of these genes results in increased risk for development of tumors. The results of this study may be a dramatic example of this process, susceptible hosts who inherit one defective allele of p53 gene can become patients when another allele is lost through later somatic mutation induced by carcinogens in cigarettes.

As stated above, family cancer history depends on many factors, such as the number of relatives,

their biologic relationship to the index case, their age distribution and the disease frequency in the population, so it can not be concluded that the positive familial history of cancer is due to genetic susceptibility, and several types of mutation do not lead to P53 accumulation and would be missed by immunohistochemistry<sup>[13]</sup>, therefore, our further study will be focusing on a larger sample collection, PCR-SSCP analysis and DNA sequencing, and the application of more efficient statistical methods.

## REFERENCES

- 1 Yu MC, Garabrant DH, Peters JM, Mack TM. Tobacco, alcohol, diet, occupation and carcinoma of the esophagus. *Cancer Res*, 1988;49(18):3843 - 3848
- 2 Minna J, Nou M, Takahashi T, Shutte J, Chiba I, Viallet J *et al*. A molecular pathogenesis of lung cancer. In: Bergsageal DE, Mak TW, eds. Molecular mechanisms and their clinical applications in malignancies. ed 1. Orlando, FL: Academic Press, 1990:63 - 83
- 3 Chiba I, Takahashi T, Nau M, Damico D, Curriel D, Mitsudomi T *et al*. Mutation in the p53 gene are frequent in primary, resected non-small cell lung cancer. *Oncogene*, 1990;5(8):1603 - 1610
- 4 Zuorong, Shi. A comparison of three immunoperoxidase technique for antigen detection in colorectal carcinoma tissues. *J Histochem Cytochem*, 1988;96(2): 371 - 372
- 5 Guo Y, Lu S, Liu Y. Overexpression of p53 gene in esophageal cancer induced by nitrosamine. *Chin J Tumor*, 1992;14(1):241 - 244
- 6 Baker SJ, Fearon ER, Nigro IM, Hamilton SR, Preisinger AG, Jessup JM *et al*. Chromosome 17 deletion and p53 gene mutations in colorectal carcinomas. *Science*, 1989;244(1):217 - 221
- 7 Hollstein MC, Metcalf RA, Welsh JA, Montesano R, Harris CC. Frequent mutation of the p53 gene in human esophageal cancer. *Proc Natl Acad Sci USA*, 1990;87(12):9958 - 9961
- 8 Benchimol S, Pim D, Crawford L. Radioimmunoassay of the cellular protein p53 in mouse and human cell lines. *EMBO*, 1982;7(5):1055 - 1062
- 9 T Soussi, Y Legros, R, Lubin K. Multifactorial analysis of p53 alteration in human cancer: a review. *Int J Cancer*, 1994;57(1):1 - 9
- 10 Reich NC, Levine AJ. Growth regulation of a cellular tumor antigen p53 in nontransformed cells. *Nature*, 1984;308(1):199 - 201
- 11 Hollstein M, Slidransky D, Vogelstein B. p53 mutation in human cancers. *Science*, 1991;253(1):49 - 53
- 12 Harris CC, Hollstein M. Clinical implication of the p53 tumor suppressor gene. *N Engl J Med*, 1993;329(5):1318 - 1327
- 13 Moll UM, Riou G, Levine AJ. Two distinct mechanisms alter p53 in breast cancer mutation and nuclear exclusion. *Proc Natl Acad Sci*, 1992;89(2):7262 - 7266

# Relationship between HBV viremia level of pregnant women and intrauterine infection: nested PCR for detection of HBV DNA

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**Subject headings** hepatitis B; DNA, viral/analysis; radioimmunoassay; polymerase chain reaction; intrauterine infection

## Abstract

**AIM** To determine the incidence of hepatitis B virus (HBV) in trauterine infection and to explore the relationship between HBV viremia level of pregnant women and HBV intrauterine infection.

**METHODS** Sixty-nine pregnant women were divided into three groups. Group A, 41 HBsAg positive patients, 14 of them were HBeAg positive (group A1), and 27 HBeAg negative (group A2); Group B, 12 HBsAg negative patients, but positive for anti-HBs and/or anti-HBe and/or anti-HBc; and Group C, 16 patients negative for all HBV markers. Blood samples of mothers were taken at delivery, samples of their infants were collected within 24 hours after birth (before injection of HBIG and HBV vaccine). All the serum samples were stored at - 20°C. HBV serum markers were tested by radioimmunoassay and HBV NDA were detected by nested polymerase chain reaction.

**RESULTS** In group C, all of 16 newborns were negative for HBsAg and HBV DNA. In group A, 7 infants were HBsAg positive (17.1%), and 17 (41.5%) were HBV DNA positive ( $P < 0.05$ ). The incidence of intrauterine HBV infection was much higher in group A1 than that in group A2 (HBsAg 42.9% vs 3.7%, HBV DNA 92.9% vs 14.8%,  $P < 0.05$ ). The incidence of HBV intrauterine infection was significantly different between high and low HBV viremia of mothers (93.3% vs 42.9%,  $P < 0.05$ ).

**CONCLUSION** The incidence of HBV intrauterine infection is high when HBV DNA in newborns detected with nested PCR is used as a marker of HBV infection. It is related to HBV viremia level of mothers.

## INTRODUCTION

The incidence of chronic HBV infection is high in China, more than 120 million people in China are carriers of HBV, 40% to 60% of them catch HBV infection from their mothers. So the key strategy for controlling HBV infection in China is to prevent HBV transmission from mother to infant. Transmission from mother to infant takes place in uterine, during delivery, and after birth. Vaccination after birth is of efficacy in preventing infant from HBV infection during delivery and after birth, but it can not interrupt HBV intrauterine infection. Previous studies showed that the HBV intrauterine infection rate was low (2.1% - 8.0%). However, recent investigations indicate that the rate is as high as 35% - 50%, indicating that intrauterine infection is the main route for HBV transmission from mother to infant<sup>[1-3]</sup>. We detected HBV DNA in the sera of newborns to determine HBV intrauterine infection rate and to explore its relation to HBV viremia level of mothers.

## SUBJECTS AND METHODS

### Subjects

Sixty-nine pregnant women and their newborns were investigated. All pregnant women were confirmed to be HBsAg positive by solid phase radioimmunoassay (spRIA), followed up and delivered at our hospital. They were divided into 3 groups. Group A, 41 cases positive for HBsAg, among them 14 were HBeAg positive (group A1) and 27 HBeAg negative (group A2). Group B, 12 cases negative for HBsAg but positive for anti-HBs and/or anti-HBe and/or anti-HBc (independent or combinant presence). Group C, 16 cases negative for all HBV markers.

None of the pregnant women had histories of hepatitis, symptoms and signs of hepatitis, threatened abortion, threatened premature delivery, and edema-hypertension-proteinuria syndrome. There was no significant difference in age, week of pregnancy at delivery, gravidity and parity among the three groups.

### Methods

Blood samples of gravida were collected at delivery and the samples of newborns were taken within 24 hours after birth (before injection of hepatitis B vaccine and hepatitis B immunoglobulin). All serum

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Received 1997-09-02

samples were stored at -20°C.

Serum HBV DNA was tested by nested polymerase chain reaction. Primers were designed according to the S region of HBV genome, synthesized by the Shanghai Institute of Cellular Biology. The sequences of primers are shown in Table 1.

**Table 1 Sequences of primers**

Number	Position	Sequence (5' - 3')
1	300-321	CATCTTCTTGTTGGTTCTTCTG
2	715-695	TTAGGGTTTAAATGTATACCC
3	421-441	TCTATGTTCCCTCTTGTGC
4	626-605	ACCACATCATCCATATATCTG

\*1, 2 outer primers; 3, 4 inner primers

The product of first amplification was electrophoresed on 1.8% agarose gel, appearance of 416bp band was considered as strong positive for HBV DNA. If first amplification is negative, the product was used as plate for second amplification. The product of second PCR was electrophoresed on agarose gel, appearance of 206bp band was considered as weak positive for HBV DNA. Each sample was examined twice, there were positive, negative, and blank controls in each test.

Serum HBV markers were tested with solid phase radioimmunoassay (kits from 3V Company).

X2 test and direct calculation of probability on fourfold data were used for statistical analysis.

## RESULTS

Sixteen mothers in group C were negative for serum HBV DNA and their newborns were all negative for HBsAg and HBV DNA. The positive rates of HBsAg and HBV DNA in groups A and B are shown in Table 2.

In group A, the intrauterine infection rate was 17.1% (7/41) when HBsAg was used as a marker of intrauterine infection, the rate was high up to 41.5% (17/41) when HBV DNA was used, the difference was significant,  $P < 0.05$ . The HBV intrauterine infection was closely related to the mothers' HBeAg status (Table 2).

**Table 2 Detection rate of HBsAg and HBV DNA**

Group	Cases	Mother		Newborn	
		HBV DNA+ (%)	HBV DNA+ (%)	HBsAg+ (%)	HBsAg+ (%)
A1	14	14 (100.0)	13 (92.9)	6 (42.9)	
A2	27	7 (25.9)	4 (14.8)	1 (3.7)	
B	12	1 (8.3)	0	0	

HBV intrauterine infection was related to mothers' status and levels of serum HBV DNA (Tables 3 and 4).

**Table 3 Relationship between HBV intrauterine infection and mothers' HBV DNA status**

Mothers' HBV DNA	No. of neonates	HBV DNA+	Intrauterine infection rate (%)
+	22	17	77.2
-	31	0	0

<sup>b</sup> $P < 0.01$  vs mothers' HBV DNA positive

**Table 4 Relationship between HBV intrauterine infection and mothers' serum HBV DNA levels**

Mothers' HBV DNA level	No. of neonates	HBV DNA+	Intrauterine infection rate (%)
Strong positive	15	14	93.3
Weak positive	7	3	42.9

<sup>a</sup> $P < 0.05$  vs mother HBV DNA weak positive.

## DISCUSSION

Nested polymerase chain reaction (n-PCR) for HBV DNA detection is a sensitive and specific method for determining HBV intrauterine infection. The incidence of HBV intrauterine infection reported by different researchers is greatly discrepant, ranging from 2.1% to 50%, due to different sensitivities of methods used<sup>[3]</sup>. Yi *et al* detected HBV antigen in the liver of artificially aborted fetus with immunohistochemical assay and immuno-electromicroscopy, 43.75% of fetus from HBsAg positive pregnant women were infected with HBV<sup>[4]</sup>. Tang *et al* found that the HBV intrauterine infection rate was 44.4% (12/27) when HBV DAN in liver of fetus from HBsAg positive mothers was detected with Southern Blot, but the rate was only 18.5% when HBV antigen in fetal liver was detected by immunohistochemical method<sup>[5]</sup>. In this study, the HBV intrauterine infection rate was 17.1% when HBsAg in newborn serum detected by spRIA was used as a marker for diagnosis of intrauterine infection, but the rate was 41.5% when HBV DNA in newborn serum detected with n-PCR was used as a marker for diagnosis ( $P < 0.05$ ). The results indicated that n-PCR for detecting HBV DNA in serum of newborn was a sensitive and specific method for diagnosis of HBV intrauterine infection. The sensitivity of spRIA for HBsAg was at ng level, and tissues and cells of fetus were not mature, resulting in low expression level of HBV antigen. So detection of HBsAg in newborn serum underestimated the incidence of HBV intrauterine infection<sup>[5]</sup>. The sensitivity of PCR for HBV DNA detection ranged from 10 fg/ml to 1 ag/ml. Nested PCR prevented the "plateau" of one-time amplification, and increased the sensitivity and specificity by changing the primers and plates<sup>[6]</sup>. Nested PCR for detection of serum HBV DNA in newborn had a more practical value than the methods used by Yi<sup>[4]</sup> and Tang<sup>[5]</sup>, it can be used in clinical research into the mechanism of HBV intrauterine infection and in evaluation of the effect of methods for interrupting HBV intrauterine infection.

There is a closely positive correlation between the level of HBV viremia in mother and HBV intrauterine infection. HBeAg is a serum marker indicating HBV active replication. It has been reported that the HBV infection risk of infants from HBeAg positive mothers is 80% - 90%<sup>[1,7]</sup>. In our study all 14 HBeAg positive mothers had HBV DNA in serum, the HBV DNA detection rate of their newborns was as high as 92.9% (13/14). However, only 25.9% of HBeAg negative mothers were positive for HBV DNA, 14.8% (4/27) of their newborns were positive for HBV DNA in serum. These results show that the risk of HBV intrauterine infection is much higher in HBeAg positive mothers than that in negative ones ( $P < 0.05$ ).

The presence of HBV DNA is a direct marker of HBV active replication. The incidence of HBV intrauterine infection was much higher in newborns of mothers with HBV DNA than that of mothers without HBV DNA (77.3% *vs* 0,  $P < 0.01$ ), and that the incidence was higher in newborns of mothers with high level of serum HBV DNA (strong positive for HBV DNA) than that of mothers with low level (weak positive for HBV DNA) of HBV DNA (93.3% *vs* 42.9%,  $P < 0.05$ ). These results confirmed that HBV intrauterine infection was

positively related to the level of HBV replication in the mothers.

In conclusion, the incidence of HBV intrauterine infection is high, and it is positively related to the level of HBV replication in mothers. In order to control the epidemic of hepatitis B in China, it is important to explore the mechanism of HBV intrauterine infection and to develop effective methods for interrupting intrauterine infection. We have finished a prospective control trial, confirming that multiple injections of hepatitis B immunoglobulin during pregnancy can prevent fetus from HBV infection effectively (unpublished data).

## REFERENCES

- 1 Zhang SL, Li YF. Interrupting mother-to-child transmission of hepatitis B virus: control epidemic of hepatitis B. *Foreign Medicine (section of woman and child health care)*, 1995;6(2):61-65
- 2 Hu LN, Gu ML. Interuterine infection and mother to child transmission of hepatitis B virus. *Practical J Applied Obstet Gynecol*, 1995;11(2):59 - 61
- 3 Mituda T, Yokota S, Mori T. Demonstration of mother-to-infant transmission of hepatitis B virus by means of polymerase chain reaction. *Lancet*, 1989;i(8499): 886-888
- 4 Yi JR, Wang JW, He NX. Hepatitis B virus markers were detected in fetuses aborted from HBsAg-positive mothers. *Acta Virol*, 1985;1(2):100 - 104
- 5 Tang SX, Yu GL, Cheng CR. Study on the mechanisms and influential factors of intrauterine infection of hepatitis B virus. *Chin J Epidemiol*, 1991;12(6): 325 - 326
- 6 Yang DL, Wang BC (eds). *Technics of DNA amplication and its use in medicine*. Jinan: Shandong Science and Technology Publishing House, 1992:210 - 212
- 7 Zhang SL, Li YF. The clinical significance and advance in detection of antigens and antibodies of hepatitis B virus. *J Clin Intern Med*, 1993;10(4):14 - 15

# Immunohistochemical detection of HCV infection in patients with hepatocellular carcinoma and other liver diseases \*

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**Subject headings** Hepatitis C; carcinoma, hepatocellular; immunohistochemistry; liver neoplasms; liver diseases

## Abstract

**AIM** To detect HCV infection in patients with HCC and other liver diseases by the immunohistochemical method.

**METHODS** The expression of HCV antigen was identified by means of LSAB (labelled streptavidin-biotin) method using anti-NS3 monoclonal antibody.

**RESULTS** The positive rates of HCV antigen in the three groups of HCC, liver cirrhosis and hepatitis were 13.5% (7/52), 12.5% (2/16), and 10% (4/40) respectively, while in the samples from patients with constitutional jaundice and normal liver samples, no HCV antigen was found. HCV antigen could be seen in the nuclei and/or cytoplasm of carcinoma cells and/or pericancerous hepatocytes. In HCC, HCV antigen was more often seen in nuclei than in cytoplasm. The positive rate of HCV antigen in pericancerous tissues was higher than that in cancerous tissues.

**CONCLUSION** HCV is associated with HCC, and HCV infection enhances the development of liver diseases. HCV affects the initiative period of HCC and induces the malignant phenotypic alteration of hepatocytes.

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\*Project supported by grants from the China Medical Board of New York, No. 93-582 and the National Natural Science Foundation of China, No. 39470648.

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Received 1997-07-10

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the common malignant tumors which ranks the third in cancer mortality in our country, while its etiology and carcinogenesis are still far from clearly identified. The association of hepatitis C virus (HCV) infection with HCC has been indicated by serosurvey<sup>[1,2]</sup>, but studies at cellular level in detecting HCV antigen in liver tissues to demonstrate this association have so far been rare. The significance of results from a few immunohistochemical studies reported is quite limited because of the use of polyclonal antibodies and a small number of cases. In order to reveal the HCV infection status in HCC and other liver diseases and to explore the relationship between HCV and HCC at cellular level, we detected immunohistochemically the HCV antigen expression in cancerous tissues and liver tissues of 116 cases of different liver diseases (mainly HCC) using LSAB (labelled streptavidin-biotin) method and monoclonal antibody against NS3 antigen of HCV.

## MATERIALS AND METHODS

### Patients and samples

Liver tissue samples were obtained from inpatients during resection of their HCC in the Department of Abdominal Surgery of Affiliated Tumor Hospital of Sun Yat-Sen University of Medical Sciences in the period from April 1993 to February 1994 and the preserved paraffin embedded samples by liver biopsy in the Department of Pathology of First Affiliated Hospital of Xinjiang Medical College from 1986 to 1990 were also collected for study. The clinical data are shown in Table 1. The diagnosis was made according to pathological examination, clinical data and laboratory assay.

**Table 1 Clinical data of 116 cases of liver diseases**

Groups	Cases	Male/female	Age (years) ( $\bar{x}\pm s$ )	ALT (IU/L) ( $\bar{x}\pm s$ )
HCC	52	41/11	42±2	126±93
Cir.	16	10/6	42±6	171±68
CH	40	28/12	35±9	269±51
CJ	8	5/3	23±1	76±45

HCC: hepatocellular carcinoma; Cir: cirrhosis; CH: chronic hepatitis; CJ: constitutional jaundice

### Reagents

The monoclonal antibody against NS3 antigen of HCV, prepared by Bionikes Co. U. S. A. , was kindly provided by the Virology Institute of Chinese Academy of Preventive Medicine; LSAB (labelled streptavidin-biotin) kit and monoclonal antibody to HBsAg were obtained commercially (LSAB kit from DAKO, anti-HBs from Clinical Immunology Lab of Tongji Medical University).

### Immunohistochemical methods

The tissue sections were dewaxed routinely and then treated as follows: 0.3% H<sub>2</sub>O<sub>2</sub>-methanol blocking for 20min, phosphate-buffered NaCl solution (PBS) washing, incubation with monoclonal antibody against NS3 of HCV overnight at 4°C, continuing the following procedure according to the instruction of LSAB kit, DAB staining.

The negative controls included: ①substitution of monoclonal antibody against NS3 of HCV with unrelated antibody (anti-HBs) or PBS, ②exclusion of incubation with antibody against NS3 of HCV, and ③normal liver samples as controls.

## RESULTS

### *The positive rates of HCV antigen in the groups of different liver diseases*

In the groups of HCC, cirrhosis and chronic hepatitis, the positive rates of HCV Ag were 13.5% ( 7/52 ), 12.5% ( 2/16 ) and 10% ( 4/40 ) respectively, while in the samples from constitutional jaundice and normal liver no HCV Ag was found. No positive staining was shown in the negative controls except with anti-HBs as the first antibody ( the HBsAg staining was located differently, as compared with HCV antigen).

### *Location and distribution of HCV antigen in tissues*

HCV Ag could be seen in the nuclei and/or cytoplasm of cancer cells and/or pericancerous hepatocytes of HCC. In the 7 positive cases of HCC, HCV Ag was more often seen in nucleus than in cytoplasm (5 cases in nuclei, only 2 cases in cytoplasm). This difference was not significant in the other groups. The HCV Ag positive cells were scattered singly or gathered in small groups in some parts. The detecting rate of HCV Ag in cancer tissues was 5.8% ( 3/52 ), whereas in the pericancerous tissues it was as high as 12.5% (6/48), significantly higher than that in the former.

## DISCUSSION

Since the application of ELISA and PCR techniques in the detection of HCV antibody and HCV RNA,

the view that HCV infection was an important risk factor for the genesis of HCC was supported by many studies which were mainly carried out in HBV non-endemic areas such as Japan and Europe<sup>[3,4]</sup>. Even in China, as an HBV hyperendemic country, the association of HCV infection with HCC was also preliminarily demonstrated, but the published investigations up to now were almost all serological studies. Detection of HCV antigen at cellular level in liver tissues to demonstrate this association has been reported rarely. The specificity of the results from a few immunohistochemical studies reported was quite limited because of the use of polyclonal antibody derived from patient's serum, and the consistency of the results from different studies was quite poor<sup>[5]</sup>. We detected HCV antigen expression in cancerous tissues and pericancerous tissues of HCC using LSAB method and monoclonal antibody against NS3 antigen of HCV, and our results supported the association of HCV infection with HCC. The positive rate of HCV antigen in pericancerous hepatocytes was higher than that in cancer tissues, similar to the results of HCV RNA detection reported by Ohkoshi<sup>[6]</sup>, and this phenomenon gives us two hints: HCV probably affects mainly the initiative period of HCC development and enhances malignant phenotypic alteration of normal hepatocytes; and as soon as the malignant alteration has taken place, the compatibility of hepatocytes to HCV decreases. The positive rates of HCV antigen in cases of HCC and cirrhosis were higher than those in cases of chronic hepatitis, constitutional jaundice and normal liver tissues and this result suggests that HCV infection may enhance the development of liver diseases. It is common knowledge that HCV, as a non-reverse transcription RNA virus, completes its proliferation cycle in cytoplasm, and so the expression of HCV antigen in nucleus is not related with the proliferation cycle itself. Whether HCV NS3 antigen, as a "counter-modulating" factor, gets into the nucleus to influence expression and regulation of host cell genes and to induce malignant alteration of cells remains to be studied further in the future.

## REFERENCES

- 1 Caporaso N, Romano M, Marmo R. Hepatitis C infection is an additive risk factor for development of hepatocellular carcinoma in patients with cirrhosis. *J Hepatol*, 1991;12(3):367 - 371
- 2 Nalpas B, Driss F, Pol S. Association between HCV and HBV infection in hepatocellular carcinoma and alcoholic liver disease. *J Hepatol*, 1991;12(1):70 - 74
- 3 Okuda K. Hepatocellular carcinoma, Recent progress. *Hepatology*, 1992;15(5): 948 - 951
- 4 Zhou YP, Peng WW, Yao JL. Detection of plus and minus strands of HCV in tissues of HCC. *Acad J SUMS*, 1994;15(3):14 - 16
- 5 Shi MX, Zhu SN, Lu SL. Staining of HCV antigen in tissues of human liver diseases. *Acad J Shanghai Med Univ*, 1992;19(2):94 - 96
- 6 Ohkoshi S. Detection of hepatitis C virus RNA in sera and liver tissues of non-A, non-B hepatitis patients using the polymerase chain reaction. *Jpn J Cancer Res*, 1990;81(9):862 - 866

# A new rat model of portal hypertension induced by intraportal injection of microspheres

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**Subject headings** portal vein; hypertension, portal; disease model, animal; latex microsphere

## Abstract

**AIM** To produce a new rat model of portal hypertension by intraportal injection of microspheres. **METHODS** Measured aliquots of single or different-sized microspheres (15,40,80 $\mu$ m) were injected into the portal vein to block intrahepatic portal radicals. The resultant changes in arterial,portal,hepatic venous and splenic pulp pressures were monitored. The liver and lungs were excised for histological examination.

**RESULTS** Portal venous pressure was elevated from basal value of 0.89 - 1.02 kPa to a steady-state of 1.98 - 3.19 kPa following the sequential injections of single- or different-sized microspheres, with a markedly lowered mean arterial pressure. However, a small-dose injection of 80 $\mu$ m microspheres ( $1.8 \times 10^5$ ) produced a steady-state portal venous pressure of  $2.53 \times 0.17$  kPa, and all rats showed normal arterial pressures. In addition, numerous microspheres were found in the lungs in all experimental groups. **CONCLUSION** Portal hypertension can be reproduced in rats by intraportal injection of microspheres at a small dose of 80  $\mu$ m ( $1.8 \times 10^5$ ). Intrahepatic portal-systemic shunts probably exist in the normal rat liver.

## INTRODUCTION

Portal hypertension is associated with gross haemodynamic disturbances in portal and systemic circulations. Animal models are still important for research into portal hypertension. One of the most popular models used is the partial portal venous ligation model in the rat<sup>[1]</sup>, but this can only achieve extrahepatic portal venous occlusion and is not representative of intrahepatic portal hypertension as seen clinically. Carbon tetrachloride induced models of cirrhosis closely resemble the major features of the human disease<sup>[2]</sup>, but take a long time to develop and are associated with a high mortality and a wide heterogeneity in the stage and development of cirrhosis<sup>[3,4]</sup>. In the present study, a new rat model of portal hypertension was successfully induced by intraportal injection of microspheres.

## MATERIALS AND METHODS

### Animals

Thirty-eight Sprague-Dawley rats weighing 250 g - 350 g were randomly divided into six groups. Groups 1 ( $n = 6$ ) and 2 ( $n = 6$ ) received sequential injections of single-sized microspheres of 15 and 80  $\mu$ m diameters respectively. Groups 3 ( $n = 6$ ) and 4 ( $n = 6$ ) were given sequential injections of different-sized microspheres in order of size 15, 40 and 80  $\mu$ m and 80, 40 and 15  $\mu$ m, respectively. According to the results of the four groups above, two bolus injections of 80  $\mu$ m microspheres were selected as the suitable dose for the induction of portal hypertension in the model and these were given to Group 5 ( $n = 8$ ) for further observation. Rats in Group 6 ( $n = 6$ ) were injected with saline and served as controls.

### Measurement of pressures

The animals were anaesthetised with fentanyl/fluanisone (0.3 ml/kg, subcutaneously) and midazolam (0.3 ml/kg, subcutaneously). Mean arterial pressure (MAP) was monitored using a catheter in the left carotid artery. The abdomen was opened via a midline incision and the portal vein was cannulated through an ileocolic vein for measurement of portal venous pressure (PVP) and injection of microspheres. The splenic pulp pressure (SPP) was measured through a 23G butterfly scalp needle. Wedged hepatic venous pressure (WHVP) was measured in Groups 1,2,5 and 6. All of the cannulae were connected to P23XL (Viggo Spectramed Inc.) pressure transducers, and

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\*Supported partially by the King's College Medical Research Trust and the Central Research Fund of the University of London.

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Received 1997-04-15 Revised 1997-06-14

permanent recordings were made on a polygraph recorder (Grass Instruments Inc., USA). When steady basal pressures had been achieved for at least 5 minutes, injections of microspheres or saline started. Before injection, latex microspheres (Coulter Electronics Ltd., England) were agitated for 60 seconds. In each injection, microspheres were given iav the portal venous catheter in a volume of 0.2 ml and immediately followed by 0.2 ml saline injection to flush the catheter. Only when a steady PVP has been achieved for at least 5 minutes was the next injection given. In Group 5 there was no interval between the two injections. The numbers of microspheres used are shown in Table 1. Following completion of the injection, animals were observed for 10 - 30 minutes until the final steady PVP had been reached. Finally a vascular clamp was applied to the portal vein at the liver hilus and the pressures monitored. After this, the animals in Group 6 received partial portal venous ligation. At the end of the experiment, all rats were killed by opening the chest. The liver and lungs were taken and fixed in 10% formal-saline for histological examination.

**Table 1** Intraportally injected aliquots of microspheres

Group	Sphere diameter (µm)	No of aliquots	No of spheres/aliquot	Total No of spheres
1	15	6	$5.6 \times 10^6$	$3.4 \times 10^7$
2	80	5	$9.0 \times 10^4$	$4.5 \times 10^5$
3	15,40,80	15µm×2	$5.6 \times 10^6$	$1.1 \times 10^7$
		40µm×2	$2.4 \times 10^5$	$4.8 \times 10^5$
		80µm×2	$9.0 \times 10^4$	$1.8 \times 10^5$
4	80,40,15	80µm×2	$9.0 \times 10^4$	$1.8 \times 10^5$
		40µm×2	$2.4 \times 10^5$	$4.8 \times 10^5$
		15µm×2	$5.6 \times 10^6$	$1.1 \times 10^7$
5	80	2	$9.0 \times 10^4$	$1.8 \times 10^5$

Note: Pressures showed no changes after the 4th and 5th injections in Group 2. Therefore the 6th injection was not given.

### Statistics

Results were expressed as mean  $\pm$  standard error. Comparisons were made by means of *t* test. Results

were considered statistically significant at  $P < 0.05$ .

### RESULTS

MAP decreased by approximately 45% following the first injection of microspheres in all experimental groups (kPa,  $12.95 \pm 1.66$  vs  $7.32 \pm 0.68$ ,  $P < 0.001$ ). MAP in Group 5 eventually returned to normal levels after approximately 40 minutes following the two injections of 80 µm microspheres. No change in MAP was observed in the control rats during saline injections and after partial portal venous ligation. However, portal venous occlusion in control rats produced a significant reduction in MAP (kPa,  $12.86 \pm 1.65$  vs  $6.88 \pm 1.14$ ,  $P < 0.001$ ), similar to that seen after microsphere injections in the experimental groups ( $P > 0.05$ ).

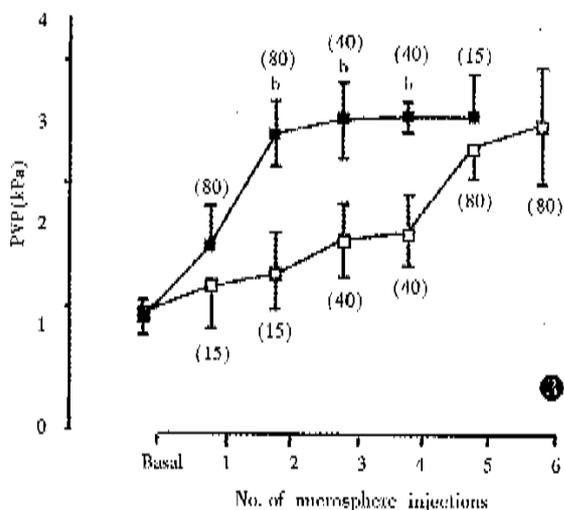
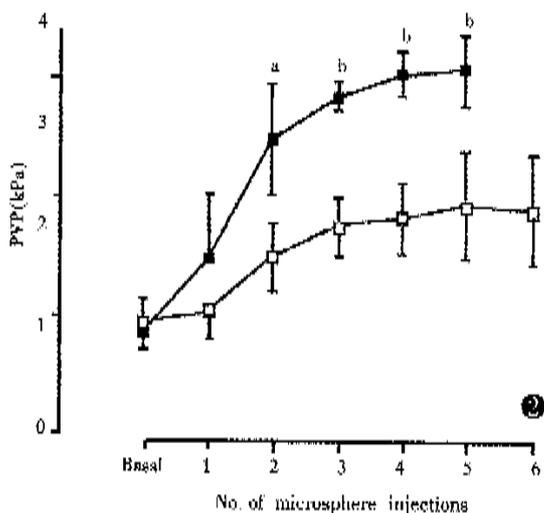
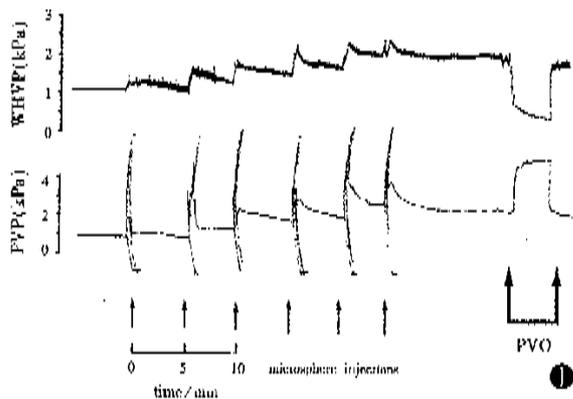
PVP rose gradually following the microsphere injections and, in Group 1, synchronous increase in WHVP was found (kPa,  $0.93 \pm 0.13$  vs  $1.65 \pm 0.24$ ,  $P < 0.01$ ) (Figure 1). In Group 2, 80 µm diameter microspheres produced a large, rapid increase in PVP (Figure 2) with a significant reduction in WHVP (kPa,  $0.89 \pm 0.09$  vs  $0.47 \pm 0.09$ ,  $P < 0.01$ ). In Groups 3 and 4, a marked increase in PVP was only observed after injection of 80 µm diameter spheres (Figure 3). Four rats in Group 4 died within 2 - 17 minutes after the 6th injection of microspheres. In Group 5, two sequential bolus injections of 80 µm microspheres elicited an immediate reduction in WHVP (kPa,  $0.92 \pm 0.13$  vs  $0.47 \pm 0.09$ ,  $P < 0.01$ ) and an immediate increase in PVP to  $2.53 \text{ kPa} \pm 0.17 \text{ kPa}$ , which remained elevated during the observation period of 150 minutes. There were no significant changes in PVP in the control group after injection of an equivalent volume of saline aliquots. Results are compared in Table 2.

Portal venous occlusion with a clamp produced a further large, rapid rise in PVP (Figure 1) to  $5.16 \text{ kPa} \pm 0.76 \text{ kPa}$ ,  $4.87 \text{ kPa} \pm 0.79 \text{ kPa}$ ,  $4.95 \text{ kPa} \pm 0.99 \text{ kPa}$ ,  $5.55 \text{ kPa} \pm 0.89 \text{ kPa}$  and  $5.75 \text{ kPa} \pm 0.32 \text{ kPa}$  in groups 1,2,3,5 and the control respectively. There was no significant difference between the values in these groups ( $P > 0.05$ ). Partial portal venous ligation in control rats induced a significant increase in PVP to  $2.75 \text{ kPa} \pm 0.07 \text{ kPa}$  and was similar to that in Group 5 ( $P > 0.05$ ).

**Table 2** Comparison of changes in portal venous pressure (PVP) and splenic pulp pressure (SPP) in different groups after microsphere injections

Group	PVP(kPa)				SPP(kPa)			
	Basal	Post-infusion	Basal-Post	% increase	Basal	Post-infusion	Basal-Post	% increase
1	$0.98 \pm 0.21$	$1.98 \pm 0.48^a$	$1.00 \pm 0.37^a$	$102.2 \pm 35.6^a$	$1.17 \pm 0.39$	$2.01 \pm 0.51^a$	$0.84 \pm 0.44^a$	$90.9 \pm 41.8$
2	$0.89 \pm 0.19$	$3.19 \pm 0.29^a$	$2.30 \pm 0.36^a$	$272.2 \pm 78.0^a$	$1.44 \pm 0.43$	$3.03 \pm 0.33^a$	$1.60 \pm 0.60^a$	$139.3 \pm 51.7$
3	$1.02 \pm 0.11$	$2.65 \pm 0.49$	$1.64 \pm 0.52$	$162.8 \pm 60.1$	$1.24 \pm 0.16$	$2.49 \pm 0.45$	$1.24 \pm 0.48$	$103.1 \pm 48.5$
4	$0.98 \pm 0.13$	$2.70 \pm 0.13$	$1.72 \pm 0.20$	$178.6 \pm 41.0$	$1.16 \pm 0.19$	$2.63 \pm 0.13$	$1.49 \pm 0.23$	$133.5 \pm 39.2$
5	$1.04 \pm 0.12$	$2.53 \pm 0.17$	$1.45 \pm 0.23^c$	$140.3 \pm 32.0^c$	$1.44 \pm 0.19$	$2.47 \pm 0.19$	$1.04 \pm 0.28^c$	$74.9 \pm 26.6^a$
6(control)	$1.04 \pm 0.07$	$1.02 \pm 0.05$			$1.36 \pm 0.20$	$1.37 \pm 0.19$		

<sup>a</sup> $P < 0.05$ , vs other groups; <sup>c</sup> $P < 0.05$ , vs groups 3 and 4. Student's unpaired *t* test.

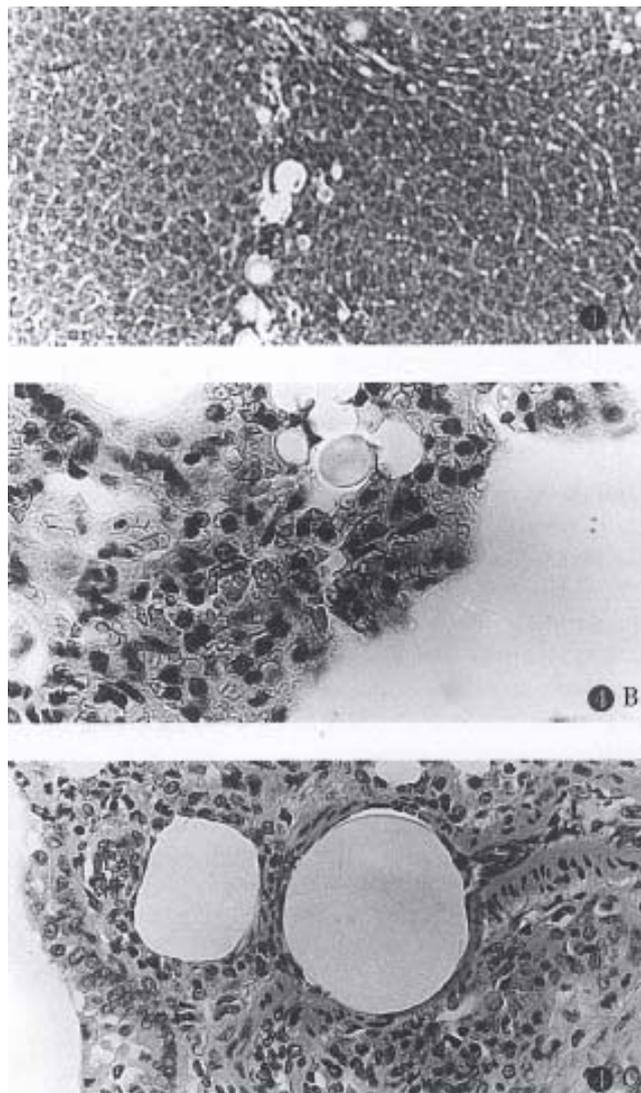


**Figure 1** Portal venous pressure (PVP) and wedged hepatic venous pressure (WHVP) recordings obtained from a rat in Group 1 during intraportal microsphere injections and subsequent portal venous occlusion (PVO).

**Figure 2** Changes in portal venous pressure (PVP) following intraportal microsphere injections in Group 1 (□) and Group 2 (■).

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, Groups 2 vs Group 1.

**Figure 3** Changes in portal venous pressure (PVP) following intraportal microsphere injections in Group 3 (□) and Group 4 (■). Four rats in Group 4 died after the 6th injection and therefore steady PVP recordings could not be obtained after this time. <sup>b</sup>P<0.01, Group 4 vs Group 3.



**Figure 4** Histological sections showing (A) 15, 40 and 80 μm spheres lodged in liver (Group 3, original magnification × 10), (B) 15 μm spheres lodged in lung (Group 1, original magnification × 40) and (C) 80 μm spheres lodged in lung (Group 2, original magnification × 16).

Histological examinations showed that in Groups 1 and 2 almost all of the portal radicles identified in the fields were blocked by the injected microspheres, with 15 μm spheres (Group 1) lodged in the terminal portal venules and 80 μm spheres (Group 2) lodged in the large portal radicles. In Group 3, the large and small portal radicles were simultaneously blocked by the different sized spheres injected. However, in Group 4, portal venules were mainly obstructed by the 80 μm spheres, while many 15 and 40 μm spheres injected after the 80 μm diameter spheres were trapped over the 80 μm spheres. The results in Group 5 were similar to Group 2, but with fewer spheres than those observed in Group 2. Numerous microspheres were found in the lungs of all experimental groups (Figure 4).

## DISCUSSION

Anatomically the intrahepatic portal tract branches into progressively smaller radicles until the sinusoids are reached. Therefore, intraportal injection of microspheres can block the intrahepatic portal radicles and lead to presinusoidal portal hypertension. The present study confirmed that the PVP was elevated to a steady state 100% - 270% above the basal value following microsphere injections. Obviously, the increase in PVP was related to the size and numbers of microspheres used: the 80  $\mu\text{m}$  spheres caused a large, rapid rise in PVP compared to 15  $\mu\text{m}$  spheres (Figure 2). As shown by the histology, the 80  $\mu\text{m}$  spheres were trapped in large portal radicles and therefore smaller numbers would produce a marked PVP raising effect. The results of sequential injections of different-sized microspheres showed that the final PVP was dependent upon the effect of the largest spheres (Figure 3).

When intrahepatic portal radicles were blocked by the microspheres injected, the presinusoidal increase in resistance produced not only an elevated PVP, but also a markedly lowered MAP because of extensive mesenteric pooling of portal vein blood. Therefore, both the PVP and MAP should be considered into the reproduction of this model. From the results of the first four groups, it was found that two injections ( $1.8 \times 10^5$ ) of 80  $\mu\text{m}$  spheres created an augmentation in PVP with the recovery of MAP. Furthermore, all rats in Group 5 showed a normal MAP and high PVP of  $2.53 \text{ kPa} \pm 0.17 \text{ kPa}$  which was comparable to the value of  $2.75 \text{ kPa} \pm 0.07 \text{ kPa}$  in the control group after partial portal venous ligation, and to the value of  $1.8 \text{ kPa} \pm 0.2 \text{ kPa}$  found in our cirrhotic model previously induced by carbon tetrachloride<sup>[2]</sup>. These suggest that  $1.8 \times 10^5$  of 80  $\mu\text{m}$  spheres is the suitable dose for induction of portal hypertension in the model.

Clinically, portal hypertension is mainly caused by different forms of chronic liver disease, and is characterized by an increased resistance in the liver and by the formation of portosystemic collaterals. Though in early studies the site of the increased resistance was thought to be postsinusoidal, recent works have proved that the presinusoidal resistance is also increased in cirrhotic liver and contributes to the development of portal hypertension<sup>[5,6]</sup>. In particular, the presinusoidal block is the main cause of the idiopathic portal hypertension and the portal hypertension resulted from chronic biliary obstruction<sup>[7,8]</sup>. Therefore, this model, in regard to the location of increased intrahepatic resistance, possesses the general features of clinical cases. Moreover, the fact that many microspheres appeared in the pulmonary vascular bed suggested the opening of portosystemic collaterals during the procedure, with a diameter of more than 15  $\mu\text{m}$  - 80  $\mu\text{m}$ .

Originally we believed that the presence of microspheres in the lung is only due to the opened extrahepatic collaterals, and this seems to be supported by the observation that the SPP was slightly lower than PVP. However, in this study although the PVP in groups 1 and 2 did not increase significantly after the 3rd injection, which implied that the intrahepatic portal radicles had been saturated by the microspheres injected, the final PVP achieved was substantially less than that obtained by portal vein occlusion. Consideration of the simultaneous changes of PVP and WHVP in Group 1 strongly suggested the existence of intrahepatic portal-systemic shunts in the normal rat liver. Opening of these shunts would permit portal blood flowing directly into the hepatic veins, leading to an elevation of WHVP and preventing further increase in PVP. When the portal vein occlusion was performed extrahepatically, the function of intrahepatic shunts was deprived, and, as a result, PVP could rise to an extremely high level. The intrahepatic shunts have not been described in the normal liver, but have been reported in cirrhotic livers in rats<sup>[9]</sup> and in humans<sup>[10]</sup>. It has been suggested that the frequency of large shunts (diameter > 25  $\mu\text{m}$ ) is relatively low and this is probably responsible for the reduction in WHVP after 80  $\mu\text{m}$  microsphere injections in this study.

This model of portal hypertension is intrahepatic and can be induced rapidly, with the opening of intra- and extra-hepatic portal-systemic shunts. A major advantage of this model is that while the intrahepatic presinusoidal block is achieved acutely, the normal liver architecture remains. This may be particularly beneficial to the research in the actions of mechanical obstruction or some humoral substances, related to liver dysfunction<sup>[2]</sup>, in the pathogenesis of portal hypertension. In the experimental cirrhotic model, it is difficult to differentiate these two actions.

## REFERENCES

- Geraghty JG, Angerson WJ, Carter DC. Portal venous pressure and portosystemic shunting in experimental portal hypertension. *Am J Physiol*, 1989; 257 (Gastrointest Liver Physiol. 20):G52 - G57
- Li XN, Huang CT, Wang XH, Leng XS, Du RY, Chen YF *et al.* Changes of blood humoral substances in experimental cirrhosis and their effects on portal hemodynamics. *Chin Med J*, 1990; 103(12):970 - 977
- Tamayo RP. Is cirrhosis of the liver experimentally produced by CCl<sub>4</sub> an adequate model of human cirrhosis. *Hepatology*, 1983; 3(1):112 - 120
- Sieber CC, Lopez-talavera JC, Groszmann RJ. Role of nitric oxide in the in vitro plasmic vascular hyporeactivity in ascitic cirrhotic rats. *Gastroenterology*, 1993; 104(6):1750 - 1754
- Shibayama Y, Nakata K. Localization of increased hepatic vascular resistance in liver cirrhosis. *Hepatology*, 1985; 5(4):643 - 648
- Shibayama Y, Nakata K. On the pathogenesis of portal hypertension in cirrhosis of the liver. *Liver*, 1988; 8(1):95 - 99
- Fukuda Y. Pathological study on Banti's syndrome. *Acta Pathol Jpn*, 1968; 18(4):457 - 472
- Nayak NC, Ramalingaswami V. Obliterative portal venopathy of the liver. *Arch Pathol*, 1969; 87(4):359 - 369
- Wood AJJ, Villeneuve JP, Branch RA, Rogers LW, Shand DG. Intact hepatocyte theory of impaired drug metabolism in experimental cirrhosis in the rat. *Gastroenterology*, 1979; 76(6):1358 - 1362
- Hoefs JC, Reynolds TB, Pare P. A new method for the measurement of intrahepatic shunts. *J Lab Clin Med*, 1984; 103(3):446 - 461

# Tumor radioimmunoimaging of chimeric antibody in nude mice with hepatoma xenograft \*

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**Subject headings** liver neoplasms, experimental; carcinoma, hepatocellular; chimeric antibody; mice, nude; hepatitis B virus; disease models, animal; radioimmunodetection; radioimmunotherapy

## Abstract

**AIM** To study the radioimmunoimaging (RAII) using the human/mouse chimeric Ab to evaluate its targeting activity in animal models.

**METHODS** To chimeric Ab was labeled with <sup>131</sup>I. RAII was performed at different intervals after injection of radio-labeled Abs in nude mice with human hepatoma xenograft, and tissue distribution of radioactivity was measured. Comparison was made in the chimeric Ab between the single segment Ab and previous murine mAb against HBxAg.

**RESULTS** The experimental objects developed tumor-positive image after 2 days of radio-labeled Abs injection, and the peak accumulation of radioactivity fell on the 7th day. The tumor/liver ratioactivity of the chimeric Ab, single segment Ab, anti-HBx mAb, and the control group was 281±0.21, 2.44±0.16, 4.60±0.19, and 0.96±0.14, respectively.

**CONCLUSION** The genetic engineering Abs have a considerable targeting activity which can be used as a novel humanized vector in the targeting treatment of liver cancer.

## INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China. Patients with such a tumor can not, by and large, be diagnosed in its early stage and, therefore, lose the chance for operation. The purpose of this study was to explore the potential value of chimeric human/mouse Ab applied to the treatment of HCC in targeting therapy. Clinical evidence showed that the anti-HBx mAb prepared by HCC-related hepatitis virus X protein was effective vectors<sup>[1-3]</sup>. However, because of its murine-derived nature, the protein had certain immunogenicity, thus hindering its clinical applications in targeting therapy considerably. In order to circumvent the obstacle, modifications have been made to humanize the Abs using genetic engineering techniques. The light- and heavy-chain variable region (VH, VL) gene of the cloned anti-HBx mAb were ligated with the constant region (CH) gene of human IgG, resulting in the construction of a human/mouse chimeric Ab gene and its expression in the prokaryotic vector<sup>[4]</sup>. In this study, we carried out the radioimmunoimaging (RAII) on nude mice models bearing human HCC using <sup>131</sup>I-labeled chimeric Ab, aiming at characterizing the localization of the chimeric Ab in the HCC, and evaluating its potential use as a vector in targeting therapy.

## MATERIAL AND METHODS

### *Preparation of the chimeric human/mouse Ab and the single segment Ab*

The chimeric Ab gene was cloned into pUC19, and subcloned into expressing plasmid PBLMVL<sub>2</sub>, which was a gift from Professor Wu (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, China). A single colony from a fresh plate was incubated in a 3 ml culture and grown overnight at 30°C; then transferred into fresh medium, incubated at 30°C for 2 h-3 h until A<sub>600</sub> = 0.5, and then induced in a medium containing 15 mM MgSO<sub>4</sub> at 42°C for 3 h. The bacteria were collected, cytolysed by ultrasonication, and aspirated. The supernatant was dried by vacuum and stored at -20°C.

### *Labeling of the Abs*

The chimeric Ab, single segment Ab, anti-HBx mAb and expression protein of *E. coli* (TG1) were labeled with <sup>131</sup>I (provided by the Chinese Atomic Energy Institute) by the Iodogen method. Unincorporated <sup>131</sup>I was separated from the bound

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\*Project supported by the National Natural Science Foundation of China, No.39370681.

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**Received** 1997-09-20

iodine by gel filtration on a Sephadex G-50 column. The whole process of labeling was a bacteria free and pyrogen free event<sup>[5]</sup>.

**Animal model**

22 LTNM4 nude mice models bearing human HCC by subcutaneous implantation were established in the authors' institute. Of these models, 5 mice were injected with chimeric Ab, 5 with single segment Ab, 5 with anti-HBx mAb as positive control, 5 with <sup>131</sup>I as negative control, and 2 with TG1. A small amount tumor sample (2 mm<sup>3</sup> - 3 mm<sup>3</sup>) of HCC tissue originated from a nude mouse was transplanted subcutaneously into the 22 nude mice, and tumors measuring 1 cm in diameter were used for the immunoimaging study<sup>[6]</sup>.

**Radioimmunoimaging and biodistribution analysis**

The experimental and control groups were injected intraperitoneally with <sup>131</sup>I-labeled human/mouse chimeric Ab, single segment Ab, anti-HBx mAbs, TG1 and <sup>131</sup>I respectively at a dose of 250 μCi per mouse. And γ imaging was operated on the 1st, 5th, and 7th day after injection. All animals were killed on the 7th day following the injection in order to determine the biodistribution of their radioactivity.

**RESULTS**

**Immunoactivity assay of the radio-labeled Abs**

The human/mouse chimeric Ab, single segment Ab, anti-HBx mAb, and TG1 were labeled by the Iodogen method. After separation of unincorporated <sup>131</sup>I from bound iodine, the binding activity of the chimeric Ab, single segment Ab, and anti-HBx mAb remained unchanged by ELISA (Figure 1).

**Radioimmunoimaging of nude mice models bearing HCC**

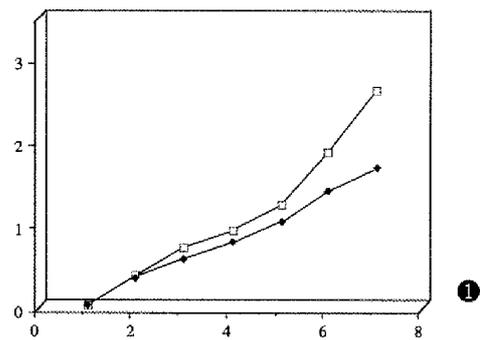
One day after injection of the <sup>131</sup>I-labeled human/mouse chimeric Ab and single segment Ab, the experimental animals showed distinct radio-accumulation in their abdominal cavities, whereas a faint -image was developed in tumor region. From the 5th day following injection, the distinct background image gradually disappeared, and an obvious radio-accumulation developed in tumor region. This phenomenon lasted until the 7th day. The positive control displayed tumor-positive image on the 5th day following injection of anti-HBx mAb, and even more clear tumor image was obtained on the 7th day which was in conformity with the previous reports. No accumulation was found in the negative control with injection of TG1 and <sup>131</sup>I. Since the thyroid glands of the nude mice were not blocked, they showed obvious radio-accumulation in the experiment (Figure 2).

**Biodistribution of the radio-labeled Abs**

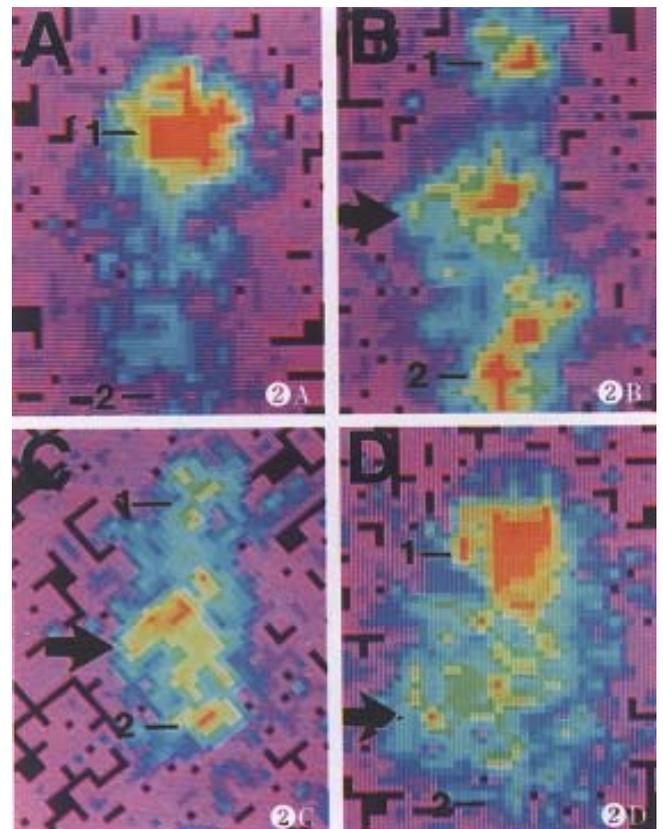
Table 1 shows the radioactivity of tumor versus

other tissues expressed as T/NT ratio following the injection of radio-labeled Abs after 7 days.

The tumor/liver ratio in human/mouse chimeric Ab and single segment Ab was 2.81 and 2.44, respectively in the experimental group, which was significantly higher than that of <sup>131</sup>I and TG1 in the negative control group (0.96 and 0.41). But they are lower than the tumor/liver ratio of murine derived mAb (4.6) in the positive control group. In addition, there has been a high accumulation of radioactivity in blood, gastrointestinal tract and kidney, especially predominant in the human/mouse chimeric Ab.



**Figure 1** The binding activity curve of chimeric Ab by ELISA. MoAbs: anti-HBx mAb; sfv: anti-HBx human/mouse chimeric Ab



**Figure 2** The localization of <sup>131</sup>I-labeled chimeric Ab in nude mice bearing human HCC.

- A: <sup>131</sup>I-TG1
- B: <sup>131</sup>I-chimeric Ab
- C: <sup>131</sup>I-single segment Ab
- D: <sup>131</sup>I-anti-HBx mAb
- Arrow: the tumor image
- 1. The thyroid gland area
- 2. The bladder area

**Table 1** T/NT ratio of <sup>131</sup>I in nude mice models bearing human HCC ( $\bar{x}\pm s$ )

	<sup>131</sup> I-labeled chimeric Ab	<sup>131</sup> I-labeled segment Ab	<sup>131</sup> I-labeled anti-HBx mAb	<sup>131</sup> I	<sup>131</sup> I-TG1
Blood	1.81±0.35	3.26±0.09	1.70±0.05	0.76±0.14	1.18
Liver	2.81±0.21	2.44±0.12	4.60±0.20	0.96±0.14	0.41
Kidney	0.48±0.09	2.22±0.07	2.66±0.15	0.42±0.05	0.69
Spleen	0.64±0.09	1.59±0.13	1.74±0.01	0.97±0.09	0.37
GI	1.06±0.03	0.40±0.08	2.67±0.33	0.90±0.01	0.07
Heart	3.84±0.37	3.23±0.23	6.10±0.51	1.86±0.15	2.39
Bone	4.25±0.20	5.71±0.11	6.70±0.24	1.84±0.13	1.97
Muscle	10.86±0.76	11.39±0.26	10.38±0.16	3.62±0.22	4.07

## DISCUSSION

Targeting therapy has been applied to the clinical treatment of liver cancer as a powerful means to kill tumor cells for more than a decade. In this context, mAbs were usually the first choice. However, because of the murine origin of these mAbs, their repeated use often led to generation of anti-antibodies which would markedly reduce their bio-efficiency. Although several methods were adopted such as changing species and varieties of antibodies' origin, adding immunosuppressors, etc., the incidence of human anti-mouse Abs (HAMA) was 63% - 95% after the treatment with murine derived antibodies. The incidence of HAMA was 34.4% in our report<sup>[7]</sup>. The ultimate approach to solving this problem is, perhaps to reduce the immunogenicity of antibodies, that is to humanize the antibodies.

Based on our previous results, the expression level of X gene of hepatitis B virus was much higher than that of s gene and c gene, the T/NT ratio of anti-HBx- mAb approached 4.5 in the nude mice models, clinical targeting therapy trial confirmed that anti-HBx mAb was an exciting vector, and a humanized chimeric Ab gene had been established<sup>[4,5,8]</sup>, we are trying to evaluate its potential value in the targeting therapy as a novel vector.

The results indicate that a distinct accumulation of tumor radioactivity developed on day 5 after injection of radio-labeled chimeric Ab, and an even more obvious accumulation was obtained on day 7. In bio-distribution analysis, the T/NT ratio on day 7 of the chimeric Ab, single segment Ab, positive control anti-HBx mAb, negative control TG1 and <sup>131</sup>I was 2.81, 2.44, 4.60, 0.41 and 0.96, respectively. This promising result suggests that recombinant Abs do have affinity to liver cancer tissues. Therefore, they can specifically direct against liver cancer tissues.

Although the chimeric Ab displayed certain high T/NT ratio, it did not surpass that of murine derived mAb. The reason behind this may be as follows: ①The Ab used in this experiment was the expression product of *E. coli*. When it was extracted from the bacteria by sonic cytolysis, the extracts were none other than primary yields of *E. coli*. They contained not only the heat-induced Ab protein, but also other impure proteins derived from the expression of *E. coli* itself. For fear of possible inactivity of the Ab by gel filtration method, the expression product was not further separated and

purified. This may account for the relatively low T/NT ratio in *E. coli*; ②The chimeric Ab was the product of prokaryotic expression vector. It is short of post-translational processing toward foreign protein by a eukaryotic system, such as glucylation, methylation, carboxylation, etc.. This may result in the production of an imperfect protein from which the experimental results may suffer.

In regard to the relatively high accumulation of radioactivity in blood, kidney, spleen, etc., the possible answer may be due to the impure Ab protein containing foreign substances which were apt to be uptaken by mononuclear cells, leading to the increase in the nonspecific binding.

The labeling ability was quite low for the chimeric Ab and single segment Ab in this experiment. Initially, we attributed this fact to fewer lysine groups of the recombinant Ab than those of murine derived mAb, for the principle of Iodogen method is to incorporate iodine with lysine groups of protein. Later, we found the amount of lysine groups of the genetic engineering Ab were no less than those of murine derived mAb. Therefore, our conclusion in terms of the low labeling ability of the recombinant Ab was the impurity of the protein with excess of foreign substances, and the lack of modification during post-translational processing. In a word, recombinant chimeric Ab has the advantages of low immunogenicity and high bio-efficiency of antibody-mediated ADCC and CDC, which certainly approve its qualification in clinical use. There is, however, much to be desired in terms of the practical use of chimeric Ab in clinical targeting therapy. Currently, work is underway in our institute to further separate and purify these chimeric Ab, make it expressed in eukaryotic systems in order to obtain intact secretory Ab.

In line with the advance of novel technologies in biomedical sciences, the increasing use of phage display antibody library technique, the antibodies, derived from the total antibody library, can be constructed *in vitro* without immunization *in vivo*. It can be well predicted that in the near future, certain active proteins with high affinity will be produced to benefit the mankind.

## REFERENCES

- 1 Liu KD, Tang ZY, Lu JZ, Fan Z, Yu YQ. Evaluation of radioimmunotherapy in the multimodality treatment of hepatocellular carcinoma (HCC). *Chin J Oncol*, 1992;14(6):430 - 432
- 2 Liu KD, Tang ZY, Fan Z, Lu JZ, Yu YQ, Zhou XD. Radioimmunotherapy in treatment of unresectable hepatoma: a report of 43 cases. *Chin J Cancer Res*, 1994;6(1):74 - 78
- 3 Sand H. Experimental studies of radioimmunodetection of cancer: an overview. *Cancer Res*, 1990;50(suppl):809 - 813
- 4 Zhou G, Liu KD, Tang ZY, Chen YH, Wu XF, Schroeder CH. Reconstruction and expression of chimeric anti HBx antibody *Escherichia coli*. *J Cancer Res Clin Oncol*, 1997;123(6):325 - 330
- 5 Li J, Tang ZY, Liu KD, Dai ZY. Application of radiolabeled anti-HBx monoclonal antibody for HCC targeting therapy. *Chin Natl J Med*, 1996;76(4):271-274
- 6 Liu KD, Yu D, Bao YM, Ma ZC, Tang ZY. Targeting study of human hepatocellular carcinoma (HCC) using human HCC model in nude mice. *Chin J Oncol*, 1988;10(6):414 - 416
- 7 Liu KD, Tang ZY, Lu JZ, Fan Z, Zeng SC, Xie H *et al.* Long-term results of targeting therapy using radiolabeled antibodies in multimodality treatment of hepatocellular carcinoma (HCC)-analysis of 75 cases. *Acta Acad Med Shanghai*, 1995;22(suppl):14 - 18
- 8 Li J, Tang ZY, Liu KD, Schroeder CH. Preparation of anti HBx MoAbs and determination of HBx antigen in liver cancer. *Chin J Med*, 1994;74(9):533 - 535

# Establishment of a nonradioactive assay for 2'-5' oligoadenylate synthetase and its application in chronic hepatitis C patients receiving interferon- $\alpha$

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**Subject headings** 2'-5' oligoadenylate synthetase/analysis; interferon-alpha; chromatography, thin layer; hepatitis C/enzymes

## Abstract

**AIM** To establish a nonradioactive assay for 2'-5' oligoadenylate synthetase (2-5 AS) and to measure the 2-5AS in peripheral blood mononuclear cell (PBMC) extracts of patients with chronic hepatitis C before IFN- $\alpha$  injection, 24 hours and one month after the first injection.

**METHODS** 2-5AS in cell extracts of PBMCs from 10 normal persons and 15 chronic hepatitis C patients were determined with PEI cellulose thin-layer chromatography.

**RESULTS** The assay of 2-5AS in human PBMC was found to be rapid, sensitive, specific and reliable. The 2-5AS activity of PBMC in normal persons was in a quite low level (2.0%), and it was increased about ten-folds after stimulation of IFN (19.7%), ( $P < 0.01$ ). In 15 chronic hepatitis C patients, the basal levels of 2-5AS before IFN treatment were higher than those of normal persons, being much higher in the group showing poor response to IFN treatment, but 24h after the first injection of IFN- $\alpha$  the 2-5AS level showed a more rapid and much greater rise in those patients with a good response.

**CONCLUSION** 2-5AS may be a useful parameter of biological response during the IFN therapy.

## INTRODUCTION

The double-stranded RNA (dsRNA) dependent enzyme, (2'-5') oligoadenylate synthetase (2-5AS), which was first discovered in interferon (IFN)-treated cells, could polymerize ATP into (2'-5') oligoadenylate (2-5A). These were probably related to the antiviral activity and perhaps antiproliferative effects of IFN, called 2-5AS system. Treatment of cells (such as peripheral blood mononuclear cells, PBMC) with IFN led to the de novo synthesis of 2-5AS; this enzyme could produce oligoadenylates from ATP in the presence of double-stranded RNA (dsRNA) according to the equation:  $(n + 1) ATP \rightarrow PPP5'A(2'P5'A)_{n+1}PPi$  ( $n \geq 1$ ). (2'-5') oligoadenylates greater than the dimeric form could activate an endoribonuclease (RNase L) which was able to cleave mRNA, leading to an inhibition of viral protein synthesis<sup>[1]</sup>.

Recently, some researchers determined 2-5AS level of PBMC in patients with chronic viral hepatitis as a biological response parameter during the therapy with IFN or in the diagnosis of diseases related to the IFN system. Now, lots of radioactive methods have been reported for measuring 2-5AS in EAT cells, Hela cells, Wish cells, rabbit reticulocytes and lymphocytes<sup>[2]</sup>. In this paper, we developed a non-radioactive assay for determining the 2-5AS in human PBMC: PEI-cellulose thin-layer chromatography.

## MATERIALS AND METHODS

### Preparation of rabbit reticulocyte lysate

Rabbit reticulocytes were prepared from adult male New Zealand rabbits (from Animal Laboratory of Beijing Medical University) by a modified method of Borsook *et al*<sup>[3]</sup>. One ml of a neutralized 2.5% aqueous solution of phenylhydrazine hydrochloride was injected subcutaneously each day, together with 0.05 mg of folic acid and 0.05 $\mu$ g of vitamin B<sub>12</sub> were injected intramuscularly daily, reticulocyte counting was performed on smears of blood drawn from ear veins and stained with brilliant cresyl blue in 0.15mol/L NS. On the 7th or 8th day, blood samples were drawn while over 90% of the circulating red cells were then reticulocytes.

The blood samples were added to a flask containing heparin, washed three times with 0.01 mol/L phosphate buffer solution (PBS) pH 7.4. After centrifugation, the supernatant was removed

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\*Supported by the grant from China Medical Board (93-582) of New York, Inc.

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Received 1997-06-18

completely and the cells were treated with equal volume of lysis buffer (20 mmol/L Tris-HCl pH 7.6, 5 mmol/L magnesium acetate, 30 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, 10% glycerol, 0.5% Nonidet P-40) at 4°C for 30 minutes. After centrifugation (15,000 × g, 10 minutes at 4°C), the 2-5 AS containing supernatant was collected and frozen at -70°C for use.

#### **Preparation of human PBMC extracts**

Human PBMC of 10 normal persons were isolated from heparinized whole blood (5-10ml) by Ficoll-Hypaque sedimentation (Lymphocyte Separation Medium). Then  $1 \times 10^7$  washed PBMC per ml were incubated 24 hours (37°C, 5% CO<sub>2</sub>) in RPMI-1640 supplemented with 20% fetal calf serum with or without adding IFN $\alpha$  (INTRON, Schering, NJ) at 1000 U/L. After washing three times with PBS, PBMC were sedimented at 9000 × g for 1 minute and then lysed by lysis buffer ( $1 \times 10^7$  cells per ml), and extracts were prepared by centrifugation of the lysed cells at 15 000 × g for 10 minutes at 4°C, then were stored at -70°C for use.

PBMC extracts from fifteen chronic hepatitis C patients were prepared as stated above, before IFN- $\alpha$  injection, 24 hours and also 1 month after the first injection of IFN $\alpha$ . They were stored at -70°C for use.

#### **Assay of 2-5AS**

Because 2-5AS can catalyze the synthesis of 2-5A, the detection of 2-5A might be a reflection of the 2-5AS activity. In this study, we used the PEI-cellulose thin-layer plates (Sigma, USA) to separate 2-5A from residual ATP. The plate can specially separate the chemical compound with over 3 molecule-phosphate residues from those with less than 3 molecule-phosphate residues by thin-layer chromatography. First, the residual ATP not polymere-rized into 2-5A was digested with digestion buffer (Hexokinase 2.52 g/L, 30 mmol/L glucose monohydrate, 10 mmol/L magnesium acetate) to transform it into ADP (with 2 molecule-phosphate residues), then the chromatography was carried out, so the 2-5A was separated from ADP. The results of chromatography could be seen under ultraviolet lamp.

Poly (I) : poly (C) agarose beads were washed with buffer A (20 mmol/L Tris-HCl pH 7.6, 5 mmol/L magnesium acetate, 30 mmol/L 2-mercaptoethanol, 1mmol/L EDTA, 10% glycerol) three times, then suspended in buffer A (1+1 by vol) and 40  $\mu$ l aliquots were transferred into Eppendorf tubes. After centrifugation and removal of supernatant, 400  $\mu$ l PBMC extracts (or 0.8 ml rabbit reticulocytes lysates) were added into the tubes, reaction continued for 1 h at 4°C. Following this binding reaction, the agarose-beads were washed four times with 800  $\mu$ l buffer A. The 2-5AS

reaction was started by addition of 25  $\mu$ l buffer B (buffer A + ATP 3 mmol/L, poly (I) : poly (C) 50 mg/L, creatine phosphokinase 0.1 g/L, creatine phosphate 6mmol/L), then incubated overnight at 37°C, the reaction was stopped by heating the mixtures (3-5 minutes at 95°C). After 2-5AS reaction, 20  $\mu$ l of incubation mixture supernatants was transferred into wells of microtiter plates, 5  $\mu$ l of digestion buffer were added (30 minutes at room temperature). Then all the samples were spotted onto a PEI-cellulose thin-layer plate (20 cm × 20 cm) 2 cm from the bottom. After drying the spots, the plate was immersed in methyl alcohol for 10 minutes with continuous shaking. After drying the plate, the chromatography was developed in chromatographic buffer (0.75 mol/L potassium phosphate tribasic anhydrous, pH 3.5) and developed to the top of the plate in this solvent, the procedure took about 10 minutes. The plate was then dried and exposed to ultraviolet ray: there was only one ultraviolet absorbing spot of "ADP" to the PBMC sample without 2-5AS activity, but there were two spots (one for "ADP"; another for "2-5A") to the sample with different 2-5AS level. Under UV analyzer, each spot was then cut out with scissors, to be immersed into 0.5 ml soaking buffer (20 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA, 1.5 mol/L NaCl) overnight, and absorbance at 259 nm was determined by the supernatant of each sample. The 2-5AS activity was calculated from the percentage of ATP turnover, according to the following formula:

$$\frac{OD_{2-5A}}{OD_{2-5A} + OD_{ADP}} \times 100\%$$

#### **Study designs**

Study 1: Each batch of rabbit reticulocyte lysate was subject to examination sixteen times.

Study 2: PBMC extracts from 10 normal persons were measured for 2-5AS with or without induction by IFN $\alpha$ , and the results were used for determining the varying responses in different normal persons to IFN $\alpha$ .

Study 3: Fifteen patients with chronic hepatitis C (11 male, 4 female; the average age for 40.7 years) who were treated with IFN $\alpha$  were studied. All the patients were positive for anti-HCV and HCV-RNA, but negative for anti-human immunodeficiency virus (HIV) and anti-hepatitis A, B and D, their diseases had lasted more than one year, and all the fifteen patients had elevated serum alanine aminotransferase (ALT) activities of at least twice the upper limit of normal before IFN $\alpha$  therapy.

The patients were given IFN $\alpha$  intramuscularly for 3 months, at a dose of 3 mega units (MU) daily during the first 4 weeks and then thrice weekly for 8 weeks. Determinations for 2-5AS of PBMC were

performed in samples obtained pre-treatment, 24 h and 1 month after the first injection. At the same time, the fifteen patients' serum samples were examined every two weeks for ALT and anti-HCV, HCV-RNA. This study was to determine the relation between 2-5AS level and the curative effect to IFN $\alpha$  for chronic hepatitis C patients.

### Statistical analysis

Statistical analysis was made with *t* test.

## RESULTS

### Digestive results of the residual ATP

To 25 $\mu$ l of the buffer B was added different volumes (0 $\mu$ l - 5 $\mu$ l per sample) of digestion buffer. After reaction, the incubated mixtures were spotted onto a plate and the chromatography was developed, the digestive percentage of ATP with different volumes of digestion buffer is shown in Table 1.

The digestive ratio of ATP was analyzed according to the similar formula of the turnover percentage of ATP:

$$\frac{OD_{ADP}}{OD_{ADP}+OD_{ATP}} \times 100\%$$

The results of Table 1 suggested that when 4 $\mu$ l of digestion buffer was added, the residual ATP in the incubation mixtures could be changed completely into ADP even if there was no 2-5AS activity in the sample. To ensure the accuracy of the assay, 5 $\mu$ l of digestion buffer was added.

### Results of rabbit reticulocyte lysates (study 1)

The results of rabbit reticulocyte lysate in the 16 determinations of a single sample for 2-5AS were 31.6  $\pm$  0.02 ( $\bar{x}$ % $\pm$ s), with CV of 6.0%.

### Results of 2-5AS in PBMC of 10 normal persons with or without induction by IFN- $\alpha$ (study 2)

PBMC extracts were examined for 2-5AS in one batch; the average levels of the cells before (Group A) and after (Group B) induction by IFN $\alpha$  are shown in Table 2.

### Results of 2-5AS in 15 chronic hepatitis C patients (Study 3)

In the fifteen patients, after IFN $\alpha$  therapy, the ALT normalization rate was 53.5% (8/15) and the ALT reduction rate (>50% decrease of baseline ALT) was 33.3% (5/15), with a total improvement rate (normalization plus reduction rate) of 86.6% (13/15); clearance rate of HCV-RNA was 60.6% (9/15) and of anti-HCV was 20.0% (3/15).

Before IFN $\alpha$  therapy, the 2-5AS average activity in PBMC of 15 patients was 12.6%, significantly higher than that of the normal group ( $P$ <0.01); the 2-5AS levels of patients 24 h and 1 month after the first injection was 64.9% and 40.4% respectively, an increase of 7.0 and 4.7

folds of that before IFN $\alpha$  injection.

These 15 patients were divided into two groups. In group A (9 patients), HCV-RNA disappeared after IFN $\alpha$  therapy, and group B (6 patients) HCV-RNA remained detectable after treatment. Their 2-5AS levels are shown in Table 3.

**Table 1 The digestive results of ATP**

The volume of digestion buffer ( $\mu$ l)	0	1	2	3	4	5
Digestive percentage of ATP (%)	0	3.1	18.3	34.1	100	100

**Table 2 2-5AS levels in different groups**

	Group A		Group B	
	Before IFN $\alpha$ stimulation	After IFN $\alpha$ stimulation	Before IFN $\alpha$ stimulation	After IFN $\alpha$ stimulation
<i>n</i>	10		10	
<i>x</i> (%)	2.0		19.7	
<i>s</i>	0.02		0.09	
<i>P</i>	<0.01			

**Table 3 2-5AS levels in groups A and B patients ( $\bar{x}$  $\pm$ s)**

Group	<i>n</i>	2-5AS level				
		pre <sup>a</sup>	24h <sup>b</sup>	24h/pre	1 month <sup>c</sup>	1 month/pre
A	9	9.3 $\pm$ 4.7%	72.1 $\pm$ 15.3%	9.4 $\pm$ 4.3	42.3 $\pm$ 8.2%	6.2 $\pm$ 5.0
B	6	17.5 $\pm$ 5.9%	54.1 $\pm$ 14.2%	3.4 $\pm$ 1.6	37.5 $\pm$ 7.0%	2.4 $\pm$ 0.8
<i>P</i>		<0.05	<0.05	<0.01	>0.05	>0.05

<sup>a</sup>pre: pre-treatment; <sup>b</sup>24 h: 24 h after first injection; <sup>c</sup>1 month: 1 month after first injection.

## DISCUSSION

Chronic viral hepatitis is one of the major infectious diseases that endangers the public health seriously and thus is an important world wide problem. At present, there is no effective medication other than interferon (IFN). It is known that IFN has antiviral, antiproliferative, and immunoregulating properties, and its therapeutic effect depends to some extent on the dose, schedule and route of administration. So the determination of the optimal dose, schedule and route of administration is an important way to improve the response of patients treated with IFN. But the concentration of IFN in peripheral blood is very difficult to determine because of its low blood concentration and short half-life. Recently, some people determined the interferon-induced enzymes (such as 2'-5' oligoadenylate synthetase, 2'-5' oligoadenylate phosphodiesterase and protein kinase *et al*) as a biological response parameter of IFN<sup>[4]</sup> therapy.

Since the assay of 2-5AS was first reported in 1979, lots of methods had been introduced<sup>[5-8]</sup>. In this study, a new nonradioactive assay of 2-5AS was developed, the results of 2-5AS in rabbit reticulocyte lysates showed that this assay was a sensitive, rapid and specific method. The assay of

2-5AS in PBMC of 10 normal persons with or without IFN induction showed the following results: ①The 2-5AS activity of PBMC in normal person was at a quite low level (2.0%), and the individual difference was obvious (0% - 7.1%,  $s = 0.02$ ); ②The 2-5AS level increased about ten-fold after stimulation of IFN (19.7%), and the difference of 2-5AS levels before and after IFN stimulation was significant ( $P < 0.01$ ); ③ There was much differences in the response of IFN stimulation in different persons (9.5% - 36.6%,  $s = 0.09$ ) and this might be a reason to explain a variable therapeutic effect of IFN in different patients.

Now, many reports have shown the relationship between 2-5AS level and the therapeutic effect of IFN in chronic hepatitis B patients<sup>[9,10]</sup>, but we have not seen any report about chronic hepatitis C. In our study, the newly developed assay of 2-5AS (PEI-cellulose thin-layer chromatography) was used to determine 2-5AS in 15 chronic hepatitis C patients treated with IFN. The results showed that: ①The 2-5AS activity of PBMC in patients with chronic hepatitis C before treatment was much higher than that of normal persons (12.6% *vs* 2.0%,  $P < 0.01$ ). In patients with effective treatment (HCV-RNA disappeared after IFN therapy), the 2-5AS activity before IFN therapy was lower than that in patients with ineffective treatment (HCV-RNA remained detectable after IFN therapy) (9.3% *vs* 17.5%,  $P < 0.05$ ). This might be associated with the endogenous IFN system, which has already been activated in patients with poor response to IFN therapy; the use of large doses of external exogenous IFN might inhibit the activated host IFN system. ②Twenty-four after the first injection, the 2-5AS of all 15 patients increased in different degrees, compared with its basal levels (average 7.0 fold increase), but the 2-5AS level in patients with effective treatment increased more significantly than in patients with ineffective treatment (72.1% *vs* 54.1%,  $P < 0.05$ ; 9.4 folds

*vs* 3.4 folds,  $P < 0.01$ ). This suggested that a greater increase of 2-5AS in PBMC 24 h after the first injection of IFN might be associated with the effectiveness of IFN therapy. ③One month after the first injection, 2-5AS level was also higher than its basal level (average 4.7 fold increase), but there was no difference between patients with good response and those without (42.3% *vs* 37.5%,  $P > 0.05$ ; 6.2 fold *vs* 2.4 fold,  $P > 0.05$ ).

In summary, the results of our study suggest that: ①The developed assay in this study (PEI-cellulose thin-layer chromatography) is a reliable way to determine 2-5AS of PBMC. ②In patients with chronic hepatitis C, a less elevated basal level of 2-5AS of PBMC before IFN therapy and a rapid and greater increase in 2-5AS 24 h after the first injection of IFN may anticipate a better response to IFN therapy.

## REFERENCES

- 1 Ferbus DJ, Justesen J, Bertrand H, Thang MN. (2'-5') Oligoadenylate synthetase in the maturation of rabbit reticulocytes. *Mol Cellular Bio*, 1984;62(1):51 - 55
- 2 Bruchelt G, Beck J, Schilbach-Stuckle K, Koscielniak E, Treuner J, Niethammer D. Methods for the determination of the interferon-induced enzyme 2'-5' oligoadenylate synthetase in mononuclear blood cells. *J Clin Chem Clin Biochem*, 1987;25(12):879-888
- 3 Borsook H, Deasy CL, Haagen-Smit AJ, Keighley G, Lowy PH. Incorporation in vitro of labeled amino acids into proteins of rabbit reticulocytes. *J Biol Chem*, 1952;96(3):669 - 694
- 4 Merritt JA, Borden EC, Ball LA. Measurement of 2'-5' oligoadenylate synthetase in patients receiving interferon-alpha. *J Interferon Res*, 1985;5(1): 191 - 198
- 5 Johnston MI, Preble OT, Imai J, Jacobsen H, Torrence PF. A sensitive immunoenzymometric assay for 2'-5' oligoadenylate. Detection of elevated 2'-5' oligoadenylate synthetase in human peripheral mononuclear cells. *J Immunol Methods*, 1983;65(1):123 - 135
- 6 Merlin G, Revel M, Wallach D. The interferon-induced enzyme oligoadenylate synthetase: rapid determination of its in vitro products. *Anal Biochem*, 1981; 110(1):190 - 196
- 7 Miele MB, Liu DK, Kan NC. Fractionation and characterization of 2'-5' oligoadenylates by polyacrylamide gel electrophoresis: an alternative method for assaying 2'-5' oligoadenylate synthetase. *J Interferon Res*, 1991;11(1):33 - 40
- 8 Schattner A, Merlin G, Wallach D, Rosenberg H, Bino T, Hahn T *et al.* Monitoring of interferon therapy by assay of (2'-5') oligoadenylate synthetase in human peripheral white blood cells. *J Interferon Res*, 1981;1(4):587 - 594
- 9 Furuta M, Akashi K, Nakamura Y, Matsumoto K, Yamaguchi H, Takamatsu H *et al.* 2'-5' Oligoadenylate synthetase activity in peripheral blood lymphocytes as a clinical marker in interferon therapy for chronic hepatitis B. *J Interferon Res*, 1987;7(1):111 - 119
- 10 Shindo M, Okuno T, Matsumoto M, Takeda M, Takino T, Sokawa J *et al.* Serum 2'-5' oligoadenylate synthetase activity during interferon treatment of chronic hepatitis B. *Hepatology*, 1988;8(4):366 - 370

# Simplified isolation and spheroidal aggregate culture of rat hepatocytes

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**Subject headings** liver cell; cell isolation; tissue culture; aggregates

## Abstract

**AIM** To explore a simplified method for isolation of hepatocytes and establish a method of primary hepatocyte culture with more aggregates and longer persistence.

**METHODS** Wistar rat hepatocytes were isolated by a single extracorporeal two-step perfusion method, and the cells were seeded on poly-HEMA coated flasks and cultured with hormonally defined medium and gentle shaking at regular intervals.

**RESULTS** The total yield of isolating hepatocytes amounted to  $10^8$  cells for each rat liver with the viability of more than 90% in all isolations. Under the nonadherent environments, the cells were found to attach to each other and form multicellular aggregates rapidly, and the aggregates became spheroidal shape after two days in culture. The morphologic characteristics and albumin synthetic function of the multicellular spheroidal aggregates can be maintained for one month.

**CONCLUSION** The simple and reliable isolation as well as large scale and longer time culture of hepatocytes can be used for experiments in liver cell transplantation and bioartificial liver support system.

## INTRODUCTION

Hepatocyte culture, as an optimal model in vitro, plays an important role in the studies of liver disease<sup>[1]</sup>. However, the collagenase perfusion technique in situ can not be performed easily, the usual culture of hepatocytes, seeded on tissue culture dish under classical culture conditions can not adapt to large scale and high density culture and can not maintain liver-specific functions for longer time, thus limiting the development and application of hepatocytes culture. In this study, we used a simplified two-step perfusion method and some effective measures to isolate the rat hepatocytes in vitro and culture hepatocyte as spheroidal aggregates.

## MATERIALS AND METHODS

### Animals

Wistar rats weighing 150 g - 200 g, were provided by the Experimental Animal Centre of Third Military Medical College. The animals have received standard laboratory diet.

### Cell isolation

Liver cells were isolated by an adaptation of the two-step perfusion method<sup>[2]</sup>. Briefly, the animals were anesthetized with barbital (30 mg/kg, b. w, intraperitoneally) and their livers were removed intact. The liver was first perfused in vitro via the portal vein with warmed (37°C)  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free Hanks balanced salt solution at a flow rate of 5 - 8 ml/min for 10 - 15 ml/min, and then perfused with 0.05% collagenase (Sigma, Type IV) in the same solution supplemented with 5 mM  $\text{CaCl}_2$  and 50 mM HEPES. The reperfusion with collagenase solution lasted 20min at a rate of 5 ml/min at 37°C. After 10min of incubation (37°C) with gentle shaking, the suspension was filtered and hepatocytes were sedimented at 50 g for 3min.

### Aggregate culture

Freshly isolated liver cells ( $4 \times 10^5$  in 1 ml of RPMI 1640 medium) were seeded in culture flasks precoated with poly (2-hydroxyethyl methacrylate) (poly-HEMA, Sigma) and cultured in a humidified atmosphere of 5%  $\text{CO}_2$  and 95% air at 37°C. In order to inhibit cells attachment on wells and promote cells aggregation, the culture flasks were rotated at a regular interval. After 4 h of incubation, RPMI 1640 medium was changed by a hormone defined medium (HDM) composed of Williams medium E supplemented with 10 mg/L insulin, 10  $\mu\text{g/L}$  HGF, 50  $\mu\text{g/L}$  EGF, 100  $\mu\text{g/L}$

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\*Project supported by the National Natural Science Foundation of China, No. 39370189.

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Received 1997-05-20

hydrocortisone, 100 µg/L glucagon, 50 mg/L linoleic acid, 100 U penicillin, 0.1 mg/ml streptomycin, 1 mg/L amphotericin B and 10% FCS (mainly reagents were purchased from Sigma). The medium was changed every day.

### Histology

Cell morphology in the culture was observed under Olympus phase contrast microscope. For electron microscopy the spheroidal aggregates of hepatocytes cultured for different time periods were fixed at 4°C for 4 h with 3% glutaraldehyde in 0.1 M phosphate buffer and post-fixed for 1 h with 1% OsO<sub>4</sub>, dehydrated in graded ethanol solution and embedded in Epon 618. Ultrathin sections were prepared conventionally and observed with a JEM-2000SX transmission electron microscopy (TEM). Scanning electron microscopy (SEM) sample was dehydrated by the same method and further dehydrated by critical point-drying. A thin-layer of gold was deposited on the cell surface by a sputtering system and examined in AMRAY 1000B SEM.

### Albumin production

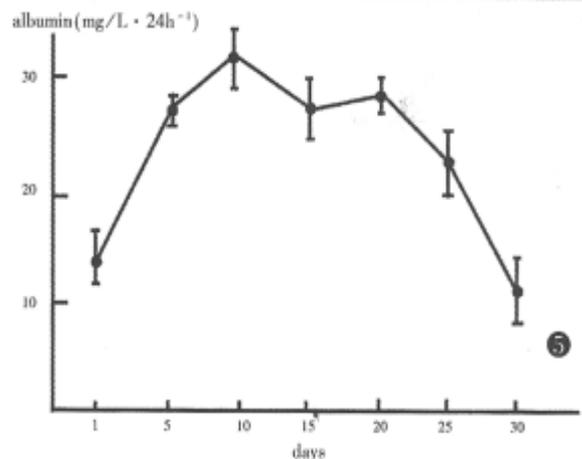
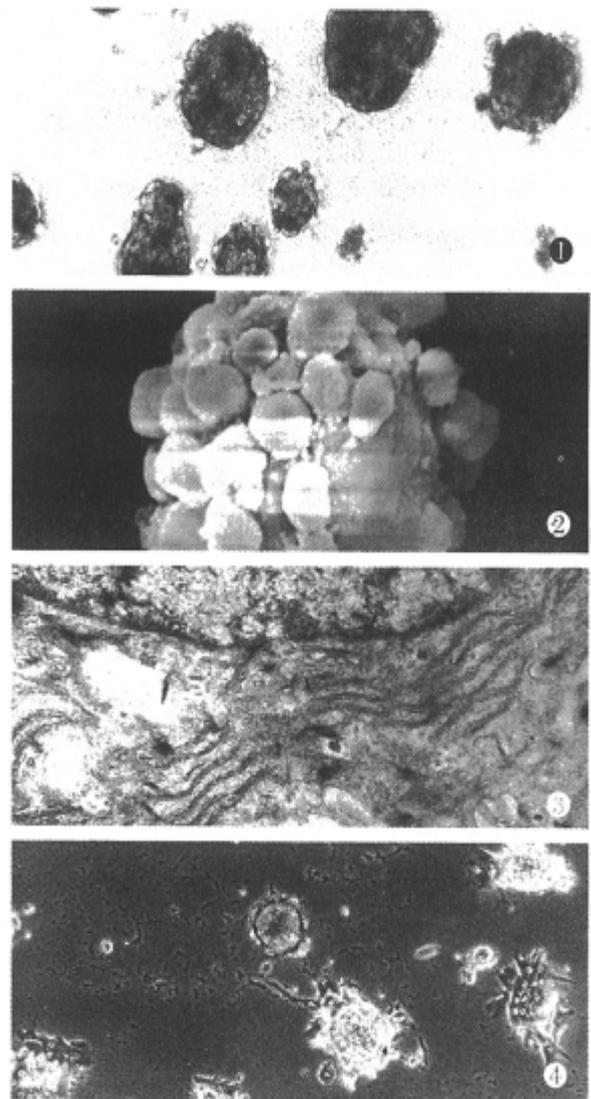
The albumin synthesis by hepatocytes in culture media was determined on the Beckman CX-7 Synchron Clinical System.

## RESULTS

The total yield of isolated hepatocytes by the simplified two-step perfusion method amounted to 2 - 4 × 10<sup>8</sup> cells for each liver. The viability of hepatocytes as judged by the trypan blue test was estimated as 90% - 98% in all isolations.

Under the defined culture environment and repeated gentle shaking, the freshly isolated hepatocytes attached to each other within 4 h - 8 h and multiple aggregates of different sizes were loosely formed and the multiplicity of aggregates increased along with time. Up to 48 h, a lot of regular spheroidal aggregates were seen in flasks. The aggregate hepatocytes spheroids appeared very tight and dense in centre, and on the surface of the spheroids attached a lot of single cells as flower (Figures 1,2). Through action of single cells, some spheroids were also attached to each other. The characteristics of spheroidal aggregates can be maintained until the 30th day of culture. At the same time, TEM revealed that the cultured hepatocytes had a large round nucleus and abundant cytoplasmic organelles as observed in normal ones (Figure 3).

When the aggregate culture stopped, cell spheroids were transferred into collagen-coated wells. After 24h of incubation (37°C, 5% CO<sub>2</sub>) approximately, 74% - 81% spheroids were seen to attach on substratum and many cells incorporated into spheroids migrated out forming a monolary (Figure 4).



**Figure 1** Phase-contrast microscopic feature of multicellular spheroidal aggregates of hepatocytes at day 4. (× 200)

**Figure 2** Scanning electron microscopy of spheroidal aggregates at day 14. (× 1000)

**Figure 3** Transmission electron microscopy of hepatocyte spheroids cultured for 25 days, showing abundant organelles in hepatocytes. (× 10 000)

**Figure 4** Phase-contrast photomicrograph of hepatocyte spheroids attached to collagen-coated wells. (× 100)

**Figure 5** Albumin synthesis and secretion by aggregate hepatocytes.

Secretion of albumin by hepatocytes was virtually detectable in the aggregate culture. By 5 days in culture, a marked increase in albumin production was seen, which can be maintained until the 25th day of culture. By 30 days, albumin secretion decreased rapidly (Figure 5).

## DISCUSSION

Until recently, hepatocytes are isolated according to the two-step perfusion method devised by Seglen. But as the method required a special apparatus and a lot of collagenase for liver perfusion in situ it is not so easy to completely dissociate the hepatocytes and to perform hepatocytes culture in ordinary laboratories. In our experiment, the rat liver was removed and perfused with collagenase in vitro. Although the yield of isolated hepatocytes is relatively low, the number still amounted to 108 cells and more than 90% of hepatocytes were viable. The equipment necessary for use was simple, the dose of collagenase was much lower than by Seglen method, and it can be adjusted according to the requirements of hepatocytes. These denote that the collagenase perfusion in vitro is a simple and reliable method for isolating hepatocytes from the biopsied samples and animal livers, which may be useful in performing hepatocytes culture widely.

Under the standard monolayer culture conditions, maintenance of survival and different functions of cultured hepatocytes have proven to be difficult. It is also difficult to provide large scale production of hepatocytes for some special use. Therefore some attempts have been made to increase the functional longevity and large scale production of hepatocytes in primary culture. For example, cultures of rat hepatocytes on Biosilon microcarriers allow one to obtain large metabolic active cells<sup>[3]</sup>, cocultures of human or rat hepatocytes with rat liver epithelial cells allow the

maintenance of several liver functions up to 2 months<sup>[4]</sup>.

But the microcarrier method and coculture are unfavourable in use. Since the culture of neonatal rat liver cells in spheroidal aggregates was described by Landry *et al*<sup>[5]</sup>, special attention has been paid to the culture method. Because the spheroids were formed in three-dimensional structures where cell-cell contacts are maximized, and the reaggregate and re-form structures resembled in some aspects of those found in vivo. Under those cultural conditions, the hepatocytes can maintain viability and functional integrity for months. Our experimental results were in agreement with that, albumin was still secreted by cell aggregates up to 30 days of culture, at the same time, the cells were still capable of attaching collagen-coated wells possibly due to the effective methods including poly-HEMA, HDM, cell growth factors and a rotating method.

Culturing of hepatocytes as spheroidal aggregates may be preferable to monolayer culture as an experimental liver model. The exciting potential applications may include the use of these hepatic spheroids for investigation in therapeutic measures for acute hepatic failure, such as hepatocytes transplantation and the construction of extracorporeal bioartificial liver support systems.

## REFERENCES

- 1 Moshage H, Yap SH. Primary cultures of human hepatocytes: a unique system for studies in toxicology, virology, parasitology and liver pathophysiology in man. *J Hepat*, 1992;15(3):404 - 413
- 2 Seglen PO. Preparation of rat liver cells. II. Effects of ions and chelators on tissue dispersion. *Exp Cell Res*, 1973;76(1):25 - 30
- 3 Shnyra A, Bocharov A, Bochkova N, Spirov V. Large-scale production and cultivation of hepatocytes on Biosilon microcarriers. *Artifit Organs*, 1990;14(6):421 - 428
- 4 Clement B, Guguen-Guillouzo C, Campion JP, Glaise D, Bourel M, Guillouzo A. Long term co-cultures of adult human hepatocytes with rat liver epithelial cells: modulation of albumin secretion and accumulation of extracellular material. *Hepatology*, 1984;4(3):373 - 380
- 5 Landry J, Bemier D, Ouellet C, Goyette R, Marceau N. Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J Cell Biol*, 1985;101(3):914 - 923

# Effect of aging on cytoskeleton system of Kupffer cell and its phagocytic capacity \*

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**Subject headings** Aging; Kupffer cell; cytoskeleton; phagocytosis

## Abstract

**AIM** To investigate the age-related alterations of cytoskeleton system in liver Kupffer cell and their relation to the changed phagocytic function.

**METHODS** The phagocytic function of Kupffer cells from rats of various ages (6 mo, 12 mo, 18 mo and 24 mo) were quantitatively evaluated by phagocytosis of polystyrene beads. The actin distribution and measurement of Kupffer cell were determined by a phalloidin-TRITC method; and the myosin and vimentin distribution and measurement with indirect immunohistochemical staining.

**RESULTS** Aging resulted in significant alterations of actin, myosin and vimentin distributions and reductions in Kupffer cell; the 3 cytoskeleton components of 24-month-old Kupffer cell were significantly decreased to 68.0%, 84.9% and 75.5%, respectively of these of 6-month-old Kupffer cell ( $P < 0.01, 0.01$  and  $0.01$ ). And these decreases had significant positive relations with the damaged phagocytosis of the aged Kupffer cell.  $\gamma$  values were 0.96 ( $P < 0.05$ ), 0.99 ( $P < 0.01$ ) and 0.95 ( $P < 0.05$ ) respectively.

**CONCLUSION** The cytoskeleton system of the aged Kupffer cell presents an evident state of senescence, which may be an important mechanism of decreased phagocytosis of the aged Kupffer cell.

## INTRODUCTION

Kupffer cells account for about 30% of nonparenchymal liver cells and constitute the largest pool of resident macrophages in the body. They play an essential role in the elimination of foreign substance derived from the systemic circulation mainly through phagocytosis. The age-related alterations in Kupffer cell function are considered to be related to the susceptibility to sepsis after trauma or infection or to tumor of the old people<sup>[1]</sup>. In this study, the distribution and contents of actin, myosin and vimentin in Kupffer cells of various ages, and a quantitative evaluation of phagocytosis of polystyrene beads by primary cultured Kupffer cells of various ages will be described.

## MATERIALS AND METHODS

### *Animals and groups*

Twenty-four Wistar rats of different ages obtained from the Chinese Herb Research Institute of Sichuan Province were rendered into 4 groups (6, 12, 18 and 24 months of age). Each group had 6 rats.

### *Kupffer cell isolation and culture*

Liver nonparenchymal cells were isolated by a collagenase-perfusion method as reported previously<sup>[2]</sup>. Briefly, after anesthesia (30 mg of barbital/kg body wt., intraperitoneally), the liver was perfused *in situ* with  $\text{Ca}^{++}$ -free Hanks balanced salt solution at 37°C for 3min. Then 0.05% collagenase (Sigma, Type IV) was added and the liver was perfused for a further 4min with Hanks balanced salt solution. After gentle shaking, the suspension was filtered and hepatocytes were sedimented at  $50 \times g$  for 3min. Non-parenchymal cells from the treatment were collected and sedimented at  $300 \times g$  for 10min. The pellet of nonparenchymal cells was resuspended and cultured with RPMI 1640 medium (containing 15 mmol/L HEPES, 0.05 U/ml insulin, 15 mmol/L L-glutamine, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin) supplemented with 10% newborn calf serum. After 30min, the non-adherent cells were deleted. The viability of the KC was greater than 90% as determined by trypan blue exclusion.

### *Distributions and content measurements of actin, myosin and vimentin of Kupffer cell*

Kupffer cells were fixed on a plastic dish and the phalloidin-TRITC (Sigma) method was used for

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\*Granted by the Military "8th Five" Research Funds.

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Received 1997-05-09 Revised 1997-08-21

staining actin by a previously reported method<sup>[3]</sup>. The samples were observed under fluorescence microscope (Olympus VANOX) and the actin contents were determined using a fluorometer (Hitachi MPF-4). Indirect immunochemical staining was performed for myosin and vimentin using anti-myosin antibody and anti-vimentin antibody(Sigma)<sup>[4]</sup>. The samples were observed by using a 40-fold objective (Olympus VANOX). The staining images were collected by means of a Panasonic CL320 videotape camera and sent into an image analyzing computer system (CMIAS007) for quantitative assessment of the gray scale.

#### Quantitative analysis of phagocytic activity

The phagocytic function of Kupffer cell was quantitatively measured by a method previously reported<sup>[4,5]</sup>. Cultured Kupffer cells were incubated with RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C for 6 h. After the incubation,  $1.7 \times 10^8$  polystyrene beads/dish (diameter 1.1  $\mu\text{m}$ , Sigma) were added to the cultures, which were maintained for a further 60min at 37°C. The cultures were washed three times with RPMI 1640 medium and the numbers of beads in Kupffer cells were counted under an inverted phase contrast microscope (XSJ-D), Chongqing Optical Electric Appliances Plant). One hundred Kupffer cells randomly selected from each of 5 different cell preparations were used for the phagocytosis study.

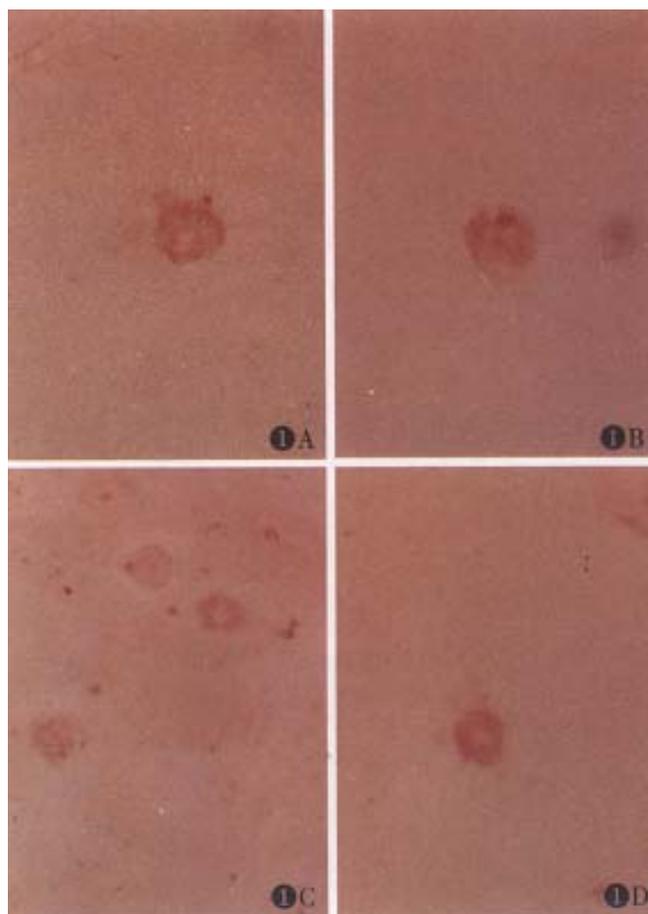
#### Statistical analyses

The results were expressed as  $\bar{x} \pm s$  and statistical analyses were made with one-way analysis of variance and Student's *t* test.

## RESULTS

#### Distribution of actin, myosin and vimentin in rat Kupffer cells at various ages

Aging caused morphological changes in cytoskeleton system of Kupffer cells are shown in Figure 1. Kupffer cells of 6-mo-old rat appeared flat and there were several pseudopodia on the surface. The myosin was shown in the pseudopodia. In fluorescent staining for actin, intense specific fluorescence was observed in both the cytoplasm and the peripheral region along pseudopodia. The distribution of actin was similar to that of myosin. Vimentin positive fibres extended throughout the whole cytoplasm and a perinuclear accumulation was also present in 6-month-old Kupffer cells. Kupffer cell of 24-month-old rat appeared small and circular and the number of pseudopodia decreased. Weak and diffuse fluorescence and staining could be observed around the pseudopodia and the nuclei of the aged cells in actin and myosin staining respectively. Weak staining could also be observed throughout the cytoplasm of the aged Kupffer cells in vimentin staining.



**Figure 1** Distribution of myosin and vimentin of 6-month-old (a,b) and 24-mo-old(c,d) Kupffer cells (ABC $\times$ 400). a and c: myosin;b and d: vimentin.

**Table 1** The actin, myosin and vimentin contents of Kupffer cells of each age group ( $\bar{x} \pm s$ )

Group	Actin (n = 6) (ng phalloidin/ $\mu\text{g}$ prot.)	Myosin (n = 5) (gray scale)	Vimentin (n = 5) (gray scale)
6 mo	8.63 $\pm$ 0.60	134.88 $\pm$ 8.14	137.50 $\pm$ 5.71
12 mo	8.24 $\pm$ 0.53	141.46 $\pm$ 5.18	129.91 $\pm$ 7.01
18 mo	6.20 $\pm$ 0.71 <sup>b</sup>	121.89 $\pm$ 7.52 <sup>a</sup>	118.13 $\pm$ 13.54 <sup>a</sup>
24 mo	5.87 $\pm$ 0.49 <sup>b</sup>	114.45 $\pm$ 5.09 <sup>b</sup>	103.08 $\pm$ 8.41 <sup>b</sup>

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01, vs 6 mo.

#### Quantitative measurement of the 3 cytoskeleton components of Kupffer cells at various ages

Aging resulted in significant reductions of the 3 cytoskeleton components mentioned above, i. e., the contents of actin, myosin and vimentin in 24-mo-old KC were significantly decreased to 68.0%, 84.9% and 75.5% respectively of those in 6-mo-old KC (*P*<0.01,0.01 and 0.01), (Table 1).

#### Phagocytic activities of Kupffer cells from each group

In the 6-mo group,  $19.6 \pm 2.1$  beads were taken up by cultured Kupffer cells during the 60min observation period. In 12-mo, 18-mo and 24-mo

groups,  $20.8 \pm 2.1$ ,  $12.7 \pm 1.5$ ,  $8.6 \pm 2.3$  beads were taken up into Kupffer cells respectively. The difference between 6-month-old group and 12-month-old group was not significant ( $P > 0.05$ ). The difference between 6-month-old group and 18-month-old group and that between 6-month-old group and 24-month-old group were significant ( $P < 0.05$ ).

### Analysis of correlation

Significant positive correlation was found between the changes of actin, myosin and vimentin contents in Kupffer cell aging and the damaged phagocytosis.  $\gamma$  values were 0.96 ( $P < 0.05$ ), 0.99 ( $P < 0.01$ ) and 0.95 ( $P < 0.05$ ), respectively.

## DISCUSSION

Kupffer cell is the main component of the host monocyte-macrophage system. It is crucially important for the host to fight against infection or sepsis<sup>[6]</sup>. The previous study showed that the decreased phagocytosis of the aged Kupffer cell was responsible for the increased severity of pathophysiological changes after endotoxemia<sup>[7]</sup>. So it is of significance to study the mechanism of decreased phagocytosis in Kupffer cell aging.

A 24-month-old rat was used in the present experiment as the aging model, which was comparable to the age of 65 - 75 in the human being, an age period consistent with the standard of old people of our country. So 24-month-old rat may serve as a qualified model for the study of Kupffer cell aging<sup>[2]</sup>.

The mechanism of phagocytosis by Kupffer cells is still not completely understood. The ruffling of cell membrane and formation of pseudopodia play an important role in the phagocytosis of Kupffer cells and this is believed to be accomplished by the cytoskeleton. In the cytoskeleton, actin-myosin interaction through the calcium-calmodulin systems plays a major role in this activity<sup>[8]</sup>. In this system, intracellular  $Ca^{2+}$  combined with calmodulin to form the active calcium-calmodulin complex, which activates an enzyme, myosin light chain kinase, for phosphorylating the light chain of

myosin. Phosphorylated myosin, but not unphosphorylated myosin, can interact with actin to induce the activity of cell membrane and pseudopodia and then phagocytosis. The process of this system is reversible, in that a phosphatase can catalyze dephosphorylation of myosin, restoring it to a form that can not be activated by actin.

Vimentin is another important cellular cytoskeleton component. It is in radial arrangement in cytoplasm, forming a frame to support the actin-myosin system and other organelles. It can prevent the cell from being injured by changing its tension, thus keeping the cellular shape.

In the current study, the distribution and determination of actin, myosin and vimentin in Kupffer cells at various ages were studied and our observations revealed that the distributions of the 3 cytoskeletons dramatically changed and their contents significantly decreased in aged Kupffer cells. The actin fluorescence and myosin staining in the area of the pseudopodia became weak in the aging Kupffer cells. The vimentin staining throughout the whole cytoplasm became weak too. These results indicate that the age-related damage of the cytoskeleton system in Kupffer cell aging is one of the important mechanism responsible for a decrease in phagocytosis.

## REFERENCES

- 1 Brouwer A, Parker SG, Hendriks HF, Gibbons L, Horan MA. Production of eicosanoids and cytokines by Kupffer cells from young and old rats stimulated by endotoxin. *Clin Sci*, 1995;88(2):211 - 217
- 2 Sun WB, Han BL, Peng ZM, Ma RL, Cheng J, Duan HC *et al.* Establishment and evaluation of Kupffer cell model for aging study. *Chin J Geriatr*, 1995; 14(6):357-359
- 3 Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T. Fluorescent phalloidin, a tool for the visualization of cellular actin. *Proc Natl Acad Sci USA*, 1979;76 (9):4498-4502
- 4 Hirose M, Watanabe S, Ueno T, Kitami N, Sato N. Pertussis toxin-induced redistribution of cortical actomyosin and inhibition of phagocytosis in rat Kupffer cells. *J Gastroenterol Hepatol*, 1993;8(2):348 - 350
- 5 Sun WB, Han BL, Chen J, Ma RL, Duan HC, Wang HZ. The effect of aging on the Kupffer cell's phagocytosis and susceptibility to endotoxin. *Chin J Expe Surg*, 1995;12(5):263 - 264
- 6 Albright JW, Albright JF. Aging alters the competence of the immune system to control parasitic infection. *Immunol Lett*, 1994;40(3):279 - 285
- 7 Knook DL, Brouwer A. Kupffer cells and the acute phase response: the effect of aging. *Immunol Invest*, 1989;18(1-4):339 - 350
- 8 Bretscher A. Microfilament structure and function in the cortical cytoskeleton. *Ann Rev Cell Biol*, 1991;7(1):337 - 374

# Pancreatic tumor: DSA diagnosis and treatment

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**Subject headings** pancreatic neoplasms/diagnosis; pancreatic neoplasms/therapy; angiography, digital subtraction

## Abstract

**AIM** To study the clinical significance of the diagnosis and catheterized interventional treatment of digital subtraction angiography (DSA) for pancreatic tumors.

**METHODS** Ninety-two patients with pancreatic tumor, 69 males and 23 females, aged from 41 to 70 years (mean 57.1 years) were diagnosed with DSA. Sixty-one patients with pancreatic cancer were treated with transcatheter celiac and superior mesenteric arterial anticancer agents (MMC 20mg, EADM 40 mg and 5-FU 2.0 g) infusion (TCSAI).

**RESULTS** The DSA diagnoses were confirmed by operations and pathological examinations, with a coincidence 82.6% rate of, and a therapeutic effective rate of 42.6%.

**CONCLUSION** DSA is of diagnostic value for pancreatic tumors, and helpful in understanding the course of the disease, judging the prognosis and selecting the therapeutic regimen, and could improve the chemotherapeutic effect as well.

## INTRODUCTION

In order to study the clinical significance of the diagnosis and catheterized interventional treatment of digital subtraction angiography (DSA) for pancreatic tumors, 92 cases of pancreatic tumor were diagnosed with DSA, and 61 cases of pancreatic cancer were treated with transcatheter celiac and superior mesenteric arterial infusion of anticancer agents (TCSAI) from Nov. 1987 to April 1997, with satisfactory results.

## MATERIALS AND METHODS

### Patients

Ninety-two patients (69 men, 23 women, aged from 41 - 70 years, averaging 57.1 years), were diagnosed as having pancreatic tumor through history of disease, B-ultrasonography, PTC and/or ERCP and CT.

### Methods

Abdominal aortography was performed in the 92 cases 8 times, selective celiac arteriography 18 times, combined angiographies of superior mesenteric artery 22 times, common hepatic artery 22 times, proper hepatic artery 15 times and gastroduodenal artery 11 times (Siemens Angiotron-CMP Bicolor DSA System) by percutaneous transfemoral-artery catheters in Seldinger's way, 10 ml - 50 ml 76% meglucamine diatrizoate for each case. Among them, 61 cases of pancreatic cancer were treated with TCSAI for a total of 93 times. The anticancer agents (MMC 20 mg - 40 mg, FADM 40 mg, 5-Fu 2 g) were divided into 2 equal parts, and infused into the celiac artery and superior mesenteric artery, respectively. The cases of early and intermediate cancer were infused after resection and the cases of late and recurrent cancer were infused immediately after arteriography. In accordance with the patient's condition and curative effect, TCSAT was performed once every 3-4 weeks, but not more than 3 times in 61 patients.

## RESULTS

### Diagnosis

DSA diagnoses of 92 cases were confirmed by operations and pathological examinations. Among them, 89 cases had pancreatic cancer, which was located in the head of pancreas in 67 cases, in body of pancreas, 18 cases and in tail, 4. Thirty-nine were complicated with metastatic hepatic cancer, 21 had metastatic celiac cancer and 3 cases had pancreatic cystadenocarcinoma. The diagnostic coincidence rate was 82.6% (76/92), without

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\*Reported at International Meeting of Exchange of HUAXIA Medical Academic Thesis, Hangzhou, China, 1997.

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Received 1997-07-22

positive misdiagnosis rate, and the negative rate was 17.4%(16/92), which may be caused by less blood supply and underdevelopment. DSA examination had no obvious adverse reactions.

### Treatment

The therapeutic effective rate (including the cases whose subjective symptoms were improved) was 42.6% (26/61). Tumors were either put under control or reduced in size in 31.1%(19/61). In 39 advanced cases of complicated with metastatic hepatic cancer, the 1-year survival rate was 51.3% (20/39), 3-year survival rate, 15.4% (6/39), and one patient died of late failure within 30 days. The main adverse reactions of TCSAI were nausea, vomiting anorexia, abdominal distention and so on, which usually disappeared in 1-3 days, and WBC decreased temporarily in 1-2 weeks. No severe complications happened.

### DISCUSSION

To define the relationship between cancer and branches of gastroduodenal, hepatic artery, superior mesenteric vessels, portal vein and abdominal aorta is the key to decide whether to perform radical resection or enlarging resection (including superior mesenteric vessels resection). The other examinations used now for pancreatic tumors include B-ultrasonography, hypotonic duodenography, PTC, ERCP, CT and so on. However, B-ultrasonography can be disturbed easily by intra-abdominal gas and obesity hypotonic duodenography, PTC and ERCP can not show the range and diffusion of pancreatic cancer and relation of the tumor to peripheral vessels and CT can reveal involved vessels, but only the cross sections and not the whole length, while DSA was of the peculiar diagnostic value, which could not be substituted by the other examinations, in understanding the relationship between cancer and vessels.

Although DSA is more advanced than common angiographies, DSA originally does not belong to selective angiography<sup>[1]</sup>, so that the crisscross and overlap of the vascular images may often hinder the diagnosis when there are many vessels in the examined size at the same time. In order to remedy this defect, we have improved the contrast techniques and methods to combine DSA with selective angiography so as to greatly raise the image quality and spatial resolution. That DSA is applied to celiac, superior mesenteric, hepatic and gastroduodenal arteriographies can continuously show the arterial,

capillary and venous phases of the tumors in turn, so as to know size, shape, infiltrative range, metastasis and vascularity of the cancer, and further define the pathologic quality, disease course and assist in the colocalization of cancers.

Pancreatic cancers belong to ischemic tumors, therefore DSA arterial phase is the most important in the diagnosis of the cancer. Capillary phase and venous phase are of the significance of assistant diagnosis, the arterial phase mainly shows abnormal flexion, irregular dilatation, plexiform aggregation or interruption of gastroduodenal artery, superior and inferior pancreaticoduodenal arteries caused by compression and erosion of the cancer. In early stage, the appearance of gastroduodenal artery is normal or mildly stiff and displaced. In advanced stage, cancer infiltrates peripheral arteries, even hepatic artery and its branches, through pancreatic capsules. Carcinoma of body and tail of pancreas usually infiltrates superior mesenteric artery and splenic artery so that they can be irregular, flexional, stiff, constrictive or interrupted. Capillary phase usually shows filling defect in the site of cancer when pancreatic parenchyma is stained. Normal pancreatic head has so rich blood circulation that the staining is deeper in the head than in the body and tail. Early carcinoma of pancreatic head makes the staining of the head light. In late stage the staining disappear. Venous phase can show that portal vein, superior mesenteric vein and splenic vein are involved in advanced cancers. Pancreatic cystadenoma is also an ischemic lesion, the peripheral blood vessels are compressed and displaced outward and dilated, without the signs of stiffness, constriction and interruption, and the tumor margin is clear.

DSA high-quality images greatly raise the accuracy, safety and chemotherapeutic effect of TCSAI. Higher concentration anticancer agents act directly on the cancer and the lymph nodes to produce cytotoxins, block the synthesis of DNA in cancer cells and reduce the ineffective distribution of the agents in general normal tissues. The infusion can also give a better therapeutic effect to hepatic metastasis<sup>[2]</sup>, resulting in inhibiting cancer growth, metastasis and recurrence, and increasing the possibility of resection.

### REFERENCES

- 1 Wu JP, Qiu FZ, Huang Jia-Si's Surgery. Fifth edition, Beijing: The People's Health Press, 1992:211
- 2 Xu HB, Zhang YJ, Wei WJ, Xiao YQ, Jia SL, Qian ZP *et al.* The role of DSA in diagnosis and treatment of inoperable liver cancer. *Chin J Clin Oncol*, 1992;19(6):451 - 453

# Bile acids in serum and bile of patients with cholesterol gallstone

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**Subject headings** cholesterol gallstone; bile acids/analysis; deoxycholic acids/analysis; chromatography, gas

## Abstract

**AIM** To analyze serum bile acids and biliary lipids of patients with cholesterol gallstone (CS) and explore the relationship between deoxycholic acid (DCA) and CS disease.

**METHODS** Analysis of bile acids in serum was done with gas-chromatography in two groups: CS group ( $n = 151$ ) and control group ( $n = 256$ ). Serum bile acids and biliary lipids were also studied in 90 matched samples.

**RESULTS** The serum DCA was  $0.955 \mu\text{mol/L} \pm 0.078 \mu\text{mol/L}$  in CS group, which was more than that of control group ( $0.696 \mu\text{mol/L} \pm 0.047 \mu\text{mol/L}$ ),  $P < 0.01$ . The ratio of DCA/chenodeoxycholic acids (CDCA) was  $1.76 \pm 0.30$  in CS group, about two times that in control group ( $0.92 \pm 0.14$ ). The mole percent of DCA in bile was positively related to cholesterol saturation index (CSI) ( $P < 0.01$ ) and the mole percent of CDCA in bile negatively to CSI ( $P = 0.01$ ). There was correlation between the mole percent of DCA, CDCA and cholic acid in bile and in serum.

**CONCLUSION** It is suggested that DCA is lithogenic and the increased amount of DCA or the ratio of DCA/CDCA in serum may be one of the features of cholesterol gallstone patients.

## INTRODUCTION

Bile acids may play a role in the formation and dissolution of cholesterol gallstones. Chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) are litholytic bile acids, which can dissolve cholesterol fraction of gallstones and used in prevention. Deoxycholic acid (DCA), however, as one of lithogenic bile acids has attracted great attention<sup>[1]</sup>. The purpose of this study was to analyze bile acids in both serum and bile and biliary lipids in cholesterol gallstone patients, and find out the relationship between bile acids and biliary lipids, especially between DCA and biliary lipids.

## MATERIALS AND METHODS

### Subjects

**Two groups were studied** The cholesterol gallstone group had 151 patients. Among them 88 had cholesterol gallstones classified by both the observation of the cross section of stones obtained during cholecystectomy and chemical analysis of their components. The other 63 asymptomatic patients had radiolucent gallstones and well-functioning gallbladders shown by oral cholecystography. The control group included 247 healthy individuals by both physical examination and B-ultrasonography and 9 patients without gallstones confirmed at the time of non-biliary surgery. None of the subjects had any evidence of hepatic or renal abnormality, or other diseases such as diabetes, hypertension and coronary heart disease. Both gallbladder bile and venous blood were obtained during elective cholecystectomy. For the nonoperative subjects, only fasting blood was available.

### Study protocol

In analysis of serum bile acids, the gallstone group comprised 63 males and 88 females, with an age range of  $52.6 \pm 11.5$  years, and the control group 169 males and 87 females, with an age range of  $50.0 \pm 10.3$  years.

In analysis of matched serum and biliary bile acids, all the subjects had undertaken abdominal operation. Eighty-one with cholecystectomy had cholesterol gallstones and 9 with non-biliary operations had no stone in the gallbladder, including 37 males and 53 females with a mean age of  $50.5 \pm 11.0$  years.

### Assay of bile acids

Bile samples were directly hydrolyzed with NaOH.

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Received 1997-09-20

Two ml blood was extracted through Sep-pak® C<sub>18</sub> cartridge and then hydrolyzed with NaOH. Bile acids from both bile and serum were treated as hexafluoroisopropyl ester trifluoroacetyl derivatives and measured quantitatively by a Shimadzu GC-9A gas-chromatograph equipped with electron capture detector<sup>[2]</sup>. A glass column packed 1.5% QF-1/Chromsorb W AW DMCS (2.1 m × 3.2 mm ID) was used. The operating procedure was as follows: injection and detector temperature, 300°C; column temperature, 240°C; and nitrogen flow-rate 60 ml/min.

#### Biliary cholesterol saturation index (CSI)

Biliary cholesterol was measured by enzymatic method and phospholipids by Bartlett's method. The total bile acids (TBA) was summed by DCA, CDCA and cholic acid (CA). CSI was calculated according to the critical table of Carey.

#### Statistical analysis

The results were expressed as  $\bar{x} \pm s_x$ . Statistical analysis included both *t* test and correlation test. *P*-values less than 0.05 were considered as significant.

## RESULTS

#### Characteristics of bile acids in serum of gallstone patients

Patients with cholesterol gallstones had significantly higher serum DCA than controls. However, there was no significant difference between the two groups in the other serum bile acids and total bile acids (Table 1). When analyzing the mole percent of serum bile acids (Table 2), we found that DCA was increased in patients with gallstones compared with controls, CDCA was decreased and the ratio of DCA/CDCA increased nearly one-fold.

#### Correlation between bile acids and CSI

Biliary bile acids were mainly composed of CA, CDCA and DCA with a small fraction of lithocholic acid (LCA). Correlation analysis of 90 samples of gallbladder bile acids showed that DCA and LCA were positively correlated with CSI whereas CDCA was negatively correlated with CSI. No correlation was found between CSI and CA (Table 3). These showed each bile acid had its own characteristics, DCA and LCA were lithogenic whereas CDCA could decrease CSI.

#### Correlation between biliary and serum bile acids

The concentration of biliary bile acids was much higher than those in serum (Table 4). Significant correlation existed between the mole percent of biliary and serum bile acids, which suggested that serum bile acids concentration might reflect the relative concentration of bile acids in the gallbladder (Table 5).

**Table 1** Changes of serum bile acids in the two groups ( $\mu\text{mol/L}$ ,  $\bar{x} \pm s_x$ )

Bile acids	Group with calculi ( <i>n</i> = 151)	Control group ( <i>n</i> = 256)
DCA*	0.955 ± 0.078	0.696 ± 0.047
CDCA	2.535 ± 0.304	2.084 ± 0.141
CA	1.202 ± 0.134	1.211 ± 0.147
TBA	4.692 ± 0.432	3.290 ± 0.267

\**P* = 0.002.

**Table 2** Mole percent of serum bile acids in the two groups (% $\bar{x} \pm s_x$ )

Bile acids	Group with calculi ( <i>n</i> = 151)	Control group ( <i>n</i> = 256)	<i>P</i>
DCA	28.12 ± 1667	23.03 ± 1.04	0.007
CDCA	45.11 ± 2.01	52.40 ± 1.32	0.001
CA	27.51 ± 1.93	24.77 ± 1.09	>0.05
DCA/CDCA	1.76 ± 0.30	0.92 ± 0.14	0.004

**Table 3** Correlation between bile acids and biliary CSI (*n* = 90)

Bile acids	Correlation coefficient	<i>P</i>
LCA	0.288	0.006
DCA	0.311	0.003
CDCA	-0.269	0.01
CA	-0.101	>0.05

**Table 4** Biliary and serum bile acids (*n* = 90,  $\bar{x} \pm s_x$ )

Bile acids	Bile (mmol/L)	Serum ( $\mu\text{mol/L}$ )	Correlation coefficient*
LCA	1.97±0.18		
DCA	24.80±2.12	1.08±0.11	0.12
CDCA	76.93±4.01	2.35±0.43	0.07
CA	40.27±3.08	1.31±0.19	-0.09
TBA	143.97±7.33	4.65±0.62	-0.07

\**P*>0.05.

**Table 5** Correlation between mole percent of biliary and serum bile acids (*n* = 90,  $\bar{x} \pm s_x$ )

Bile acids	Bile (mole %)	Serum (mole %)	Correlation coefficient	<i>P</i>
LCA	1.89±0.24			
DCA	17.52±1.06	30.15±2.59	0.445	<0.001
CDCA	54.16±1.12	36.66±2.91	0.434	<0.001
CA	26.43±0.96	33.19±3.04	0.313	0.003

## DISCUSSION

Biliary cholesterol supersaturation is a prerequisite for the formation of cholesterol gallstones. A positive correlation was found between serum DCA and CSI in bile of patients with stone<sup>[3]</sup>. Therefore it might be possible to predict biliary CSI just by analyzing serum bile acids, but this study was primarily based on inpatients with gallstone. The present study was performed among normal

population, asymptomatic gallstone patients and healthy individuals, rather than inpatients<sup>[3,4]</sup>. Among the 407 samples, the mole percent of biliary DCA was found positively correlated with CSI, and serum DCA was increased by about 37% in patients with cholesterol gallstones as compared with control, and the ratio of DCA/CDCA increased nearly one-fold. Hence, there is not only possibility of prediction of CSI in bile, but also characteristic change of DCA in the cholesterol gallstone patients.

Normally, bile acids can be classified into primary bile acids, CA and CDCA, and secondary bile acid, DCA. CA is dehydrogenated to DCA by intestinal bacteria and their secretion is coupled with cholesterol. Bile acid can increase cholesterol secretion and is related to its different hydrophobicity. DCA is more hydrophobic and is followed by greater output of biliary cholesterol than other more hydrophilic bile acids, as CA, CDCA and UDCA. It had been demonstrated by DiDonato *et al*<sup>[5]</sup> that biliary DCA increased from 5.3% to 43.9% and bile CSI increased simultaneously from 0.9 to 1.34 after given a dose of 3 mg/kg of DCA orally for three to four weeks. One week after its discontinuance, the biliary CSI returned to the original level. DCA could also promote cholesterol crystal formation. The *in vitro* experiment showed that the nucleation time shortened when the amount of taurodeoxycholic acid increased<sup>[1]</sup>. Therefore, DCA had a dual effect on supersaturation and nucleation, which contributed to gallstone formation. It was further confirmed by Berr *et al*<sup>[6]</sup> that increase in

hydrophobic bile acids and decrease in its counterparts were the main metabolic disturbance for cholesterol gallstone disease.

Hepatocytes uptake most of the bile acids in the portal vein and then transport them into enterohepatic circulation. The remaining minor portion of bile acids escaped into the systemic circulation are the origin of bile acids in peripheral circulation. They are proportionally related to the composition of bile acid pool<sup>[7]</sup>. Whiting *et al* suggested that serum bile acids could reflect biliary bile acids, which was confirmed by the present study. As serum DCA is relevant to the relative concentration of bile DCA, the determination of DCA may be useful for the prediction and guiding preventive treatment of gallstone disease<sup>[8-10]</sup>.

## REFERENCES

- 1 Marcus SN, Heaton KW. Deoxycholic acid and the pathogenesis of gallstones. *Gut*, 1988;29(4):522 - 533
- 2 Han TQ, Tang WH, Wu WF, Zhang SD, Fu PB. Analysis of bile acids in bile and serum by gas liquid chromatography. *Chin J Exp Surg*, 1992;9(3):109
- 3 Han TQ, Zhang SD, Fu PB. The possibility of prediction of cholesterol saturation of gallbladder bile. *Chin J Surg*, 1990;28(5):285 - 288
- 4 Tang WH, Han TQ, Zhang SD. Serum lipids in predicting the liability to gall stone formation. *Shanghai Med J*, 1994;17(8):446 - 449
- 5 Donato P, Carubbi F, Ponz de Leon M, Carulli N. Effects of small doses of deoxycholic acid on bile cholesterol saturation in patients with liver? cirrhosis. *Gut*, 1986;27(1):23 - 28
- 6 Berr F, Pratschke E, Fischer S, Paumgartner G. Disorder of bile acid metabolism in cholesterol gallstone disease. *J Clin Invest*, 1992;90(3):859 - 868
- 7 Han TQ, Zhang SD, Fu PB. Prediction of bile composition. *Foreign Med Sci, (Sec Dig Dis)*, 1990;10(4):194 - 1978
- 8 Huang ZQ. Trend in current biliary surgery. *Chin J Surg*, 1992;30(2):69 - 70
- 9 Grundy SM, Kalser SC. Highlights of the meeting on prevention of gallstones. *Hepatology*, 1987;7(5):946 - 951
- 10 Han TQ, Zhang SD, Chen S, Yi F, Han SR, Tao Z *et al*. Prediction of cholesterol stones using Logistic regression model: a prospective study. *Chin J Dig*, 1995;15(6):313 - 316

# Current status of gene therapy in gastroenterology

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**Subject headings** gastroenterology; gene therapy; gene transfer; gene expression; DNA, antisense; genes, suppressor, tumor; oncogenes; neoplasms

## INTRODUCTION

The potential role of genetic intervention extends from diseases caused by single gene defects, through severe viral infections, to polygenic disorders, such as diabetes mellitus and arteriosclerosis. However, gene therapy can be defined as the introduction and expression of an exogenous gene in human cells for therapeutic benefit, and is conventionally restricted to human diseases associated with single gene defects. There are wider opportunities for genetic intervention and these include strategies to reduce or block gene expression as well as the introduction of nonmammalian genes. The rapid progress in our understanding of some of the molecular mechanisms involved in the pathogenesis of cancer and metabolic disorders, coupled with the development of gene delivery vector technology, has urged us to consider novel genetic approaches to digestive diseases.

There is no shortage of ideas and applications for genetic intervention in human diseases, but there are great limitations not only with the efficiency and targeting of the present generation of gene transfer vectors but also with our incomplete understanding of transcription control.

## GENE TRANSFER TECHNOLOGY

The gene delivery technology is advancing rapidly and there have been specific developments that could be translated into gene based therapies for gastroenterological diseases. For example, *ex vivo* transfer methods are being studied extensively using hepatocytes obtained through liver biopsy, partial hepatectomy, and from specimens harvested for liver transplantation. Adult liver cells transiently undergo active proliferation permitting *in vitro* gene transfer even with vectors that require active cell division for entry and expression. Gene transfer may then be facilitated through a number of methods, including viruses, liposome, calcium phosphate coprecipitation, particle bombardment, naked DNA injection, and electroporation. The transfected cells are reintroduced into the host by using, for example, a microcarrier system into the peritoneum, gel beads, hepatocyte coated cell support matrix implanted next to liver tissue, or into

the spleen or portal circulation through direct injection.

The spectrum of delivery systems for *ex vivo* gene transfer is broadly applied also to the *in vivo* model. Although the transfer efficiency of liposomes is low, these lipids can be made comparatively easily to high chemical purity and have low immunogenicity, which may permit repeated administrations. They have been used successfully in an *in vivo* model, by topical administration to epithelial cells both in the airways and the intestinal tract and also by the intravascular route. A recent study showed high efficiency transfer of the APC tumor suppressor gene in liposome complexes delivered to normal mouse colonic epithelium by rectal catheter infusion. Almost 100% of epithelial cells expressed the gene for up to four days, which is consistent with the known rate of turnover of this tissue<sup>[1]</sup>. Intravenous injection of a rat insulin gene expression vector in liposome complexes results in uptake primarily by the liver and spleen. Improvement in hepatocyte uptake can be achieved by incorporating lactosyl ceramide into the phospholipid bilayer; this galactosyl terminal asialoganglioside is specifically recognized by a receptor highly selective for hepatocytes. Many different lipid agents are now being explored for efficacy of DNA transfer and it seems likely that the composition of the complex will have to be optimized for different targets and different routes of administration.

Of the available methods of gene delivery, viruses have been proved the most efficient so far. Achieving viral gene transfer to specific organs for clinical application will be difficult, however, particularly as viral titres 10 to 1000 times higher than those usually attained (typically 10<sup>6</sup> infectious units per milliliter) will be necessary for *in vivo* strategies. There is now extensive experience with retroviruses whose main advantages include their small size and easy manipulation, and with stable colinear integration with host genome. They are comparatively non-toxic and are efficient for gene transfer. Retroviruses persist in up to 5% of hepatocytes three months after injection of an infected hepatocyte cell suspension into the portal vein after partial hepatectomy. The small intestinal epithelium is an attractive target for gene therapy because of its large surface area, easy accessibility, and the presence of stem cells with known locations. Although few studies have yet targeted the intestinal system *in vivo*, marker genes have been transferred to the epithelial surface with retroviral vectors in animal models. Clearly, unless the therapeutic or marker gene is transferred to the stem cells, the rapid turn-over of this specialized

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epithelium would seriously limit potential benefits of delivered genes. Retroviruses have a number of disadvantages, notably the requirement for cells that are actively dividing to permit viral DNA integration, the ability to carry only small DNA sequences, and a small but finite risk of causing insertional mutagenesis as a result of random integration.

Currently alternative viral vectors with potential advantages over retroviruses in specific applications are under development. Adenoviruses can infect non-dividing cells, can be concentrated to high titres, and are comparatively highly efficient vectors. Adeno-associated viruses are ubiquitous and non-pathogenic in humans and can also infect non-replicating cells, but, like retroviruses and adenoviruses, are limited in the size of the foreign gene that can be inserted. This last problem may be overcome by the use of herpes simplex group viruses and possibly even vectors based on hepatitis B virus, which has potential additional advantages of hepatotropism and an ability to integrate with host genome *in vivo*.

#### GENE THERAPY IN GASTROENTEROLOGY

Strategies for genetic intervention can be divided into five main aspects: replacement or augmentation of gene expression; reduction of expression of genes by antisense or ribozyme technology; genetic prodrug activation; augmentation of immune responses; and polynucleotide vaccination.

Some strategies can be achieved by *ex vivo* gene transfer into isolated human cells, which can then be reimplanted into the host, while others require delivery and expression of genes to target cells *in vivo*—a major challenge with current vector technology. In this review we consider important clinical applications within these categories and outline the directions of study that should lead to clinical trials in the near future.

##### *Cystic fibrosis*

Replacement strategies for disorders resulting from a single gene defect are attractive candidates for gene therapy. Inheritance of two mutated copies of the cystic fibrosis transmembrane conductance regulator (CFRT) gene on chromosome 7q22 causes this common autosomal recessive disorder<sup>[2]</sup>. The CFRT functions primarily as a cyclic adenosine monophosphate activated chloride channel in epithelial cell. The CFRT gene has a complex mechanism of regulation and is expressed mainly in particular classes of epithelial cell. In humans these sites include certain cells of the lung, crypt cell in the ileum, duodenum and colon, the pancreatic ducts, and gall bladder. There is a recent evidence of endogenous CFRT gene expression in intrahepatic biliary epithelial cells, which is consistent with clinical findings of cystic fibrosis induced biliary damage.

Despite recent molecular genetic information regarding the CFRT gene and its product, the exact cause of the mucosal abnormalities in cystic fibrosis is unclear. The changes are not fully explained

simply on the basis of loss or poor functioning of a small cAMP activated chloride conductance in the apical membranes of certain specialized epithelial cells. It is not clear how different mutations in the CFRT gene actually cause specific phenotypic presentations of cystic fibrosis.

Despite this problem, substantial progress has been made with gene therapy strategies for cystic fibrosis. The four transgenic mouse models available for study have severe intestinal disease with relative sparing of lung and pancreatic epithelia (unlike those in humans). Correction of the lethal intestinal defect has been shown by transfection of human CFRT by liposomal delivery in a vector under the control of rat intestinal fatty acid binding protein gene promoter. Treated mice survived for up to seven months, well beyond the expected four weeks of the control group, and also showed functional correction of ileal goblet cell and crypt cell hyperplasia and cAMP-chloride secretion. Gene therapy aimed at correcting lung abnormalities in human subjects has entailed the direct introduction of non-mutant CFRT cDNA into the epithelial cells of the respiratory tract in the hope that this will result in sufficient CFRT protein being made in these cells to correct the disease process. Vectors used so far include liposomes and adenoviruses, each has its own drawbacks: the liposomal route is proving relatively inefficient while immunological responses to adenovirus may limit its efficacy. Nevertheless initial data from two phase I clinical trials of cationic liposomal mediated CFRT delivery of a plasmid containing the CFRT cDNA to the nasal epithelium has resulted in over 20% correction of conductance abnormalities in nasal mucosal biopsies and the overall treatment was well tolerated. Attempts have also been made to transfer the CFRT gene into the biliary epithelium *in vivo* using adenoviruses. In one study, injection of the vector directly into the common bile duct during laparotomy resulted in gene expression in nearly all intrahepatic bile duct epithelial cells. Gene expression remained stable after 21 days in epithelial cells of small ducts. These results are encouraging particularly because gene delivery might be achieved by endoscopic retrograde cholangiopancreatography in the future<sup>[2]</sup>.

##### $\alpha_1$ antitrypsin deficiency

Approximately 1% of the British population carry the PiZ defect caused by a point mutation in the  $\alpha_1$  antitrypsin gene in chromosome 14, leading to low serum concentrations of  $\alpha_1$  antitrypsin and predisposition to emphysema and liver cirrhosis. Savransky *et al*<sup>[3]</sup> have proposed one approach to correcting this gene defect in human PiZ GM2522 fibroblasts using the technique of targeted homologous recombination to the gene locus, replacing exon V of the abnormal gene with the exon V counterpart of a normal complementary DNA. Other groups have shown a similar effect in hepatoma cell lines and hepatocytes in rat liver *in vivo* using soluble carrier systems, which utilize the asialoglycoprotein receptors on hepatocytes. It is conceivable that in due course harvested human fetal

hepatocytes identified as possessing the PiZ defect could be subjected to targeted homologous recombination *in vitro* and then reinfused into the portal vein.

#### **Low density lipoprotein (LDL) receptor deficiency**

Homozygous LDL receptor deficiency leads to familial hypercholesterolaemia, in which patients have six-to eight-fold increases in plasma LDL-cholesterol values associated with premature atherosclerotic disease and early death. This is a common disease resulting from a mutation in the genomics of the LDL receptors affecting one in 500 of the population. LDL receptors are found in most tissues but it is hepatic expression of the receptor that is most crucial in cholesterol homeostasis. This condition may respond to drug treatment in many cases but transfer of LDL receptor genes to the liver may be a treatment option for a few subjects with intractable hypercholesterolaemia for whom the only treatment option is liver transplantation. Animal studies using the model for homozygous familial hypercholesterolaemia, the Watanabe heritable hyperlipidemic rabbit, have shown successful *ex vivo* retroviral transfer of the receptor gene to 20% of cultured hepatocytes. Transduced cells then expressed receptor levels four or five times higher than normal hepatocyte controls, and after reinjection into the host liver, resulted in lowering total cholesterol value by 50%-70% of its pretreatment value, an effect that lasted over four months. Furthermore there are indications that clinical benefit may result from even partial correction of the total receptor defect, and clinical study has already been underway in the United States using retroviral *ex vivo* gene transfer.

#### **Antisense DNA oligomer treatment: suppressing HBV expression**

Using asialo-orosmucoid coupled with poly-L-lysine, both single and double stranded DNA has been delivered specifically to hepatocytes by targeting their asialoglycoprotein receptors. An antisense oligodeoxynucleotide complementary to the polyadenylation signal for human hepatitis B virus was introduced into HepG2 hepatoma cell line by Wu *et al.*<sup>[4]</sup>. This cell line has been transfected with a complete human hepatitis B virus genome and secreted infectious viral into the culture medium. At 24 hours and seven days after exposure to the antisense sequence, HBV DNA values were 80% and 95% lower than controls respectively. Furthermore, by using similar carrier systems, two separate antisense sequences injected into Beijing ducks (an animal model for HBV infection) resulted in suppression of viral replication by over 90% compared with controls.

#### **Replacing defective tumor suppressor genes**

In cell culture, malignant properties can often be reversed by inserting normal tumor suppressor genes. The difficulty for corrective strategies lies in delivering actively expressed vectors to each single tumor cell of an established and possibly

disseminated cancer *in vivo*. The same argument may not apply for prophylactic therapy to replace gene function in cells of patients with inherited abnormalities of tumor suppressor genes, where it may not be necessary to correct the constitution of every cell to significantly reduce the risk of cancer development within the lifetime of a person.

Normal (wild type) *p53* is involved in the control of cell cycle progression as well as in arresting replication to permit repair in DNA-damaged cells. It may also be involved in restricting precursor populations by mediating apoptosis or programmed cell death. Abnormal or mutant *p53* permits the accumulation of gene mutations and chromosomal rearrangements and has been associated with virtually every sporadic malignancy including gastrointestinal tumors and hepatocellular carcinoma. There is experimental evidence showing the benefits of correcting *p53* abnormalities. Replacement of wild type *p53* using retroviral expression vectors in both human lung cancer cell lines with mouse model of orthotopic human lung cancer resulted in suppression of the malignant phenotype. Furthermore, there was also evidence that the combination of restoration of *p53* function and sequential administration of the cytotoxic drug cisplatin was synergistic in reducing the malignant behavior of these cell lines<sup>[5]</sup>; this is clearly an important finding that may influence approaches to adjuvant treatment for cancer.

There could also be a role of replacement therapy for *p53* in patients with Barrett esophagus, a condition that may respond poorly to medical treatment. Adenocarcinoma arising in Barrett esophagus is often preceded by mucosal dysplasia. Several researchers have found an association between *p53* mutation and adenocarcinoma related to Barrett esophagus<sup>[6]</sup>. In addition, recent reports suggest that *p53* dysfunction may participate in the progression from dysplasia to carcinoma, and that there is a correlation between presence of mutant *p53* and increasing dysplastic features<sup>[7]</sup>. For those patients with histological evidence of progressive dysplasia who are unfit for surgery and refractory to treatment with acid suppression, correction by insertion of wild type *p53* using submucosal endoscopic injection of retroviral or adenoviral suspensions may be an alternative treatment. Clearly such a strategy will require considerable improvement in gene delivery systems and targeting mechanisms for the preneoplastic cells, as well as more sophisticated understanding of transcription control to permit appropriate expression.

The identification of the APC gene participating in familial adenomatous polyposis affecting the large and small intestine, and the DNA mismatch repair gene families (hMSH2, hMLH1, hPMS1, and hPMS2) involved in hereditary non-polyposis colorectal cancer syndrome will stimulate further interest in the replacement of tumor suppressor gene function in stem cells in organs at risk of cancer development. However, the lack of knowledge concerning the temporal and special control of expression of these genes will

delay the application of such prophylactic gene therapy.

#### **Antisense DNA oligomer treatment: suppressing oncogene expression**

Antisense oligodeoxynucleotides are short (10 - 15 bases) synthetic nucleotide sequences formulated to be complementary to specific DNA or RNA sequences. By the binding of these nucleotides to their targets, the transcription or translation of a single gene can be selectively inhibited by triggering RNase H degradation of the target RNA and interfering with the processing of pre-mRNA. Examples of antisense oligomers with significant *in vitro* antiproliferative activity include those against *c-myb* in colorectal cell lines, *c-myc* in lymphoma lines, and *bcr-abl* in chronic myeloid leukemia blast cells.

The *ras* oncogenes are obvious potential targets for antisense therapeutics as they are implicated in many solid tumors including more than 75% of pancreatic cancers and colorectal cancers. Inhibition of the *ras* signaling pathway modulates critical aspects of *ras* oncogene mediated transformation in whole cells. The resulting phenotypes include reduced anchorage dependent and anchorage independent growth and morphological reversion of the cells. Recently, reduced biological aggressiveness and loss of anchorage independent growth were reported in experiments using homologous recombination to target *k-ras*. Using antisense oligonucleotides to target regions of *k-ras* mRNA there are some antiproliferative effects in human pancreatic cancer cell lines, but there is wide variability of response rates in different cell lines and little evidence of sequence specificity<sup>[8]</sup>. Moreover, there is no correlation of antiproliferative effect with reduction in amounts of *k-ras* protein expressed and these agents theoretically directing against cellular genes act instead through unpredictable sequence independent mechanisms. Similar findings have been reported for oligonucleotides designed against *c-myc* and *c-myb* sequences<sup>[9]</sup>. The amplification of *c-myc*, *c-k-ras*, and *c-Ha-ras* oncogenes in a series of 124 gastric carcinomas did not reveal any new independent prognostic factor. On the contrary, amplification of *c-erb B-2* had a significant negative impact on overall survival. An examination of the tumors involving the thymidine labeling index highlighted the need to identify the most suitable biologic material for estimating proliferative activity in gastric carcinoma<sup>[10]</sup>.

#### **Inserting drug activating 'suicide' genes**

There are many examples of genes preferentially expressed in tumors compared with normal tissues. Genetically directed enzyme prodrug therapy (GDEPT) exploits the differences in gene expression between different cell types to increase the specificity of cell destruction by coupling the promoters of differently expressed genes to prodrug activating enzymes. Examples of useful promoters include carcinoembryonic antigen in colorectal

cancer, *erbB2* in breast and pancreatic cancer<sup>[11]</sup>, and prostate specific antigen in prostate; the most widely used prodrug activating enzymes are cytosine deaminase (which converts 5-fluorocytosine to the cytotoxic 5-fluorouracil) and herpes simplex virus thymidine kinase (which converts ganciclovir to toxic phosphorylated derivatives). The system is designed so that significant transcription of the enzyme gene is activated only in tumor cells by linking the enzyme gene to transcription control elements (a 'molecular switch') selective for a particular tumor or tissue type. Significant antitumor effects from conversion of 5-FC to 5-FU have been seen in colorectal cancer cell lines transduced with the cytosine deaminase gene under a constitutive promoter<sup>[12]</sup>. There was also significant regression of tumor volume even when as little as 2% of the tumor mass contained cytosine-deaminase expressing cells. The last effect was also seen when a CEA-HSVTK construct was used to transduce CEA expressing pancreatic cancer cells, which were then engrafted into several combined immune deficient mice. When ganciclovir was given, only 10% of the cells expressed HSVTK<sup>[13]</sup>. This important feature of the GDEPT system is probably due to a so-called 'bystander effect'—attributed to transfer of the activated drug from a cell in which it is produced to others in the vicinity lacking the enzyme. There has been some success in targeting tissue such as the gastrointestinal epithelium and pancreas using the carcinoembryonic antigen gene promoter<sup>[12]</sup>, and also targeting tumor specific transcriptional activation using the  $\alpha$ -fetoprotein promoter in hepatocellular carcinoma.

#### **Augmentation of the immune response and cancer vaccines**

The promise shown by systemic high dose immunotherapy to treat cancer in murine studies has not been fulfilled in human trials except in a few cases of renal cell carcinoma and melanoma. Indeed the initial dose regimens were extremely toxic. It may be more important to deliver a small dose of cytokine at a specific site rather than high doses systematically, which has led to two approaches to cytokine based gene therapy that augment naturally produced immune response to malignancies. The first approach entails the insertion of cytokine genes into cultured tumor infiltrating lymphocytes *ex vivo*. This subset of T cells is critical for the prevention and elimination of tumors, and their antitumor efficacy improves with the transfer of the genes for tumor necrosis factor and interleukin 2. 'Cancer vaccines'—constitute the second approach and entail the induction of cytokine expression in the tumor cells so that T cell recognition of tumor antigens is enhanced. Immunostimulatory cytokine genes are transduced into tumor cells *ex vivo*, the tumor cells are irradiated to eliminate malignant activity and reintroduced into the host. Cytotoxic T cells recognize tumor specific antigens presented on the surface of these cells. They are induced by the local secretion of the transferred cytokine gene product to expand, target, and destroy cancer cells.

In addition to cytokines, a number of other genes are also capable of inducing an antitumor response including allogeneic HLA (human leucocyte antigen) genes and costimulatory molecules such as the B7 family, B7.1 and B7.2. B7 is expressed mainly on antigen presenting cells and serves as costimulatory signals for T cells, by interacting with its ligands CD28 or CTLA-4.

Cytotoxic T cells depend on antigen present in the context of self class I MHC molecules, whereas T helper lymphocytes require activation by antigen present in the context of self class II MHC molecules. Aberrant expression of MHC is a common feature of gastrointestinal cancers. Class I antigens are frequently lost, while class II expression is often unregulated. Polynucleotide vaccination (in contrast with conventional vaccines consisting of peptides, whole tumor cell lysates) has great therapeutic potential in that delivery of genes that express unique oncoproteins such as *k-ras* or *p53* endogenously within a cell and may result in an MHC class I CD8 + response and proliferative activation of cytotoxic T cells, rather than a less effective class II CD4 + response. This may be a further means of breaking down immunotolerance to tumors, which could lead to the generation of tumor specific responses. Mutated forms of the *ras* protooncogene, in particular, contain potentially antigenic T cell epitomes specific for the malignant phenotype. Certain *k-ras* mutations that produce new peptides including consensus binding motifs could cause increased immunogenicity as a direct result of differential MHC class I binding. Carcinoembryonic antigen has been utilized for active immunotherapy.

The finding that individual tumor cells often express much higher levels of this antigen suggested that carcinoembryonic antigen may be a target for immunotherapy, initially using DNA vaccines. Although humoral immune responses were seen, there was no evidence of a more powerful cell-mediated cytotoxicity. Secondly, the injected gene was of human sequence, which will be recognized by the mouse host as a foreign antigen, while the product of a gene from the homologous species may be less immunogenic. Thirdly, the challenge with a small inoculum of tumor cells at the end of a course of vaccination is clearly different from the treatment of a patient with extensive metastatic colorectal cancer. Patients with nm23-H1 allelic deletions are 3 times as likely to develop distant metastases as patients without nm23-H1 deletions (relative risk, 3.89; 95% confidence interval, 1.39, 10.89;  $P = 0.01$ ).

## PROSPECT IN THE FUTURE

These fields are under intense investigation and real advances are likely in the next decade. In the short-term investigation, further useful improvements are possible from manipulation of the RNA and DNA viruses with which we are already familiar. Development of high efficiency viral packaging systems and refinement of the purification and concentration processes can be expected to improve the titres of viral

vectors to values that could permit gene transfer by systemic administration. Targeting of delivery should be possible by incorporating single chain antibodies to cell surface antigens or ligands for transmembrane receptors into the envelope or penton coat proteins of retroviruses and adenoviruses respectively, and there are encouraging signs that this approach can be successful. In the intermediate term, we expect the arrival of 'designer vector' incorporating the most useful elements from both viral and synthetic systems and these can be varied depending on the particular application. For instance, the inverted terminal repeats of adeno-associated virus that mediate stable chromosomal integration can be combined with a backbone of another vector with a large insert capacity such as herpes virus or a bacteriophage. Integration may be enhanced by packaging a functional recombinase enzyme with the gene expression construct in liposomal complexes targeted to a particular class of cells with an antibody. In the long term, basic research will be made into the structure and organization of mammalian chromosomes, which can carry whole clusters of genes with their natural control elements into cells.

The control of gene transcription is extremely complicated and, even for the most intensely investigated systems such as the globin genes, our understanding is still fragmental. While most protocols presently use strong viral promoters to drive expression of recombinant cDNA copies of therapeutic genes, future work must be directed to defining the genomic elements that enable temporal and spatial control of expression through a lifetime. The identification of locus control regions that can insulate gene clusters from interference by surrounding genetic influences has been an important step, and many investigators are now working to understand how the promoter and enhancer/silencer elements of a gene interact with structures within the nucleus. Advances in this area will require parallel developments in the sophistication of vector design before they can be transferred into practice.

## REFERENCES

- Westbrook C, Chimera S, Arenas R, Kim S, Otto G. Human APC gene expression in rodent colonic epithelium using *in vivo* liposomal delivery. *Hum Mol Genet*, 1994;3(9):2005-2010
- Caplen N, Alton EW, Middleton P, Dorin J, Stevenson B, Gao X. Liposomal-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med*, 1995;1(1): 39-46
- Savransky E, Hytrogrou P, Harpaz N, Thung SU, Johnson EM. Correcting the PiZ defect in the alpha-1 antitrypsin gene of human cells by targeted homologous recombination. *Lab Invest*, 1994;70(2):676-683
- Wu GY, Wu CH. Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligodeoxynucleotide. *J Biol Chem*, 1992;267(10):12436-12439
- Roth J, Mukhopadhyay T, Zhang WW, Fujiwara T, Georges R. Gene replacement strategies for the prevention and therapy of cancer. *Eu J Cancer*, 1994;30(9):2032-2037
- Symmons PJ, Linehan JM, Brito MJ, Filipe M. p53 expression in Barrett's esophagus, dysplasia and adenocarcinoma using antibody DO-7. *J Pathol*, 1994;173(1):221-226
- Hardwick RH, Shephard NA, Mooghen M. Adenocarcinoma arising in Barrett's oesophagus: evidence for the participation of p53 dysfunction in the dysplasia/carcinoma sequence. *Gut*, 1994;35(2):764-768
- Carter G, Gilbert C, Lemoine N. Effect of antisense oligodeoxynucleotide targeting KRAS expression in pancreatic cell lines. *Int J Cancer*, 1995;6(4):1105-1112
- Burgess TL, Fisher EF, Ross SL, Bready J, Qian Y, Bayewitch L et al. The antiproliferative activity of c-myc and c-myc antisense oligonucleotides in smooth muscle cells caused by a nonantisense mechanism. *Proc Natl Acad Sci USA*, 1995;92(10):4051-4055
- Amadori D, Maltoni M, Volpi A, Nanni O, Sxanrpi E, Renault B et al. Gene amplification kinetics in relation to prognosis of patients with gastric carcinoma. *Cancer*, 1997;79(2):226-232
- Harris J, Gutierrez A, Hurst HC, Lemoine NR. Gene therapy for cancer using tumour specific prodrug activation. *Gene Ther*, 1994;1(1):174-179
- Huber BE, Austin E, Richards CA, Davis ST, Good SS. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumour cells transduced with the cytosine deaminase gene. *Proc Natl Acad Sci USA*, 1994;91(20):8302-8306

Clinical Experience

# Management of severe acute pancreatitis

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**Subject headings** pancreatitis/therapy; acute disease; traditional Chinese Medicine

Severe acute pancreatitis is associated with organ failure and/or local complications as necrosis, abscess and fluid collections and pseudocysts. Shock, gastrointestinal bleeding, renal insufficiency, severe metabolic disturbance are common complications whereas adult respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC) and pancreatic encephalopathy are most serious. The degree of lesions varies in different individuals, therefore treatment should be individualized. In recent years, we managed such patients by combined traditional Chinese and western medicine according to Balthazar and Ranson's CT grading<sup>[1]</sup> with great success, no death occurred in cases with CT grade E and D, and many serious complications have become preventable. The essentials of this management are depicted below.

## Inhibiting pancreatic enzyme secretions and activities and decrease of exudation into pancreatic substance, abdominal cavity and retroperitoneal space

Severe acute pancreatitis is often caused by hemorrhage and necrosis and less commonly by interstitial edema, however, the treatment is similar. The pancreatic fluid is rich in pancreatic lipase, trypsin, chymotrypsin, elastase, phospholipase A<sub>2</sub>, RNAase and kallikrein and the exudative fluid in the abdominal cavity contained protease, bradykinin, histamine, complement component, phospholipase A<sub>2</sub> and prostaglandins, which can destroy pancreatic tissues and damage the abdominal organs and tissues. Release of cytokines such as IL-1, TNF, PAF, and TXA<sub>2</sub> ET, can cause serious damages to blood vessels with consequent increase of vascular permeability and loss of plasma.

Octreolide can inhibit pancreatic exocrine secretion and gastrointestinal hormones, reduce intraductal pressure, diminish pancreatic autodigestion, decrease PAF activity and reduce exudation from microvessels. It was administered at dosage of 0.1 mg in 20 ml 25% glucose intravenously and followed by 0.5 mg in 1000 ml glucose in saline for 24 hours continuously for 5 - 7 days. Decocting of Rhubarb mixtures (Bupleuri radix 10 g, White peony 10 g, Scutellaria 10 g, Unripe bitter orange 10 g, Magnolia bark 10 g, Refine mirabilite 10 g, Rhubarb 10 g) was used concomitantly twice a day, for most serious cases, once every six hours. Rhubarb stabilizes the lysosomal membrane, inhibits secretion and activities of pancreatic lipase, trypsin, chymotrypsin, elastase, kallikrein-kinin relaxes Oddi's sphincter; promotes colonic peristalsis, contraction and emptying, besides, it has broad

antibiotic spectrum. Bupleuri radix, Unripe bitter orange 枳实 and Refined mirabilite enhance the small intestinal propulsive function, white peony inhibits pancreatic amylase, Bupleuri radix and Scutellaria lower the elevated temperature due to necrosis. The overall action of this prescription is to promote the drainage of pancreatic fluid and retroperitoneal exudation along the pathway to pancreatic duct and intestinal tract and pass out from the anus. Rhubarb mixture and octreotide potentiate each other in inhibiting the enzymatic activities, the former also alleviates the abdominal distention and pain.

## Replenishing the diminished blood volume and correcting the hypoalbuminemia

Loss of plasma into peritoneal cavity and retroperitoneal space due to increased vascular permeability leads to hypotension and even shock, this requires early replenishment of blood volume. We infused 400 ml - 600 ml of plasma instantly and 200 ml everyday afterwards till the general condition became stable. Rhubarb can decrease vascular permeability and arrest the exudative process. In fluid replacement, colloidal and crystalline solution should be given in proper proportion, human serum albumin at a dose of 10 g given everyday and balanced solution is preferred in addition to Ringer's solution, glucose in saline and glucose water with supplements of potassium chloride to maintain water electrolyte balance at an optimum level. We give 3000 ml - 4000 ml of fluid every day and keep the urinary output over 1000ml, depending on the moistening degree of the surface of tongue, which indicates the normalization of gastrointestinal function and adequate hydration.

## Improving intestinal ileus and restoring absorptive and motility function of gastrointestinal tract

Rhubarb mixture is useful in intestinal ileus patients even when peristaltic sound is diminished or nearly absent. When the ventral surface of tongue become moistened, it indicates that the absorptive and motility function of gastrointestinal tract have been restored, then the velocity and fluid volume infusion should be reduced to avoid overloading of the heart and circulation, particularly in the elderly. We do not advocate the use of atropine or gastric decompression.

## Total parenteral nutrition and maintenance of intra and extracellular ionic balance

Rhubarb has inhibitory effect on Na<sup>+</sup>, K<sup>+</sup>, -ATPase. ATP consumption is lowered and body catabolism is kept at a lower level. When general condition is stabilized, early institution of total parenteral nutrition as amino acids mixture and intralipid are imminent sodium, potassium, calcium and magnesium salts should be replenished with sodium potassium phosphate 0.1 mol 200 ml as a loading dose, thereby 100 ml everyday for two days and 100ml once every week to maintain intra and extracellular ionic balance and to correct hypocalcemia concomitantly.

## Restoring pancreatic microcirculation and perfusion of vital organs

Pancreatic microcirculatory impairment occurs at the early onset of severe acute pancreatitis with microthrombosis in

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Received 1997-08-09

most of the cases, which is synchronous with increment of plasma TXA<sub>2</sub> and TXA<sub>2</sub>/PGI<sub>2</sub> ratio. TXA<sub>2</sub> is a potent vasoconstrictor and causes platelet aggregation and constricts the arteriole. Also due to the release of many cytokines and free radicals, the PAF constricts the blood vessels, endothelin lowers the tissue blood flow, free radicals damage the pancreatic tissues, all these augment the ischemic damage on the pancreas. Tetra-methylpyrazine can inhibit TXA<sub>2</sub> synthetase activity, decrease the TXA<sub>2</sub>/PGI<sub>2</sub> ratio. PGE<sub>1</sub> can improve pancreatic microcirculation, inhibit platelet aggregation, decrease TXA<sub>2</sub> synthesis, inhibit release of TNF- $\alpha$ , IL-1, IL-6, phospholipase A<sub>2</sub> and the free radicals liberated by neutrophils and macrophages. Exogenous PGE should be given at 300  $\mu$ g as a loading dose, and 200  $\mu$ g in 250 ml glucose solution daily thereafter for 5-7 days which might arrest further ischemic damage to the pancreas and increase the perfusion of other organs such as kidney, lung and brain. To achieve the optimum effect, whether it should be given at the onset of the disease process on admission or given afterwards for tissue repair would require further cumulation of experience.

#### Prevention and treatment of pancreatic infection

Pancreatic infection develops in 10%-20% of patients with severe acute pancreatitis, especially in those with multiple organ failure and immunocompromised cases. The organisms frequently isolated from infected necrosis and pancreatic abscess are *E. Coli*, *Klebsiella*, *Enterobacter*, *Enterococcus* and other streptococci, occasionally staphylococcus, pseudomonas, anaerobes or fungus<sup>[3]</sup>. Sources of bacteria are mostly from biliary, urinary, respiratory tract or colon, which are mostly hematogenous. Bacteremia often comes from venous or urinary catheterization, so venous catheter placement should not be over one month and urinary catheter should be avoided. On selection of antibiotics, one should choose relatively liposoluble acidic drugs with high penetrability into the necrotic tissues which have a larger distribution volume and stabilized ionization rate in basic pH. Quinolones and metronidazole can pass through blood-pancreatic barrier and by cell to cell communication or via paracellular pathway or via damaged ducts, and enter the hemorrhagic necrotizing tissues. The antibiotic level is low in ischemic tissues, because the quinolones cannot diffuse back into plasma, its concentration is relatively high in pancreatic tissues, repeated administrations maintain high tissue level and enhance the degree of penetration without serum accumulation. Imipenem penetrates into pancreatic fluid, its level is higher in necrotic fluid than in pancreatic tissues<sup>[4]</sup>. Aminoglycosides have a low penetrating ability. Metronidazole is a weak base and has a low molecular size and shows a very high rate of penetration. Therefore, clinically we frequently use ciprofloxacin or together with metronidazole or imipenem alone.

#### Prevention and treatment of complications

Omeprazole 20 mg - 40 mg by intravenous infusion should be given at the beginning to prevent stress ulcer bleeding and acidification of duodenum which might further lead to secretion of pancreatic fluid. Release of myocardial suppressive

factor by the pancreas would produce tachycardia and negative effect on the heart which can be abolished by naloxone 0.4 mg intramuscularly or 0.06 mg/kg in divided doses intravenously. Naloxone can enhance cardiac contractility and cardiac output.

Adult respiratory distress syndrome is one of the most severe complications, it often arises from delayed correction of shock. Via the circulation, the phospholipase A<sub>2</sub> and trypsin disrupt type II alveolar cell with defective production of surfactant, at the same time vacuolation of macrophages affect the defensive clearing mechanism of the lung. Furthermore, the accumulation of neutrophils produce large amounts of free radicals, TXA<sub>2</sub>, TNF- $\alpha$ , fibrin and its degraded product, inflammatory mediator as leukotrienes, all participate in the damaging effect on the lungs, resulting in increased vascular permeability and exudation, interstitial pulmonary edema with early hypoxemia and late carbon dioxide retention. Once the diagnosis of ARDS is established, bronchoalveolar lavage is carried out, first on one side then the other, instill dexamethazone 40 mg and aspirate at the same time, this procedure can washout the inflammatory mediators, complement component, coagulation-fibrinolytic products as well as vascular amines. Corticosteroid inhibits IL-1, IL-6, TNF- $\alpha$ , PAF and inflammatory mediators. Serum albumin should be kept at the lower limit of normal. With diffuse interstitial lung edema, PEEP should be used. Early institution of PGE<sub>1</sub> and cytosine diphosphocholine 30 mg/kg in glucose water can inhibit phospholipase A<sub>2</sub> and prevent ARDS.

DIC is another serious complication, small dose of heparin in the dosage 75 mg/d - 100 mg/d in divided doses can be given every six hours, anti-thrombin III concentrates 3000  $\mu$ /60 ml can be given in half an hour, then 1000  $\mu$  every six hours<sup>[5]</sup>. FDP and D-dimer would decrease, platelet count and fibrinogen increase and bleeding usually ceases.

Obstructive jaundice is usually due to edema of pancreatic head. It will resolve after subsidence of inflammatory edema, only when complicated with common duct stone and deep jaundice, then surgical drainage would be necessary.

Pancreatic encephalopathy is rare, early and energetic treatment at the start can prevent its occurrence.

Due to the damage of pancreatic  $\beta$  cells, insulin supplements should be given. The hypertriglyceridemia and hyperlactic acid dehydrogenase would resolve after recovery.

All twelve cases were managed medically, only one case with subhepatic abscess larger than 3 cm was aspirated by CT guidance. All of these recovered uneventfully in 1 and a half to 2 months without serious complications.

#### REFERENCES

- 1 Balthazar EJ, Ranson JHC, Naidich DP, Megibow AJ, Caccavala R, Cooper HM *et al.* Acute pancreatitis prognostic value of computed tomography. *Radiology*. 1985;156(3):767 - 772
- 2 Zong SW, Tu JH, Liu XL. Study the pancreatic microcirculatory functional impairment in acute pancreatitis. *J Chin Exp Surg*, 1996;13(5):257 - 258
- 3 Widdison AL. Microbiology and sources of pancreatic pathogens in acute pancreatitis. In: Pederzoli P, Cavallini G, Bassi C, Falconi M (Eds). *Facing the Pancreatic Dilemma*. Berlin: Springer Verlag, 1994:291 - 300
- 4 Minelli EB. Pharmacokinetics of antibiotics in the pancreas. *Ibid*, 1994:301 - 312
- 5 Bick RL. Disseminated intravascular coagulation objective criteria for diagnosis and management. *Med Clin North Amer*. 1994;78(30):511 - 543

# Plasma leucine enkephalin content in patients with "Liver-blood deficiency" syndrome and clinical significance\*

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**Subject headings** "Liver-blood deficiency" syndrome/pathophysiology; enkephalin/blood; radioimmunoassay

## INTRODUCTION

Recent studies revealed that the leucine enkephalin (L-EK) was distributed in rat brains with a higher concentration in hypothalamus and little in the pituitary gland<sup>[1]</sup>, but the study on the relationship of L-EK with "liver-blood deficiency" syndrome (LBDS) was lacking. We determined the plasma L-EK levels in LBDS patients with clinical features of iron deficiency anemia (IDA) and/or chronic aplastic anemia (CAA), so as to investigate their relationship.

## MATERIALS AND METHODS

### Patients and controls

The LBDS was determined by the traditional Chinese medicine (TCM) and the disease entities determined by the modern medicine; an integrated method was used in selecting the objects for study. All patients came from the departments of hematology and integrated TCM and modern medicine of our hospital, most of them were inpatients, and the diagnosis of LBDS was established by two clinicians. Among the 26 patients with LBDS, 23 were iron deficiency anemia (IDA) and 3 were chronic aplastic anemia (CAA), including 7 males and 19 females; age averaged  $39.6 \pm 9.0$  (21 - 66) years. The 30 healthy controls were employees and blood donors of our hospital, including 15 of each sex, and age averaged  $32.8 \pm 10.2$  (21-46) years.

### Diagnostic criteria

The diagnostic criteria for IDA was in conformity with the "Diagnostic criteria and curative improvement standards of clinical diseases"<sup>[2]</sup>, and those of CAA accorded with the 1987 Baoji Conference revised standard<sup>[3]</sup>. The diagnostic standard of LBDS followed the certified standard of our institute<sup>[4]</sup>, including: a. dizziness; b. decreased visual acuity and/or blurred vision; c. numbness of the extremities; d. face, lip and nails pale and malnourishment; e. tongue pale and pulse taut and thready or thready. Patients presenting symptoms of b or c with additional two symptoms and excluding those displaying Yinxu (deficiency of *yin*), Yangxu (deficiency of *yang*) and Qixu (deficiency of *qi*) were diagnosed as LBDS.

### Determination of plasma L-EK

Radioimmunoassay was used to determine the plasma level of L-EK. Three ml fasting blood samples were collected at 6-8 a.m. in test tubes containing 50  $\mu$ l proinin and 40 $\mu$ l EDTA, mixed and

centrifuged at 3000rpm for 15min immediately to isolate the plasma and stored at a -20°C for determination. The reagent kit was provided by the Department of Neurobiology of Second Military Medical University, Shanghai, and the test was performed according to the manual of the kit. The instrument was the FJ-2107PY immuno-automatic counter of Xi'an 262 Factory.

### Statistical analysis

The results were expressed as  $\bar{x} \pm s$ , and the difference was examined by Student's *t* test.

## RESULTS

In the 26 LBDS patients L-EK was  $60.83 \text{ ng/L} \pm 21.44 \text{ ng/L}$  as compared with  $43.22 \text{ ng/L} \pm 17.99 \text{ ng/L}$  in 30 healthy controls ( $P < 0.01$ ).

## DISCUSSION

The opioid peptides, or the so-called endogenous opioid substances include enkephalin, endorphine and dynorphin, the three major categories, with altogether about twenty members in this family. Enkephalins come from prepro-enkephalin A, which is composed of 267 amino acids, including six met-enkephalin and one Leu-EK molecule, which was the first isolated opioid peptide in 1975 and the most abundant opioid in the brain. In the cardiovascular system enkephalin is distributed in the atrial and ventricular conductive system and in the nerve fibers of peripheral vascular walls<sup>[5,6]</sup>. EK is a kind of neural transmitter, which plays an important role in physiologic function<sup>[6]</sup>; it affects the hypothalamus, pituitary axis functional activities significantly, including the release of luteinizing hormone (LH), the follicular stimulating hormone (FSH) and the thyroid stimulating hormone (TSH)<sup>[7]</sup>. The functions of FSH and LH on the ovary are to stimulate the production of gametes and ovarian sex hormones. In circumstances of insufficient secretion of FSH and LH, ovarian functional activities are depressed and the secretion of estrogen and progesterone is defective and ultimately results in oligomenorrhea or amenorrhea and anovulation<sup>[8]</sup>. The result of our study demonstrated a significantly increased plasma level of L-EK in patients with LBDS, and the difference from the healthy controls was very significant. It is suggested that the underlying pathophysiologic basis of oligo or amenorrhea and infertility of female patients with LBDS might be ascribed to the deficient ovarian functional activity partially induced by the obviously increased enkephalin as shown by the increased plasma level of L-EK in our study.

## REFERENCES

- Richard J, KJ Chang, Baret C. Radioimmunoassay and characterization of enkephalins in rat tissue. *J Biol Chem*, 1987;253(25):531 - 536
- Health Ministry of PLA (ed). Clinical diagnostic criteria of diseases. Beijing: People's Army Surgeon Publishing House, 1989:140 - 141
- Diagnostic Criteria of Aplastic Anemia. *Chin J Hematol*, 1987;8(8):C4
- Cheng GL, Pang QM, Zhao YQ, Cheng ZQ, Li XQ. Studies on the differential standard of the TCM liver disease signs and symptoms. *Zhongguo Yiyao Xuebao*, 1990;5(1):66 - 73
- Tang J, Tang ZQ. Secretary function of circulatory system. Beijing: Joint Publishing House of Beijing Medical University and Peking Union Medical University, 1989: 144 - 147
- Hang JS. Some problems in the research of neuropeptides. *Progress Physiol*, 1988; 19(2):173 - 182
- Zhang DM, Xu LS (eds). Neuroendocrinology. Beijing: China Medical Sciencetech Publishing House, 1991:122 - 125, 383 - 386
- Yang G. Endocrine physiology of human body and its abnormalities. Tianjin: Tianjin Sciencetech Publishing House, 1980:352-365

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\*Project supported by the National Natural Science Foundation of China, No. 39170881.

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Received 1997-08-21.

# Association between *Helicobacter pylori* and gastric cancer: current knowledge and future research

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**Subject headings** stomach neoplasms/etiology; stomach neoplasms/epidemiology; stomach neoplasms/prevention and control;DNA;mutation; Helicobacter pylori; helicobacter infection

In 1994 *Helicobacter pylori* infection was classified as a definite (class 1) carcinogen by the International Agency for Research on Cancer (IARC) on the basis of available epidemiological and clinical research<sup>[1]</sup>. However, data collected over the past 3 years has failed to provide unequivocal support for the conclusion. The mechanisms by which *H. pylori* increases the risk of developing gastric cancer remain unclear, despite many significant experimental observations.

## EPIDEMIOLOGICAL STUDIES

### *Association between incidence of gastric cancer and prevalence of H. pylori infection.*

While gastric cancer is the second most common cause of cancer death, the incidence has decreased dramatically in some developed countries over the past decades<sup>[2,3]</sup>. There is also good evidence that the prevalence of *H. pylori* infection has dropped markedly in these countries, and it is unlikely that these two events have happened coincidentally. Recent studies has shown that the geographic variation in the incidence of gastric cancer is related to the prevalence of *H. pylori* infection; an increased incidence and mortality of gastric cancer in a region is strongly associated with a high prevalence of *H. pylori* infection in the population within that region<sup>[4]</sup>. However, lack of association between *H.*

*pylori* and gastric cancer has been reported in some areas of the world, such as the Mediterranean and Africa where the prevalence of *H. pylori* is high but the incidence of gastric cancer is low<sup>[5]</sup>.

### *Case control and prospective studies*

Early case control studies provided convincing support for the association between *H. pylori* infection and gastric cancer<sup>[5,7]</sup>. In 1994, Forman summarized all available case control studies and concluded that the relative risk for development of gastric cancer was 3.8 fold higher in patients with *H. pylori* infection than those without<sup>[6]</sup>. However, more recent epidemiological studies have shown inconsistent results<sup>[5,8-13]</sup>. Two studies carried out in Chinese populations showed no significant association between *H. pylori* infection and gastric cancer<sup>[8,9]</sup>, and this finding has been replicated in recent Japanese and Korean studies<sup>[11-13]</sup>.

One limitation of current epidemiological studies is that most studies did not adjust the potential confounding factors such as dietary or socioeconomic characteristics, thus bias may exist<sup>[5]</sup>. For example, poor living conditions are usually related to a high prevalence of *H. pylori* infection and a high incidence of gastric cancer. Misclassification is another limitation in current epidemiological studies. It is widely accepted that *H. pylori* infection is lost with advancing preneoplasia (such as atrophy, intestinal metaplasia and dysplasia) and that serum titres drop correspondingly<sup>[14,15]</sup>. Cases are more likely to have advanced preneoplasia than the controls, so that *H. pylori* infection may have disappeared in a substantial proportion of cases. These cases may be classified as *H. pylori* negative since the infection is usually assessed at the time of diagnosis of gastric cancer. Misclassification may also result from the application of serological assays with low sensitivity and specificity for *H. pylori* detection, and from misdiagnosis of gastric cancer. This significantly influences the outcomes of analysis, resulting in underestimation of relative risks<sup>[14]</sup>. When the misclassification errors are taken into account, it is rare to find studies that do not link *H. pylori* infection to gastric cancer. Therefore, understanding the limitations of these

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**Received** 1998-03-05

epidemiological studies is critical in interpreting the research findings.

## EXPERIMENTAL OBSERVATIONS

### *Cytotoxic strains*

Approximately 50% of *H. pylori* strains express the CagA protein. It has been shown that persons with CagA-expressing strains are more likely to develop atrophic gastritis, intestinal metaplasia and gastric cancer than those infected with CagA negative strains<sup>[16,17]</sup>. Thus, variation in CagA prevalence among populations may explain some of the discrepancies observed in the case-control studies. In populations with a high prevalence of CagA positive strains, *H. pylori* infection is more likely to be identified as a cancer risk factor. For example, the prevalence of CagA positive strains is substantially higher in Europeans than in African-born subjects<sup>[17]</sup>. Similarly, CagA positive strains are more prevalent in blacks than in whites in the USA<sup>[17]</sup>. However, this remains controversial as studies from Asian countries, such as China, Japan and South Korea, failed to show that infection with CagA positive strains is associated with these lesions<sup>[18-21]</sup>.

### *DNA alternations*

There is no evidence, so far, that *H. pylori* infection is, in itself, directly genotoxic or mutagenic. Thus, it is unlikely that there is some direct interaction between the bacterium and host DNA which leads to mutations and transformed cell phenotypes. However, *H. pylori* produces a variety of substances that may indirectly cause DNA damage of the gastric epithelial cells<sup>[4]</sup>. These products include ammonia or ammonium-containing chemicals, phospholipases, and cytotoxin(s). All these factors may impair the host defence and render the gastric epithelial cells prone to the activity of direct-acting carcinogens.

The host's immune response to *H. pylori* may eventually result in DNA damage. In theory, stimulation of leukocyte cells by a bacterial infection should lead to the activation of both specific and non specific immune defence mechanisms that would cause limitation and resolution of the infection. However, the human immune response to *H. pylori* is not capable of eradicating the infection from the stomach, as the bacteria can avoid the human immune defence system. Thus, stimulation of the leukocyte cells continues as long as the infection persists. This toxic response may, over an extensive time period, lead to structural and biological damage to the gastric epithelium. For example, the excessive production of reactive oxygen metabolites by stimulated neutrophil polymorphs and monocytes may cause extensive DNA damage and molecular mutation<sup>[22]</sup>. It has been shown that *H. pylori* in-

fecting gastric epithelium has a significantly higher level of these compounds than normal epithelium and that eradication of *H. pylori* infection returned to normal levels<sup>[23]</sup>. Genetic damage by persistent inflammation may be mediated by other chemicals, such as oxidates of nitrogen, and lead to the formation of N-nitroso-compounds and nitric oxide which can induce DNA damage.

Cell division is vital to the development of cancer and an elevated rate of mitosis increases the likelihood of a somatic DNA mutation. Studies in varied populations have shown that *H. pylori* infection is associated with an approximately doubling of cell turnover and that the turnover level returns to the normal level following eradication of the infection<sup>[24,25]</sup>. It has also been shown that *H. pylori* increases epithelial cell proliferation *in vitro*<sup>[26]</sup>. On the other hand, *H. pylori* infection has been reported to induce epithelial apoptosis (programmed cell death) *in vivo*<sup>[27-29]</sup> and eradication of the infection results in a significant reduction in apoptosis. Moreover, a recent study showed that increased cell proliferation was not associated with a corresponding increase in apoptosis in patients infected with cagA+ strains, indicating that failure to delete cells with genetic damage may result in malignant transformation<sup>[30]</sup>.

Telomeres are specialised structures at the ends of chromosomes that are important in protection and replication of chromosomes. Normally, there is progressive shortening of telomeric repeats with each cell division, whereas germline cells compensate for the end replication problem by telomerase expression which permits the synthesis of telomeres, telomerase activity is not expressed at detectable levels in normal somatic cells. So, telomeres in somatic cells progressively shorten with each cell division. Telomere reduction could cause chromosome instability and additional genetic changes, resulting in increased cell proliferation and reactivation of telomerase. Studies have shown that telomerase activity can be detected in most gastric cancers as well as precancerous lesions such as gastric intestinal metaplasia and adenoma, suggesting that the expression of telomerase activity is an early event of gastric carcinogenesis<sup>[31-32]</sup>. A recent study from Japan showed that the level of human telomerase RNA (hTR) and telomerase activity increased in parallel with the density of *H. pylori* infection, which was also associated with the grade of intestinal metaplasia<sup>[33]</sup>. This finding suggests that *H. pylori* infection may be a strong trigger for hTR overexpression in intestinal metaplasia, which may lead to telomerase reactivation.

**Microenvironmental biophysiological changes**  
Ascorbic acid (Vitamin C) is a critical antioxidant

which has important functions as a scavenger of reactive oxygen species and inhibits N nitrosation. This antioxidant property of ascorbic acid plays a central role in the chemoprevention against gastric cancer. It has been shown that *H. pylori* infection is associated with a significant decrease in the concentration of ascorbic acid in gastric juice, and that eradication of the infection normalizes the level of ascorbic acid<sup>[34,35]</sup>. Similarly, the gastric level of  $\beta$  carotene, which is also an antioxidant, is significantly decreased in patients with *H. pylori* infection<sup>[36]</sup>.

## FUTURE RESEARCH

### Epidemiology

Current epidemiological studies are limited by their small sample size and failure to adjust potential confounders. Well designed prospective and case control studies using large populations are still needed. These may confirm or refute the established “guilty” sentence for *H. pylori* infection in the development of gastric cancer.

### Pathogenesis

The model of pathogenesis of gastric cancer has been proposed as a multi-step process; *H. pylori* infection may progress to acute gastritis, chronic gastritis, atrophic gastritis, then intestinal metaplasia, dysplasia, and eventually gastric cancer<sup>[23]</sup>. However, the role of *H. pylori* infection in each step of this progression remains unclear. Moreover, the exact “point of no return” is unknown. Therefore, investigation of the association of *H. pylori* infection and precancerous lesions may provide key answers to the issues. Recently, it has been reported that *H. pylori* infection is associated with antralization (presence of antral type mucosa) at the gastric incisura, body and fundus, which is, in turn, related to intestinal metaplasia at these sites<sup>[37]</sup>. Whether antralization is a step before intestinal metaplasia, and is reversible, needs to be clarified.

### Preventive strategies

At present, an eradication rate of up to 95% has been achieved with the triple therapy and serological or urea breath tests with high sensitivity and specificity for detection of *H. pylori* infection are available<sup>[38]</sup>. If *H. pylori* infection is proven to be an initiating factor in the development of gastric cancer, then, screening and eradication of *H. pylori* with antimicrobial therapy in large scale populations, in particular, those at high risk, should be considered. However, feasibility, safety and appropriate timing of this strategy remains to be determined. Moreover, side effects and the potential spread of drug resistant strains limits its practical application.

cal application.

Encouraging results have been reported on the vaccination against *H. pylori* infection. Studies using animal models have shown that immunization against *H. pylori* not only prevents the infection but also can eradicate it<sup>£39£Y</sup>. Vaccination as the optimal strategy in humans will be confirmed in the management of *H. pylori* infection.

## CONCLUSIONS

Preliminary epidemiological data support the link between *H. pylori* infection and the development of gastric cancer. However, well-designed prospective and case-control studies using large populations are still required. Moreover, the mechanisms through which *H. pylori* infection leads to gastric cancer are not fully understood, and studies on this issue are urgently needed. Further research should also explore the possibility of preventing gastric cancer by eradicating *H. pylori* infection, with vaccines.

## REFERENCES

- 1 International Agency for Research on Cancer, Schistosomes, Liver flukes and *Helicobacter pylori*. IARC monographs on the evaluation of carcinogenic risks to humans, 1994;61:177-241
- 2 Zheng T, Mayne ST, Holford TR, Boyle P, Liu W, Chen Y et al. The time trend and age period cohort effects on incidence of adenocarcinoma of the stomach in Connecticut from 1955-1989. *Cancer*, 1993;72:330-340
- 3 Sipponen P, Kimura K. Intestinal metaplasia, atrophic gastritis and stomach cancer: trends over time. *Eur J Gastroenterol Hepatol*, 1994;6(Suppl 1):S79-83
- 4 Xia HX, Forman D, Talley NJ. *Helicobacter pylori* infection and gastric cancer. In: Fan XG, Xia HX, eds. *Helicobacter pylori* infection: basic principles and clinical practice. Changsha: Hunan Science and Technology Press, 1997:101-108
- 5 Palli D. Gastric cancer and *Helicobacter pylori*: a critical evaluation of the epidemiological evidence. *Helicobacter*, 1997;2(Suppl 1):S50-55
- 6 Forman D, Webb P, Parsonnet J. *H. pylori* and gastric cancer. *Lancet*, 1994; 343:243-244
- 7 Talley NJ, Xia HX. Gastric cancer: an infectious disease? *Cur Therapy*, 1996; 37:17-20
- 8 Eslick GD, Talley NJ. *Helicobacter pylori* infection and gastric carcinoma: a meta analysis. *Gastroenterology*, 1997;114:(in press)
- 9 Lin JT, Wang LY, Wang JT, Wang TH, Yang CS, Chen CJ. A nested case-control study on the association between *Helicobacter pylori* infection and gastric cancer risk in a cohort of 9775 men in Taiwan. *Anticancer Res*, 1995;15:603-606
- 10 Webb PM, Yu MC, Forman D, Henderson B, Newell DG, Yuan JM et al. An apparent lack of association between *Helicobacter pylori* infection and risk of gastric cancer in China. *Int J Cancer*, 1996;67:603-607
- 11 Watanabe Y, Kurata JH, Mizuno S, Mukai M, Inokuchi H, Miki K et al. *Helicobacter pylori* infection and gastric cancer. A nested case-control study in a rural area of Japan. *Dig Dis Sci*, 1997;42:1383-1387
- 12 Kimura K, Satoh K. What remaining questions regarding *Helicobacter pylori* and associated diseases should be addressed by future research? *View from far east. Gastroenterology*, 1997;113:S155-157
- 13 Kim HY, Cho BD, Chang WK, Kim DJ, Kim YB, Park CK et al. *Helicobacter pylori* infection and the risk of gastric cancer among the Korean population. *J Gastroenterol Hepatol*, 1997;12:100-103
- 14 Parsonnet J. Lesions from the epidemiology of *Helicobacter pylori* and cancer. In: Hunt R, Tytgat G. eds. *Helicobacter pylori*: basic mechanisms to clinical cure. Dordrecht: Kluwer Academic Publishers, 1996:238-244
- 15 Xia HX, Talley NJ. *Helicobacter pylori* infection, reflux esophagitis and atrophic gastritis: an unexplored triangle. *Am J Gastroenterol*, 1998;93:(in press)
- 16 Blaser MJ, Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH et al. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res*, 1995;55:2111-2115
- 17 Parsonnet J, Friedman GD, Orentreich N, Vogelstein H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut*, 1997;40:297-301
- 18 Mitchell HM, Hazell SL, Li YY, Hu PJ. Serological response to specific *Helicobacter pylori* antigens: antibody against CagA antigen is not predictive of gastric cancer in a developing country. *Am J Gastroenterol*, 1996;91: 1785-1788
- 19 Shimoyama T, Fukuda S, Tanaka M, Mikami T, Saito Y, Munakata A. High prevalence of the cagA-positive *Helicobacter pylori* strains in Japanese asymptomatic patients and gastric cancer patients. *Scand J Gastroenterol*, 1997;

- 32:465-468
- 20 Matsukura N, Onda M, Kato S, Hasegawa H, Okawa KI, Shirakawa T et al. Cytotoxin genes of *Helicobacter pylori* in chronic gastritis, gastroduodenal ulcer and gastric cancer: an age and gender matched case control study. *Jpn J Cancer Res*, 1997;88:532-526
- 21 Miehlike S, Kim JG, Small SM, Graham DY, Go MF. Lack of association between the prevalence of the cagA gene in *Helicobacter pylori* and gastric adenocarcinoma. *Gut*, 1995;37(Suppl 1):A77
- 22 Davies GR, Simmonds NJ, Stevens TRJ, Sheaff MT, Banatvala N, Laurenson IF et al. *Helicobacter pylori* stimulates antral mucosal reactive oxygen metabolite production *in vivo*. *Gut*, 1994;35:179-185
- 23 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
- 24 Cahill RJ, Xia H, Kilgallen C, Beattie S, Hamilton H, O'Morain C. Effect of eradication of *Helicobacter pylori* infection on gastric epithelial cell proliferation. *Dig Dis Sci*, 1995;40:1627-1631
- 25 Lynch DAF, Mapstone NP, Clarke AMT, Sobala GM, Jackson P, Morrison L et al. Cell proliferation in *Helicobacter pylori* associated gastritis and the effect of eradication therapy. *Gut*, 1995;36:346-350
- 26 Fan XG, Kelleher D, Fan XJ, Xia HX, Keeling PWN. *Helicobacter pylori* increase proliferation of gastric epithelial cells. *Gut*, 1996;38:19-22.x915
- 27 Dong QJ, Liu W, Zheng X, Xiao S, Shi Y, Peng Y. Effect of *Helicobacter pylori* on gastric epithelial apoptosis. *Chin J Intern Med*, 1997;36:751-753(in Chinese)
- 28 Hahm KB, Lee KJ, Choi SY, Kim JH, Cho SW, Yim H et al. Possibility of chemoprevention by the eradication of *Helicobacter pylori*: oxidative DNA damage and apoptosis in *H. pylori* infection. *Am J Gastroenterol*, 1997;92:1853-1857
- 29 Mannick EE, Bravo LE, Zarama G, Realpe JL, Zhang XJ, Ruiz B et al. Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in *Helicobacter pylori* gastritis: effect of antibiotics and antioxidants. *Cancer Res*, 1996;56:3238-3243
- 30 Peek RM, Moss SF, Tham KT, Perez Perez GI, Wang S, Miller GG et al. *Helicobacter pylori* cagA+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. *J Natl Cancer Inst*, 1997;89:863-868
- 31 Ahn MJ, Noh YH, Lee YS, Chung TJ, Kim IS, Choi IY et al. Telomerase activity and its clinicopathological significance in gastric cancer. *Eur J Cancer*, 1997;33:1309-1313
- 32 Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y et al. Telomerase activity in gastric cancer. *Cancer Res*, 1995;55:3258-3262
- 33 Kuniyasu H, Domen T, Hamamoto T, Yokozaki H, Yasui W, Tahara H et al. Expression of human telomerase RNA is an early event of stomach carcinogenesis. *Jpn J Cancer Res*, 1997;88:103-107
- 34 Sobala GM, Schorah CJ, Shires S, Lynch DA, Gallacher B, Dixon MF et al. Effect of eradication of *Helicobacter pylori* on gastric juice ascorbic acid concentration. *Gut*, 1993;34:1038-1041
- 35 Banerjee S, Hawksby C, Miller S, Dahill S, Beattie AD, McColl KE et al. Effect of *Helicobacter pylori* and its eradication on gastric juice ascorbic acid. *Gut*, 1994;35:317-322
- 36 Zhang ZW, Patchett SE, Perrett D, Domizio P, Farthing MJG. Gastric mucosal and luminal  $\beta$  carotene concentrations in patients with chronic *H. pylori* infection. *Gut*, 1996;38(Suppl 1):A3 (abstract)
- 37 Xia HH X, Kalantar J, Ma Wyatt J, Adams S, Cheung K, Talley NJ. *Helicobacter pylori* associated antralization of proximal gastric mucosa is linked to an increased risk of development of intestinal metaplasia. *Gastroenterology*, 1998;114:(in press) (abstract)
- 38 Xia HH X, Talley NJ. Prospects for improved therapy for *Helicobacter pylori* infection. *Exp Opin Invest Drugs*, 1996;5:959-976
- 39 Cortesy Theulaz I, Porta N, Glauser M, Saraga E, Vaney A C, Haas R et al. Oral immunization with *Helicobacter pylori* urease B subunit as a treatment against *Helicobacter* infection in mice. *Gastroenterology*, 1995;109:115-121

# Analysis of N-*ras* gene mutation and p53 gene expression in human hepatocellular carcinomas\*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; genes, p53; genes, ras; mutation; gene expression; polymerase chain reaction; immunohistochemistry

## Abstract

**AIM** To study the relationship between N-*ras* gene mutation and p53 gene expression in the carcinogenesis and the development of human hepatocellular carcinomas (HCC).

**METHODS** The N-*ras* gene mutation and the p53 gene expression were analyzed in 29 cases of HCC by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and immunohistochemistry.

**RESULTS** Thirteen cases of HCCs were p53 positive (44.8%), which showed a rather high percentage of p53 gene mutation in Guangxi. The aberrations at N-*ras* codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumorous liver tissues. More than 2 point mutations of N-*ras* gene were observed in 22 cases (75.86%). Twelve cases (41.37%) of HCCs showed both N-*ras* gene mutation and p53 gene expression.

**CONCLUSIONS** N-*ras* gene and p53 gene may be involved in the carcinogenesis and the development of HCC. That 38% of HCCs with N-*ras* gene mutation did not express p53 protein indicates that some other genes or factors may participate in the carcinogenesis and the development of HCC.

## INTRODUCTION

The hepatocellular carcinogenesis is presumably a multiple-step process and is influenced by many factors. The accumulation of genetic changes is necessary for emergence of hepatocellular carcinoma (HCC). Activation of pro-oncogenes and inactivation of tumor suppressor genes might be related to the carcinogenesis and the development of HCC. It has been reported that there was mutation of tumor suppressor gene p53 and overexpression of N-*ras* oncogene in human HCC<sup>[1-3]</sup>, but there have been few reports on the relationship between N-*ras* gene mutation and p53 gene expression. We analyzed the N-*ras* gene mutation by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and p53 protein by immunohistochemistry in 29 cases of human HCC in order to explore the relationship between these two kinds of genetic changes and human HCC.

## MATERIALS AND METHODS

### Clinical samples

Twenty-nine HCC surgical resected specimens were collected in Guangxi Cancer Institute during the period of 1987-1992, 28 of them had HCC adjacent liver tissues. All samples were fixed with 10% formalin, embedded in paraffin and stained with haematoxylin and eosin (HE).

### Immunohistochemistry

Immunostaining was performed using a streptavidin-biotin immunoperoxidase method. p53 protein was detected with monoclonal anti p53 antibody (Oncogene Science, PAb DO1) and Strept ABC kit (DAKO A/S Denmark). The tissues were firstly microwaved 5min, 4 times and then used for immunohistochemistry.

### DNA extraction

Genomic DNA was prepared by the proteinase K-Phenol-Chloroform extraction method.

### PCR-SSCP

Oligomers that flank codon 2-37 of N-*ras* genes was synthesized as a primer by the Department of Molecular Medicine in King's College Hospital, UK. One of them was 5'-end labelled with r-<sup>32</sup>P ATP by T4 Polynucleotide Kinase reaction. The

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\*Project supported by the National Natural Science Foundation of China, No.39060032 and the Sino British Friendship Scholarship.

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Received 1997-07-10

primersets were: 5'-GACTGAGTACAACTGGTGG-3', 5'-GGGCCTCACCTCTATGGTG-3'.

The amplified product obtained by the PCR is 118bp. 0.1µg DNA taken from tissues, which was added with 9µl PCR mixture (containing 1pmol/L primer, 0.2mmol/L dATP, dGTP, dCTP, dTTP, 0.25U Taq DNA polymerase, 50mmol/L KCl, 10 mmol/L Tris, 2.5mmol/L MgCl<sub>2</sub> and 0.45% Tween 20), covered with mineral oil. PCR reaction underwent 5min denature at 94°C, then went to 35 cycles. One cycle included 30sec denature at 95°C, 1min annealing at 55°C, 1min 30sec extension at 72°C, and finally 10min extension at 72°C.

Two µl PCR product was added to 2µl dilution containing 95% formamide, 20mmol/L EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol dye. The mixture was denatured 5min at 95°C, then applied to a 6% polyacrylamide gel (21cm×40cm×0.4cm) with 1×TBE buffer at 45mA current and 45 Watt in cold room (4°C) for 4-5 hours. The gel was dried at 80°C and autoradiographed at -70°C.

## RESULTS

### *Pathologic histology*

The tumor specimens were all diagnosed as hepatocellular carcinomas. The histological diagnosis of tumor adjacent liver tissues were: 14 (50%) cases of chronic hepatitis, including chronic active type (CAH) and chronic persistent type (CPH); 6 of them had liver cirrhosis with CAH, 6 had liver cirrhosis and 2 cases acute hepatitis.

### *Point mutation of N-ras gene*

The N-ras PCR-SSCP showed only 3 bands in normal control samples including human liver tissues (2 cases) and human placenta DNA (1 case). These 3 normal bands were also clearly discerned in all HCC and their adjacent tissues. Some of detected specimens presented mobility shift bands, which was the point mutation in N-ras gene. The aberrations at N-ras codon 2-37 were found in 79.31% of HCCs and 80.77% of adjacent non-tumor liver tissues, respectively. There was no significant difference between them ( $\chi^2$  test,  $P>0.05$ ). Twenty-two of 29 HCC (75.86%) showed 2-5 mobility shifts.

### *p53 protein detection*

p53 protein was mainly localized in HCC cell nucleus. Both nuclei and cytoplasmic staining were observed in 75% of p53 protein positive HCC. Non-tumor liver tissues showed only cytoplasmic type of p53 protein. p53 protein positive rate was 44.8% (13/29), 12 (41.37%) of them had N-ras mutation as well.

## DISCUSSION

PCR-SSCP analysis is based on single strand DNAs

of the same nucleotide length in which the nucleotide sequences different at only one or some positions can be separated by polyacrylamide gel electrophoresis. DNA polymorphisms at a variety of positions in a fragment could cause a difference in its conformation and result in change in mobility of the single strands on gel electrophoresis.

In HCC, N-ras was first proved as one of the transforming genes<sup>[4]</sup>, which belongs to G protein family. When it is converted to active oncogene by point mutation, chromosome rearrangement or gene amplification, the signal transmission of cell membranes may change, which drives cell division, results in abnormal differentiation and finally forms neoplasm. Enhanced expression of N-ras gene in human HCC has been reported<sup>[3]</sup>. The 'hot spot' mutation at codon 12, 13 or 61 in the N-ras gene has been described in many kinds of human cancers<sup>[5]</sup>. But no point mutation around these 'hot spot' was found in HCC from South Africa<sup>[6]</sup> and Japan<sup>[5]</sup>. In China, in FANG Dian Chun's study<sup>[7]</sup>, 37.2% (16/43) of HCC showed N-ras gene point mutation at codon 12 by PCR-restriction fragment length polymorphisms (PCR-RFLP). In our study, the PCR product contained codon 12, 13 of N-ras. This gene point mutation was found in 79.31% (23/29) of HCC, indicating that the frequency is rather high in HCC from Guangxi region. In contrast to the reports about no N-ras gene point mutation or only one mutation at codon 12 in HCC, we found that 75.86% (22/29) of HCC had 2-5 point mutations around codon 2-37. This data showed that the mutation positions were not limited at codon 12 or 13. Therefore, if we only investigate the 'hot spot' mutation of N-ras gene, there is a possibility of missing mutations in other regions of the gene. The mobility shifts scattered at different positions. No clustering position could be considered as a hot spot in this study. We do not know the exact codon of mutation in this fragment by PCR-SSCP analysis. They could be confirmed by DNA sequencing.

SSCP bands can be clearly discerned in HCC (79.31%) and their adjacent liver tissues (80.77%). There was no significant difference between them,  $P>0.05$ . Most of these non-tumorous liver tissues presented as chronic hepatitis and/or liver cirrhosis, which was indicative of a close correlation with HCC. Our result indicates that N-ras gene mutations are involved in the carcinogenesis and development of HCC.

Many studies have shown that p53 abnormalities were involved in the genesis of HCC<sup>[1-2]</sup>. One of the most common mechanisms leading to functional defect of this tumor suppressor gene in carcinogenesis is point mutations and expression of a conformationally altered protein that is immunohistochemically identifiable. Wild-type (normal) p53

protein has a very short half-life<sup>[8]</sup> and can not be identified immunohistologically. In contrast, most mutant p53 protein becoming more stable with an extended half-life (4-5 hours) and being overexpressed in cells were readily detected by immunohistochemistry. We examined 29 HCC for p53 protein by ABC immunohistochemistry, 13 cases were found positive, accounting for 44.8% of HCC, which showed a high frequency of p53 gene mutation in HCC in Guangxi area.

It has been evidenced that mutation of the tumor suppressor gene p53 can convert *ras* gene into oncogene<sup>[9]</sup>. The mutant p53 gene cooperation with mutant *ras* gene in cell transformation has been proved<sup>[10]</sup>. We noticed that 12/29 (41.37%) cases of HCC had both mutations of N-*ras* gene and p53 gene. The result indicates that the mutations of N-*ras* gene and p53 gene may play an important role in the carcinogenesis and maintenance of HCC. It is interesting that 38% of HCC with N-*ras* mutations was p53 protein negative. The explanation might be that some other genes or factors except N-*ras* and

p53 participated in the formation and maintenance of HCC in Guangxi region.

## REFERENCES

- 1 Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa. *Nature*, 1991;350(6317): 429-431
- 2 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinoma. *Nature*, 1991;350(6317): 427-428
- 3 Farshid M, Tabor E. Expression of oncogenes and tumor suppressor genes in human hepatocellular carcinoma and hepatoblastoma cell lines. *J Med Virol*, 1992;38(4):235-239
- 4 Gu JR, Hu LF, Wan DF, Tian PK, Guo C, Huang LH. Study on N-*ras* gene: the human primary liver cancer transformation gene. *Tumour*, 1985;5(2): 52-55
- 5 Tada M, Omata M, Ohto M. Analysis of *ras* gene mutations in human hepatic malignant tumors by polymerase chain reaction and direct sequencing. *Cancer Res*, 1990;50(4):1121-1124
- 6 Leon M, Kew MC. Analysis of *ras* gene mutations in hepatocellular carcinoma in Southern African blacks. *Anticancer Res*, 1995;15(3):859-861
- 7 Fang DC, Luo YH, Lu R, Xiao WH, Liu WW, Men RP *et al.* Studies of the point mutation of *ras* oncogene and suppressor gene p53 in 43 cases of hepatocellular carcinoma. *Acta Acad Med Militaris Tertiae*, 1994;16(5):340-343
- 8 Slagle BL, Zhou YZ, Butel JS. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus positive hepatocellular carcinomas. *Cancer Res*, 1991;51(1):49-54
- 9 Harris AL. Telling changes of base. *Nature*, 1991;350(6317):377-378
- 10 Hinds P, Finlay C, Levine AJ. Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. *J Virol*, 1989;63(2): 739-746

# Partial isolation and identification of hepatic stimulator substance mRNA extracted from human fetal liver\*

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**Subject headings** fetal liver tissue; RNA, messenger; hepatic stimulator substance; hepatocyte proliferation

## Abstract

**AIM** To partially isolate and identify hepatic stimulator substance mRNA from human fetal liver tissues.

**METHODS** The poly (A)mRNA was extracted from human fetal liver tissues of 4-5 month gestation, fractionated by size on sucrose gradient centrifugation, translated into protein from each fraction *in vitro* and then its products were tested for HSS activity.

**RESULTS** Twenty-two 500 µg total RNA was obtained from human fetal liver tissues and pooled. mRNA of 420 µg was yielded, processed by oligo (dT)-cellulose column chromatography, then was size-fractionated by ultracentrifugation on a continuous sucrose density gradient (5%-25%), and separated into 18 fractions. Translated products of mRNA in fraction 8 and 9 could produce a two-fold increase in the incorporation of <sup>3</sup>H-TdR into DNA of SMMC-7721 hepatoma cells and in a heat resistant and organ-specific way.

**CONCLUSION** The partially purified HSS mRNA was obtained and this would facilitate the cloning of HSS using expression vectors.

## INTRODUCTION

Besides hormones such as insulin and glucagon, growth factors may be implicated in the complex regulation of a constant liver mass in the adult animal. Transforming growth factor (TGF)- $\alpha$ , hepatocyte growth factor (HGF) and epidermal growth factor (EGF) are important stimulators of hepatocyte proliferation, but their activities are not liver specific. On the contrary, hepatic stimulator substance (HSS) is a hepatic specific regulator found in the weanling and regenerating livers from rat, rabbit and pig<sup>[1]</sup>. We and others<sup>[2,3]</sup> have demonstrated the presence of stimulator factors in human fetal livers. Its purification and characterization have been reported<sup>[4,5]</sup>. Furthermore, we also have demonstrated that human HSS is the product of gene expression of fetal liver tissues<sup>[6]</sup>. But the sequencing of HSS so far has been unsuccessful. To facilitate cDNA cloning of human HSS, we approached the partial characterization of HSS mRNA extracted from human fetal liver tissues, with the technic of fractionation of sucrose gradient centrifugation and translation *in vitro*.

## MATERIALS AND METHODS

### Materials

RPMI 1640 was from Gibco Lab, Grand Island, NY; oligo(dT)-cellulose column was from Pharmacia Ltd, Milwaukee, WI; wheat germ translation system; cell titer 96<sup>(TM)</sup> non-radioactive cell proliferation assay were from Promega Ltd, USA.

**Cells lines and culture.** The following cells were maintained in monolayer (or suspension) culture in RPMI 1640 medium containing 10% (vol/vol) newborn calf serum: HTC, rat hepatoma cell line; SMMC-7721, human hepatoma cell line; HL-7704, human adult hepatocyte line; HL-60, human promyelocytic leukemic cell line; K562, human erythroid leukemic cell line. Primary tissue cultures (liver, spleen and kidney) were prepared as described<sup>[6]</sup>.

**Preparation of poly (A)+mRNA.** Total RNA was extracted from human fetal liver tissues of 4-5 month gestation by a modified procedure from Chomczynsky *et al*'s<sup>[7]</sup>. The extracted RNA was ap-

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\*Project supported by the National Natural Science Foundation and High-Tech Program of China, No.863-102-08-51.

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Receive 1997-07-10 Revised 1997-08-21

plied onto an oligo(dT)-cellulose column to obtain purified mRNA.

**Sucrose gradient fractionation of poly(A)+mRNA.** Poly(A)+mRNA was fractionated on 5%-25% sucrose gradient on SW41 rotor essentially as described by Buell GN *et al.*<sup>[8]</sup>. Briefly, poly(A)+mRNA samples were lyophilized to remove ethanol and dissolved in 0.5 ml of TE buffer, heat-treated at 65°C for 10min and cooled rapidly in ice. The poly(A)+mRNA of up to 100µg per gradient was layered directly on 5%-25% (w/v) linear sucrose gradients made in TE buffer and centrifuged in an SW41 rotor at 41 000 rpm for 15 h at 20°C. Eighteen fractions (each 1ml) were collected and mRNA in each fraction was precipitated by ethanol.

**Translation in wheat germ system.** mRNA 10 mg/L was heated at 67°C for 10min, immediately cooled on ice and added to the cell-free protein synthesis system (Promega LTD) according to the manufacturer's instructions. Then the wheat germ extract components were heated at 95°C for 15min, centrifuged at 12 000×g for 20min and the supernatant was transferred to a 0.5ml micro-centrifugation tube and assayed for bioactivity.

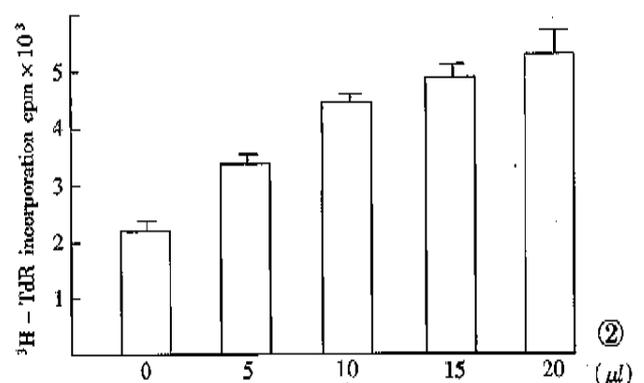
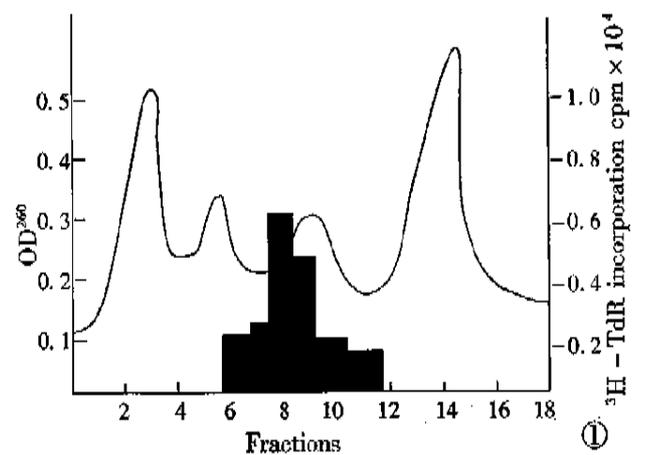
**Stimulation assay of hepatocyte proliferation.** Cells were counted and adjusted to  $5 \times 10^4$  cell/ml, and 100µl of cell suspensions were inoculated into the 96-well plastic plates and incubated for 6 h at 37°C at a humidified 5% CO<sub>2</sub> atmosphere, the medium was then replaced by a fresh one such as human partially purified HSS, translated components, culture supernatant or cytosolic fraction of transfected cos-7 cells. After 24 h of culture, 0.5µCi of [<sup>3</sup>H] thymidine per well was added and incubated for 3 h, and the cells were then collected to filters, and radioactivity was determined by a liquid scintillation counter. The activity was estimated by stimulation index (SI). SI=cpm of experimental cells/cpm of control cells.

## RESULTS AND DISCUSSION

A total of 22 500 µg RNA was obtained from human fetal liver tissues and pooled. Of this RNA, 22 000 µg was processed by oligo(dT)<sup>22</sup>cellulose column chromatography and 420 µg mRNA was yielded. This mRNA was then size-fractionated by ultracentrifugation on a continuous sucrose density gradient (5%-25%), and separated into 18 fractions, and the aliquots of each fraction were translated *in vitro* as described in the section of methods, and its products for biological activity were tested. As shown in Figure 1, the translated products of mRNA in fraction 8 and 9 could produce a two-fold increase in the incorporation of <sup>3</sup>H-TdR

into DNA of SMMC-7721 hepatoma cells.

Hepatoma cells are common targets for bioassay of hepatic stimulatory activity<sup>[9]</sup>. In the present experiment, human HSS directly prepared from fetal liver cells according to the previously described method<sup>[10]</sup> produced a two-fold increase in the incorporation of <sup>3</sup>H-TdR into DNA of SMMC-7721 hepatoma cells. Although the stimulating activity was not observed when the translated products of unfractionated mRNA was tested (Table 1), the dose-response effect of the translated products of fraction 8 and 9 mRNA (specific mRNA) on the stimulation of DNA synthesis of SMMC-7721 cell showed a positive relationship (Figure 2). The failure in detecting stimulating activity of total translated products directed by unfractionated mRNA was similar to that of our previous experiment, which might be caused by the presence of cell growth inhibitors<sup>[6]</sup>.



**Figure 1** Sucrose density gradient centrifugation analysis of HSS mRNA.

**Figure 2** Dose-response effect of SMMC-7721 cells to translated product of specific mRNA. Data are presented as  $\bar{x} \pm s$  and based on the result of five experiments (four samples for each).

**Table 1 Effect of translated products directed by different mRNA fractions on DNA synthesis of SMMC-7721 hepatoma cells**

Fractions	cpm	SI
Control	2450±452	
Purified hHSS	5384±602	2.1 <sup>a</sup>
Unfractionated mRNA	2430±292	1.0
Fraction 6	2202±389	0.9
Fraction 8	5013±382	2.0 <sup>a</sup>
Fraction 9	4123±452	1.7 <sup>a</sup>
Fraction 10	1894±172	0.7

Data are presented as  $\bar{x} \pm s$  and based on the results of four experiments (four samples for each). <sup>a</sup> $P < 0.05$ , showing stimulating effect.

**Table 2 Organ specificity of translated products of specific mRNA ( $\bar{x} \pm s$ )**

Test system	Control	Translated product of specific mRNA	Purified hHSS
SMMC-7721	1245±274	4703±236 <sup>a</sup>	4279±193 <sup>a</sup>
HFL	346±79	758±102 <sup>a</sup>	693±87 <sup>a</sup>
HFK	308±74	298±70	378±92
HFS	248±63	304±74	238±75
K562	2438±256	2058±217	3072±287
HL-60	4308±362	4703±298	3994±304

HFL, human fetal liver primary culture; HFK, human fetal kidney primary culture; HFS, human fetal spleen primary culture; hHSS, purified human hepatic stimulation substance. <sup>a</sup> $P < 0.05$ , showing stimulating effect.

**Table 3 Heat resistance of translated products of specific mRNA ( $\bar{x} \pm s$ )**

Treatment	Translated product of specific mRNA (fraction 8, 9)		Purified hHSS	
	cpm	SI	cpm	SI
Control	2034±148		1876±138	
NO heating	4703±236	2.3	4279±193	2.2
65°C for 10min	4306±287	2.1	5268±294	2.9
95°C for 10min	4579±263	2.2	5438±306	3.0

Data are based on the results of three experiments (four samples for each). The target cell was SMMC-7721 hepatoma cell.

To rule out the possible existence of the known nonspecific stimulators, we determined the target specificity and heat-resistance of the translated

product of specific mRNA. Table 2 shows that the bioactivity of translated product was liver-specific, i.e., only stimulating liver cell DNA synthesis, rather than spleen, kidney, HL-60 and so on. Table 3 indicates that the translated products were resistant to heating.

The effect of the various stimulators such as interleukin-6, fibroblast growth factor and insulin-like growth factor on the stimulation of hepatic DNA synthesis have been reported, but these stimulators could stimulate not only hepatocytes or hepatoma cells but also HL-60, K-562 cells. Unlike these stimulators, the translated products of fractionated mRNA were a liver-specific stimulator. The fact that translated stimulator of specific mRNA is resistant to heating and shows organ-specificity of action, strongly suggests that the partially purified human HSS mRNAs have been obtained. Because of the failure in amino acid sequence determination of HSS, the enrichment and fractionation of human HSS here should greatly facilitate the cloning of HSS cDNA using functional screening.

**REFERENCES**

- 1 LaBrecque DR. Hepatic stimulator substance: discovery, characteristics and mechanism of action. *Dig Dis Sci*, 1991;36(5):669-673
- 2 Tu Q, Wu CT. Biological activities of hepatocyte growth factor of human fetal liver origin. *Chin J Applied Physiol*, 1990;6(3):199-203
- 3 Yao Z, Yang W, Zhang W, Chen Y, Yang F. Human hepatic regenerative stimulator substance: partial purification and biological characterization of hepatic-stimulator substance from human fetal liver cell. *Hepatology*, 1990;12(3): 1144-1151
- 4 Tu Q, Wu CT. Experimental studies on human hepatocyte growth factor of fetal liver organ in the treatment of acute hepatic failure in mice. *Chin J Pathol*, 1991; 7(6):554-557
- 5 Tu Q, Wu CT. Studies on the purification and biological peculiarities of human hepatocyte growth factor of human fetal liver origin. *Chin J Pathol*, 1991;7(5): 537-541
- 6 He FC, Wu CT, Tu Q, Xing GC. Human hepatic stimulator substance: a product of gene expression of human fetal liver tissue. *Hepatology*, 1993;17(2): 225-229
- 7 Chomczynsky P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 1987;162(1): 156-159
- 8 Buell GN, Wickens MP, Payvar F, Schimke RT. Synthesis of full length cDNAs from four partially purified oviduct mRNAs. *J Biol Chem*, 1978;253(7): 2471-2482
- 9 LaBrecque DR, Wilson M and Fogerty S. Stimulation of HTC hepatoma cell growth in vitro by hepatic stimulator substance (HSS). *Exp Cell Res*, 1984;150 (2):419-422
- 10 LaBrecque DR, Pesch LA. Preparation and partial characterization of hepatic regenerative stimulator substance (SS) from rat liver. *J Physiol*, 1975;248(1): 273-284

# Inhibitory effects of two oligosaccharides on murine melanoma experimental liver metastasis\*

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**Subject** liver neoplasms, experimental; **headings** melanoma; oligosaccharides; neoplasm metastasis; disease models, animal

## Abstract

**AIM** To observe the effects of a chemically synthesized tetrose and a natural yeast mannan on experimental liver metastasis of mouse melanoma.

**METHODS** After treated with 4mg tetrose (tetrose group) or 4mg mannan (mannan group) for 30 minutes at 37°C, 0.5ml 1×10<sup>6</sup> B16-MBK melanoma cells were injected into the spleen of mice. Fifty-five days later, melanoma metastatic nodes on the surface of the liver and in other organs as well as mouse survival time were observed.

**RESULTS** Of the 6 mice in control (B16 cell+PBS) group, 4 died naturally within 55 days, and 2 were killed on the 55th day. All of the 6 mice had metastases in livers, the total number of the melanoma nodes on each liver surface ranged from 2 to 30, with the largest one merging into the whole liver. One mouse had a neoplasm in the remnant site of injection, and 3 had metastases in lungs. In contrast, of the 6 mice in tetrose group, only one died on the 50th day after injection, with 3 metastases in the liver, the largest being 10 mm in diameter, the other 5 mice survived until being dissected on the 55th day after injection and had no liver metastasis, but 3 of them had neoplasms in their remnant sites of injection. In mannan group, all of the 6 mice survived and no metastasis was seen except for 2 liver nodes in one mouse with the largest diameter of 1 mm. Neither tetrose nor mannan group had metastasis out of the liver, and the weight of liver in the two groups was significantly lower than those in the control group.

**CONCLUSION** Both tetrose and mannan had the effects of preventing melanoma cells from experimental metastasis to and out of the liver, and prolonging the survival time of the mouse.

## INTRODUCTION

The saccharide structures on the surface of tumor cells, particularly of the highly metastatic cancer cells, have certain peculiarities, and may play vital roles in the process of metastasis. We have demonstrated that some glycopeptides can significantly inhibit experimental metastasis of mouse melanoma and Lewis lung carcinoma cell lines towards the lungs and livers<sup>[1-3]</sup>, and the antimetastatic effects of the glycopeptides lie in their carbohydrate moieties. In order to explore the structural peculiarity associated with the metastasis-blocking function, and to develop new antimetastatic drugs, we designed and chemically synthesized a tetrose with a special structure, and chose the natural mannan to compare the effects of carbohydrate structures on experimental liver metastasis of mouse melanoma cells.

## MATERIALS AND METHODS

### *Animals and reagents*

Eighteen male Balb/c mice with similar body weight, obtained from the Animal Center of Beijing Medical University, were divided randomly into control group, tetrose group and mannan group, respectively, with 6 mice in each group. B16-MBK melanoma cell line (B16 cell for abbreviation) was provided by the Department of Cell Biology, Basic Medical Research Institute of Chinese Academy of Medical Sciences. Chemically synthesized tetrose was produced by the Department of Organic Chemistry in Beijing Medical University. RPMI 1640 was provided by JR Scientific Company, USA. Other reagents were A.R. or C.P. grade made domestically.

### *Methods*

**Induction of liver metastases.** Lafremiere's method was adopted<sup>[4]</sup>. Briefly, with the use of sterile instruments and gloves, Balb/c mice, after anaesthetized by ether inhalation, were set in the right lateral position, and a 1-cm incision was made at the left subcostal region. The spleen was gently retracted, and the short gastric vessels and gastrosplenic ligament at the upper pole of the spleen were identified, ligated and cut, thus freeing the spleen at its pedicle. This procedure allowed the spleen to be exposed outside the abdominal cavity. B16 cell suspension 0.5 ml (2×10<sup>6</sup> cells/ml) in serum-free 1640 medium was injected through a 27-gauge needle positioned in the spleen through its upper pole. The

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\*Project supported by the National Natural Science Foundation of China, No.39370169.

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Received 1997-09-02

spleen pedicle was clipped with a medium hemoclip, then the spleen was removed, and the spleen pedicle repositioned intraperitoneally. The abdominal cavity was closed in one layer. The animals were fed with standard mouse chow and water ad libitum. Fifty-five days later, the mice were killed and their livers were harvested, the tumor metastasis nodes were counted on the surface of the livers.

**Treatment of the B16 cells**

Fourty-eight hours after passage culture, the B16 cells were detached by brief digestion with 0.125% trypsin (in PBS without Ca and Mg), the digestion was stopped by 1640 medium with 10% fetal bovine serum and then the cells were resuspended in serum-free 1640 medium, readjusted to the cell concentration at  $2 \times 10^6$ /ml. After incubated respectively with 4mg tetrose (tetrose group) or 4mg mannan (mannan group) for 30 minutes at 37°C in a humidified air atmosphere of 5% CO<sub>2</sub>, the cell viability was assessed > 95% by 0.2% trypan blue exclusion test, and the cells were injected as described above in the spleen. In the control group, the cells were treated with PBS substitutionally.

**Statistical analysis**

The significance of differences among groups was determined by the Student's *t* test or  $\chi^2$  test. Two-tail *P* values were presented for all experiments.

**RESULTS**

**The melanoma experimental hepatic metastasis model**

With  $1 \times 10^6$  cells injected intraspleen, 4 mice in the control group died naturally within 55 days after the injection of B16 cells, and 2 mice were killed on the 55th day (Figure 1). All the mice in the control group had metastases in the livers, the number of the metastatic nodes ranged from 2 to 30, with the largest one merged into the whole liver. The liver weights were  $3.8 \text{ g} \pm 1.5 \text{ g}$ . Of the 6 mice, 3 had lung metastases simultaneously and one mouse was found to have a neoplasm in the injected area.

**The effects of oligosaccharide on melanoma liver metastasis**

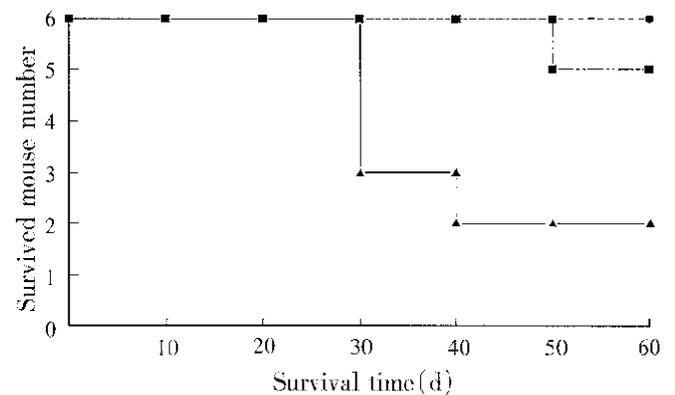
**Tetrose group.** One of the 6 mice died on the 50th day of injection with 3 melanoma nodes on its liver

surfaces, the largest diameter was 10mm, the other 5 were killed on the 55th day, 3 of which had neoplasms in their remnant sites of injection, but without metastases either in or out of the livers. The weights of livers ( $1.4 \text{ g} \pm 0.1 \text{ g}$ ) were significantly lower than the control group (Table 1).

**Mannan group.** All of the 6 mice survived until the dissection day, and only one mouse had 2 metastases in the liver with the node diameters < 1mm, others had no metastases either in or out of the livers. The livers weighted  $0.85 \text{ g} \pm 0.02 \text{ g}$ , being significantly lower than the control ( $P < 0.05$ ).

**DISCUSSION**

In our experimental liver metastasis model, intrasplenic injection of B16 cells was used to induce the cells into portal circulation, and allow the tumor cells to form liver metastases. The fact that all mice in the control group developed metastases in the livers indicated that the model was highly reproducible, carried a high metastatic rate, and therefore was a preferable model for experimental liver metastasis. Complete ligation of the gastric and other vessels and prevention of direct spleen clamping were critical, and leakage should be avoided, if it appears, the mouse should be given up.



**Figure 1** Mouse survival curve in each group after injection of B16 cell. -▲- Control ...●... Mannan -■- Tetrose

**Table 1** Effects of two oligosaccharides on experimental liver metastasis of B16 cells

Groups	Total No. mice	Liver metastases			Metastases out of liver (mice No.)
		Mice No.	Node No.	Node max. diameter	
Control	6	6	2-30	Fused to whole liver	3 <sup>△</sup>
Tetrose	6	1 <sup>a</sup>	0-3	10mm-8mm	0 <sup>a</sup>
Mannan	6	1 <sup>a</sup>	0-2	<1mm	0 <sup>a</sup>

$\chi^2$  test <sup>a</sup> $P < 0.05$  vs control;  $\Delta$ : metastases were found in lungs.

Recently, Dean *et al*<sup>[5]</sup> successfully inhibited experimental metastasis to lungs using synthetic multivalent lactosyl clusters. Tsukada *et al*<sup>[6]</sup> proved the antimetastatic and growth inhibitory effects of N-acetylchitohexaose in mouse Lewis lung carcinoma. The tetrose and mannan in the present study, with the structure differing from the above two carbohydrates, are strikingly effective in inhibiting experimental liver metastases. The tetrose we designed exists in the saccharide chains of tumor cell surface, its action suggested that the carbohydrate with this structure may block the metastatic process of tumor cells. The antimetastatic effect of mannan is also inspiring. Chandrasekaran *et al*<sup>[7]</sup> demonstrated *in vitro* that the spreading of B16 cells on basement membrane depends on the N-linked high mannose carbohydrate structure. The present study showed that after incubated with mannan, the B16 cells were blocked to metastasize toward the liver, probably by the blocking effect of mannan on the interaction, including spreading of B16 cells and basement membrane. As we know, spreading is a prerequisite of a series of biological processes (such as secreting proteinase, migration and proliferation) in invasion of tumor cells.

It should be noted, however, that the tetrose suppressed experimental liver metastases without inhibiting the neoplasia, since there were neoplasms in the remnant sites of injection in some mice in the tetrose group. The findings also proved that the antimetastatic effect was not due to the cytotoxic effect of the oligosaccharides, as all cells were fully viable after the treatment with tetrose or mannan. All these demonstrated that these two oligosaccharides selectively inhibited the experimental metastasis of mouse melanoma cells.

## REFERENCES

- 1 Zhao Y, Zhou RL. The anti-metastatic effect of laminin glycopeptides in mouse B16-MBK melanoma experimental metastasis. *J Beijing Med Univ*, 1992;24(5):404-406
- 2 Zhang QY, Zhou RL, Zhang CY. The pathological observation of anti-metastatic effect of glycopeptides in mouse Lewis lung carcinoma experimental metastasis. *J Biochem*, 1991;7(6):719-721
- 3 Liu YP, Zhou RL, Zhang S. Laminin glycopeptides inhibit mouse melanoma liver metastasis. *J Beijing Med Univ*, 1996;28(2):91-92
- 4 Lafremiere R, Rosenberg SA. A novel approach to the generation and identification of experimental hepatic metastasis in a murine model. *JNCL*, 1986;76(2):309-315
- 5 Dean B, Oguchi H, Cai S, Otsuji E, Tashiro K, Hakomori S *et al.* Synthesis of multivalent  $\beta$ -lactosyl clusters as potential tumor metastasis inhibitors. *Carbohydrate Res*, 1993;245:175-192
- 6 Tsukada K, Matsumoto T, Aizawa K. Antimetastatic and growth-inhibitory effects of N acetylchitohexaose in mice bearing Lewis lung carcinoma. *Jpn J Cancer Res*, 1990;81(2):259-263
- 7 Chandrasekaran S, Tanzer ML, Giniger MS. Oligomannosides initiate cell spreading of LN-adherent murine melanoma cells. *J Biol Chem*, 1994;269(5):3356-3366

# Detection of hepatoma cells in peripheral blood of HCC patients by nested RT-PCR\*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; RNA, messenger/blood; alpha-feto-proteins polymerase chain reaction; neoplasm metastasis

## Abstract

**AIM** To offer a more simple method with a high sensitivity and specificity for detection of hepatoma cells in peripheral blood of the patients with HCC.

**METHODS** Improved nested RT-PCR method was used to detect the expression of AFP mRNA in nuclear cells separated from peripheral venous blood.

**RESULTS** AFP mRNA contained in ten hepatoma cells was detected from 2mL peripheral blood.

**CONCLUSION** The improved nested RT-PCR assay for AFP mRNA expressed in cancer cells in peripheral blood might be a valuable method for clinical diagnosis of HCC.

## INTRODUCTION

The metastatic potential of hepatocellular carcinoma (HCC) is not only directly correlated with prognosis of the patients with HCC, but also extremely valuable for selection of adequate therapies for HCC. Detection of circulating hepatoma cells in peripheral blood might be an indicator of metastatic potential of hepatocellular carcinoma. Human  $\alpha$ -Fetoprotein (AFP) is a well-known marker for hepatoma cells. Although the serum AFP level could be one of the useful biochemical indicators for diagnosis of HCC, the conventional measurements for serum AFP concentration is not so sensitive to identify an extremely small amount of cancer cells in peripheral blood. Based on the nested RT-PCR method for detecting AFP mRNA in the circulation reported recently<sup>[1,2]</sup>, we merged the reverse transcription reaction and the first PCR into a single step, and then combined it with nested PCR. By this way, AFP mRNA contained in 10 human hepatoblastoma cells (HepG-2) could be detected in 2mL peripheral venous blood. Being highly sensitive and specific, the improved method requires relatively a small amount of blood sample, and less time to obtain similar satisfactory results.

## MATERIALS AND METHODS

### Materials

HepG-2 was derived from the Institute of Virology, Chinese Academy of Preventive Medical Sciences. The AFP primers<sup>[3]</sup> purified by HPLC were obtained from Cybersyn, BJ. The external primers were: 5' ACT GAA TCC AGA ACA CTG CAT AG3' and 5' TGC AGT CAA TGC ATC TTT CAC CA3'. The internal primers were: 5'TGG AAT AGC TTC CAT ATT GGA TTC3' and 5'AAG TGG CTT CTT GAA CAA ACT GG3'. Taq DNA polymerase and AMV reverse transcriptase were purchased from Sino-American Corp. and Promega Inc., respectively.

### Methods

**Separation of nuclear cells from peripheral blood.** Fresh healthy peripheral venous blood was collected into a sterilized tube containing 0.5% EDTA. One thousand HepG-2 cells were mixed with 2mL healthy blood and serial dilution of cancer cells was then prepared. Each dilution sample contained 1000, 100, 10 and 1 HepG-2 cells per 2mL of blood, respectively. Nuclear cells were separated from peripheral venous blood using routine method by Ficoll

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Received 1997-10-28 Revised 1997-12-01

isolation.

**Extraction of total RNA from nuclear cells.** Total RNA of nuclear cells separated from whole blood was extracted using single-step method by acid guanidinium thiocyanate extraction<sup>[4]</sup>.

**Reverse transcription and the first PCR.** A total amount of 50 $\mu$ L reaction solution contained 5 $\mu$ L 10 $\times$ PCR buffer, 0.2mmol/L of each dNTPs, 20 pmol-50pmol of each external primers, 50 U RNAase inhibitor, 30 U AMV reverse transcriptase, 3U Taq DNA polymerase and RNA template. The mixture solution was incubated at 42 $^{\circ}$ C for 30min, and followed by initial denaturation at 94 $^{\circ}$ C for 2min. The PCR procedure was carried out as follows: denaturation at 94 $^{\circ}$ C for 30s, annealing at 52 $^{\circ}$ C for 30s and extension at 72 $^{\circ}$ C for 40s, and the thermal cycles were repeated 32 times.

**Nested PCR.** Reaction mixture of 50 $\mu$ L contained 5 $\mu$ L of 10 $\times$ PCR buffer, 0.2mmol/L of each dNTPs, 20pmol-50pmol of each internal primers, 3U Taq DNA polymerase and 10 $\mu$ L of the first PCR product. The PCR procedure was: denaturation at 94 $^{\circ}$ C for 30s, annealing at 52 $^{\circ}$ C for 30s and extension at 72 $^{\circ}$ C for 40s; and the cycles were repeated 32 times as well.

**Gel electrophoresis.** The final amplification product was electrophoresed on 2% agarose gel and stained with ethidium bromide for the specific band of 101bp.

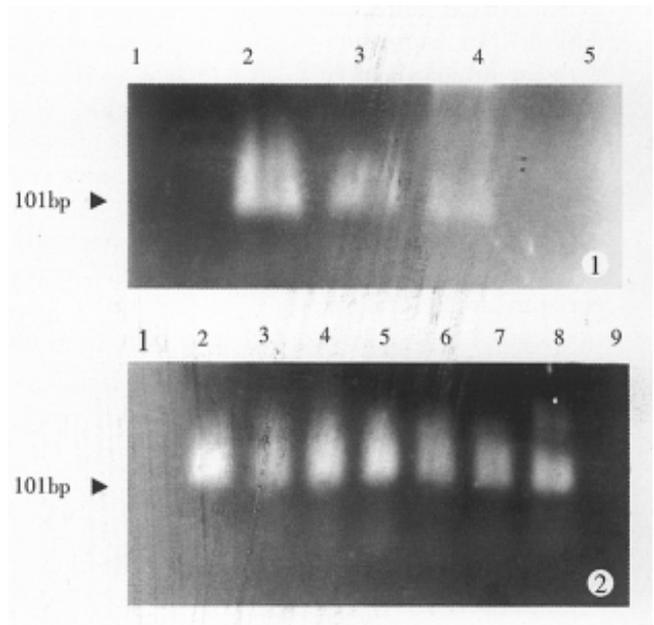
**Internal standard control assay.** Total RNA extracted from HepG-2 cells was quantified and diluted serially with DEPC-treated water, which was used as the internal standard control of this assay. Each of internal standard control samples contained 5, 1, 0.5, 0.05, 0.005, 0.0005 and 0.00005  $\mu$ g of RNA template, respectively. The diluted samples were applied to nested RT-PCR amplification following the same procedure described above.

## RESULTS

The amplification template used in this method was total RNA derived from HepG-2 cells. To seek a rapid and simple detecting method for AFP mRNA expression in hepatoma cells, we merged the reverse transcription reaction and the first PCR amplification into a single step and then combined with nested PCR. Gel electrophoresis analysis showed that specific bands (101bp) of AFP mRNA were not observed in nuclear cells from healthy peripheral venous blood. When 1000, 100, 10 HepG-2 cells were added into 2mL blood, specific bands of 101bp were demonstrated in all RNA samples from nuclear cells in each of diluted samples. However, RNA from the diluted blood containing 1 HepG-2 cells was in-

sufficient for detection (Figure 1).

As indicated in Figure 2, internal standard control assay demonstrated that the intensity of the specific bands for AFP mRNA was significantly correlated with the amount of total RNA templates. No specific bands for AFP were detected in the sample of  $5 \times 10^{-6}$  $\mu$ g RNA from HepG-2 cells by nested RT-PCR. With regard to negative control with absence of RNA from HepG-2 cells, it was also impossible to detect such specific bands.



**Figure 1** Amplification of AFP mRNA expressed in nuclear cells in peripheral blood by nested RT-PCR.

Lane 1: Blood from healthy volunteers.

Lanes 2-5: Titration of HepG-2 cells. Each lane includes (from left to right) 1000, 100, 10, 1 HepG-2 cells in 2 mL normal blood, respectively.

**Figure 2** Amplification of AFP mRNA in HepG-2 cells by nested RT-PCR.

Lane 1: Negative control.

Lanes 2-9: Titration of total RNA extracted from HepG-2 cells. Each lane includes (from left to right) 5, 1, 0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000005 $\mu$ g total RNA, respectively.

## DISCUSSION

Primary hepatocellular carcinoma is one of the most common malignant tumors in China, with the death rate ranking the first in the urban population and the second in rural areas of China. Prognosis of HCC is not only closely associated with early diagnosis and optimal therapy, but also with some important characteristics of hepatoma cells, such as the potential of intrahepatic metastasis, extrahepatic metastasis and recurrence. How to estimate the metastatic potential of hepatoma cells has remained a major puzzle for many years. AFP is expressed by

hepatoma cells and then secreted into circulating blood, thus being regarded as a useful marker for hepatocellular carcinoma. Hepatoma with the potential of intrahepatic or extrahepatic metastasis often shed hepatoma cells into portal venous blood and peripheral blood.

It was reported that the human albumin mRNA could be used as a marker of circulating hepatocytes in HCC<sup>[5]</sup>, and that AFP mRNA expression could be detected in a tiny amount of hepatoma cells in circulating blood of patients with HCC<sup>[1,2]</sup> by nested RT-PCR. Furthermore, they found that the level of AFP mRNA expression was significantly correlated with the prognosis of HCC. RT-PCR is a very sensitive technique for amplifying nucleic acids, which could be often used to detect tiny amounts of mRNA copies. Since nested RT-PCR utilizes a couple of internal primers to reamplify the specific PCR product, it exhibits higher sensitivity, stronger specificity and lower false positive occurrence as compared to single RT-PCR.

In this study, we attempted to provide a more

simple nested RT-PCR method with high sensitivity and specificity for detecting a tiny amount of hepatoma cells in circulating blood of patients with HCC. By this method, AFP mRNA could be detected when 10 HepG-2 cells were present in 2mL peripheral blood or portal venous blood, suggesting that the sensitivity of this method is similar to that reported in literature. Thus, it might be a desirable assay for clinical evaluation of metastatic potential of hepatocellular carcinoma.

## REFERENCES

- 1 Komeda T, Fukuda Y, Sando T, Kita R, Furukawa M, Nishida N *et al*. Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. *Cancer*, 1995;75(9):2214-2219
- 2 Matsumura M, Niwa Y, Hikiba Y, Okano K, Kato N, Shiina S *et al*. Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. *Biochem Biophys Res*, 1995;207(2):813-818
- 3 Morinaga T, Sakai M, Wegmann TG, Tamaoki T. Primary structures of human alpha-fetoprotein and its mRNA. *Proc Natl Acad Sci USA*, 1983;80(15):4604-4608
- 4 Chromczynski P, Acchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt Biochem*, 1987;162(1):156-159
- 5 Hillaire S, Barbu V, Boucher E, Moukhtar M, Poupon R. Albumin messenger RNA as a marker of circulating hepatocytes in hepatocellular carcinoma. *Gastroenterology*, 1994;106(1):239-242

# Hepatic adenylate energy charge levels in patients with hepatoma after hepatic artery embolization\*

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**Subject headings** ketone bodies; energy metabolism; liver/metabolism; liver neoplasms/therapy; carcinoma, hepatocellular/therapy; embolization, therapeutic; postoperative complication; liver function test

## Abstract

**AIM** To evaluate hepatic energy charge levels of the patients with hepatoma after hepatic artery embolization and its relation to postoperative complications.

**METHODS** Sixty-nine patients with hepatoma were continuously measured for their arterial blood ketone body ratio (AKBR) and compared with their postoperative clinical course or conventional liver function test after various hepatic artery embolization.

**RESULTS** AKBR in high radiation dose or jaundice group drastically decreased at 1-3 days and recovered slowly. Patients were classified into three groups according to the value of AKBR: group A (35 cases), AKBR remained higher than 0.7; group B (31 cases), AKBR had transiently dropped to 0.4-0.7 and then increased to preoperative value; and group C (3 cases), AKBR decreased steadily to below 0.4. The occurrence rate of various complications were 5.7%, 32.3% and 100% in the three groups, respectively ( $P < 0.005$ ).

**CONCLUSION** The AKBR which reflects hepatic mitochondria redox state is morereliable as a direct indicator to assess hepatic tolerance for embolization than routine liver function test.

## INTRODUCTION

Hepatic artery embolization has been the first choice for advanced hepatomas in recent years, the therapeutic effect depends upon not only thoroughness of chemoembolization or radioembolization, but also closely correlated with liver function reserve<sup>[1]</sup>. It has been shown that conventional liver function tests could not give a direct and accurate evaluation of postoperative metabolic abnormality, while the arterial blood ketone body ratio (AKBR) which reflects hepatic mitochondria redox state could serve as a relatively more reliable parameter to assess the extent of hepatic injury and vitality<sup>[2]</sup>. This study investigates the effects of various embolization methods on hepatic energy charge level and its relation to postoperative complication.

## MATERIALS AND METHODS

### Subjects

Sixty-three patients with unresectable primary hepatocellular carcinoma, 3 metastatic liver carcinoma and 3 haemangioma confirmed by B-type ultrasonography, hepatic angiography, computed tomography and operation were included in this study. There were 66 males and 3 females, aged from 21 to 64 years with a mean age of 44.3 years. Liver cirrhosis was found in 62 patients (89%). Preoperative child classification of liver function was A stage, 53 cases; B stage, 15 cases; and C stage, 1 case and increased total bilirubin was detected in 20 patients.

### Hepatic artery embolization

Intraoperative transcatheter arterial embolization was accomplished via selective right hepatic artery (39 cases) or nonselective common hepatic artery (30 cases). Eight patients received simple embolization with Gelfoam stripes. Chemoembolization with emulsion of lipiodol 10mL-15mL containing adriamycin 20mg and mitomycin C 10mg was applied to 30 patients. Radioembolization was performed on 31 patients using phosphorus 32 glass radiomicrospheres provided by the Chinese Academy of Atomic Energy. The microspheres of 0.75mg-5.6mg (radioactivity, 2.16mCi/g-11.78mCi/g, mean 61  $\mu$ m) was equal to a mean 33Gy of hepatic absorbed radiation doses used in this study.

### AKBR assay

For the assay of ketone bodies, 4ml arterial blood

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\*Funded by the China Medical Board, NY, USA

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Received 1997-10-28 Revised 1997-12-15

samples were obtained with a heparinised syringe via the femoral artery 1 day preoperatively, and 1, 3, 5 and 7 days postoperatively. When blood samples were taken, all patients were receiving intravenous infusion with 10% glucoses to maintain their blood glucose levels at 6.7mmol/L - 11.2mmol/L. The blood samples were immediately mixed with the same volume of ice-cold 10%w/v perchloric acid. The suspension was centrifuged at 10 000×g for 15min at 0°C. The supernatant was adjusted to a pH of 7.0 with cold 65% w/v potassium carbonate and recentrifuged at 10 000×g for 5min at 0°C. The final supernatant was used to measure the AKBR (AKBR = acetoacetate/ β-hydroxybutyrate), spectrophotometrically by the standard methods of Mellanby and Williamson<sup>[3]</sup>.

**Statistical analysis**

The AKBR of intergroups was compared by analysis of variance and the positive rates by Chi-square test. *P* value < 0.05 was considered to be statistically significant.

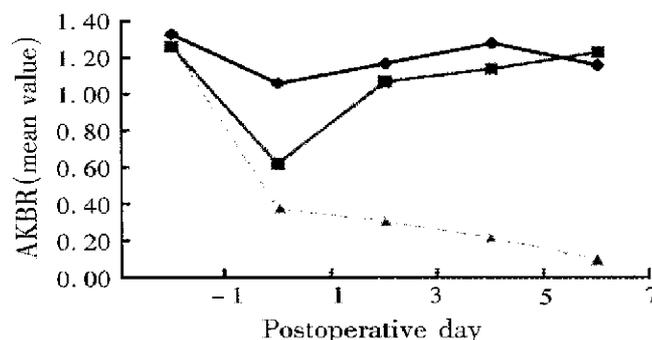
**RESULTS**

As shown in Figure 1, the patients were classified into three groups according to the postoperative changes in their AKBR. In group A (35 patients), the AKBR was above 0.7 after operation and returned to near the preoperative level in 3-7 days. In group B (31 patients), the AKBR remained between 0.4 and 0.7 after operation and thereafter gradually rose to above 0.7. In group C (3 patients), the AKBR decreased to below 0.4 and even dropped to below 0.25 without restoration.

The major postoperative complications in each group are summarized in Table 1, the occurrence

rate was 5.7% (2/35), 32.3% (10/31) and 100% (3/3) respectively (*P*≤0.005), with no significant statistical difference among the groups (*P*<0.005). However, no difference was found in postoperative changes in serum enzyme activities, albumin and total bilirubin concentrations among the three groups.

As shown in Table 2, the radioembolization at large doses had marked effect on the AKBR levels, which declined rapidly after operation and recovered slowly along with repeated occurrence of complications. The preoperative AKBR in the group with elevated bilirubin was lower than that of normal bilirubin (*P*<0.02), and most of the cases fell to below 0.7 within 1-3 postoperative days and were accompanied by a higher morbidity of complications. Liver dysfunction and hemorrhage occurred in six patients with low preoperative AKBR level (0.69-0.85), and two of them eventually died of multiple organ failure, whereas only two of other fourteen patients with AKBR of 0.97-1.43 pre-operatively suffered from cholecystitis and wound dehiscence.



**Figure 1** Postoperative changes of AKBR in three groups.

**Table 1** The relationship between changes in their AKBR and postoperative complications

Groups	Simple embolization			Chemoembolization			Radioembolization		
	Cases	Complications	Class	Cases	Complications	Class	Cases	Complications	Class
A	7	0	0	15	1	2	13	1	2
B	1	0	0	14	3	4	16	7	6
C	0	0	0	1	1	5	2	2	7

The class of complication includes: liver dysfunction, hemorrhage, multiple organ failure, cholecystitis, wound dehiscence, coagulation defects, pulmonary infection, renal insufficiency, pancreatitis, enteritis and sepsis.

**Table 2** Effects of radiation doses, embolic area and with or without jaundice on the AKBR

Groups	Cases	AKBR value					Complications
		Preoperation	First day	Third day	Fifth day	Seventh day	
High doses	8	1.26±0.41	0.66±0.15 <sup>a</sup>	0.76±0.24 <sup>a</sup>	0.81±0.18 <sup>a</sup>	0.71±0.29 <sup>a</sup>	63 <sup>b</sup>
Moderate doses	12	1.21±0.39	0.97±0.26 <sup>a</sup>	1.09±0.15 <sup>a</sup>	1.17±0.12 <sup>a</sup>	1.11±0.23 <sup>a</sup>	25
Low doses	11	1.23±0.25	0.89±0.12	0.98±0.19	1.13±0.20	1.08±0.11	18 <sup>b</sup>
With jaundice	20	0.99±0.26 <sup>a</sup>	0.72±0.19 <sup>a</sup>	0.84±0.20 <sup>a</sup>	0.91±0.32 <sup>a</sup>	0.89±0.21 <sup>a</sup>	40 <sup>b</sup>
Without jaundice	49	1.36±0.31 <sup>a</sup>	0.92±0.18 <sup>a</sup>	1.07±0.13 <sup>a</sup>	1.08±0.25 <sup>a</sup>	1.18±0.23 <sup>a</sup>	15 <sup>b</sup>
Inselective	30	1.26±0.24	1.01±0.32	1.03±0.25	1.18±0.11	1.03±0.29	27
Selective	39	1.33±0.20	0.98±0.41	1.12±0.10	1.04±0.27	1.16±0.18	18

Intergroup comparison: <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.05. High doses: mean 55Gy, Moderate doses: 31Gy, Low doses: 18.5Gy.

## DISCUSSION

AKBR can reflect the oxidoreduction state of free nicotinamide adenine dinucleotides (NADH) in liver mitochondria, i.e.,  $AKBR = K \times \text{free NAD}^+ / \text{free NADH}$ , which is closely linked to energy charge level ( $ATP + 1/2ADP / ATP + ADP + AMP$ )<sup>[4]</sup>. The AKBR, when it is greater than 1, 1-0.7, 0.7-0.4, 0.4-0.25 and less than 0.25, may indicate respectively normal mitochondria function (takes mainly sufficient glucose oxidation with an increased NAD<sup>+</sup>), slight mitochondria dysfunction (takes preferentially oxidized fatty acids as an efficient energy source rather than glucose such as group A), mitochondria membrane impairment (leads to inhibited pyruvate oxidation and enhanced  $\beta$ -oxidation of long-chain fatty acid, various complications may occur under the condition of constant ketogenesis, such as group B), serious mitochondria injury and death (results in an irreversible damage of the energy-generating system such as group C).

It has been found that acute ischemia, chemical toxic agent and nuclide internal radiation precipitate direct injury to mitochondria. Our results indicate that the reduced AKBR was related to radiation doses, and the radiomicrosphere affected obviously the hepatic energy charge level among the three therapeutic patterns (embolization, chemoembolization, radioembolization) and the two targets (selective or inselective). Hepatocellular hypoxia could inhibit the electron transport system in mitochondria chain and produced excessive accumulation of NADH. The range of AKBR decline has proved to be paralleled to the blocked extent and time of hepatic blood flow<sup>[5]</sup>, and the hepatic cell can be compromised by  $\beta$ -ray radiation and terminal embolic material.

Therefore, internal radiation therapy at large doses might not be an advisable procedure for patients with liver carcinomas.

Preoperative AKBR might serve as an approximate index for evaluation of energy reserve status. When patients were associated with severe cirrhosis and obstructive jaundice, chronic mitochondria dysfunction potentially preexisted because of their deprived portal flow, reduced endogenous insulin level acting on oxidative phosphorylation process, altered ATP transport rate and prohibited directly by bilirubin<sup>[2]</sup>. Therefore, the higher AKBR may indicate some acute compensatory capacity for injury, and their tolerance to embolization can be quantitatively assessed by combination of redox tolerance test (RTT)<sup>[6]</sup>, while the lower AKBR demonstrates that preoperative compensatory activity for synthesizing liver ATP is up to an extreme limit, e.g. various complications were observed in six patients with hyperbilirubinemia and preoperative AKBR of 0.69-0.85. Hence, the indication for TAE must be carefully considered in patients with those risk factors.

## REFERENCES

- 1 Nakamura H, Mitani T, Murakami T, Hashimoto T, Tsuda K, Nakanishi K *et al.* Five year survival after transcatheter embolization for HCC. *Cancer Chemother Pharmacol.* 1994;33(Suppl):89-92
- 2 Iwata S, Ozawa K, Shimahara Y, Mori K, Kobayashi N, Kumada K *et al.* Diurnal fluctuation of arterial ketone body ratio in normal subjects and patients with liver dysfunction. *Gastroenterology.* 1991;100(9):1371-1378
- 3 Williamson DH, Mellanby T. *Methods of enzymatic analysis.* New York: Academic Press, 1974:1836-1843
- 4 Ozawa K, Chance B, Tanaka S, Iwata S, Kitai T, Ikai I. Linear correlation between acetoacetate/ $\beta$ -hydroxybutyrate in arterial blood and oxidized flavoprotein/reduced pyridinenucleotide in freeze trapped human liver tissue. *Biochim Biophys Acta.* 1992;1138(6):350-352
- 5 Kajimura K, Moriyasu F, Someda H, Okuma M, Mori K, Ozawa K. Transcatheter hepatic arterial drug infusion therapy for hepato-cellular carcinoma: effect on the arterial ketone body ratio. *Scand J Gastroenterol.* 1993;28(6):522-526
- 6 Yan LN, Wu YT, Meng XQ, Cheng LL, Li XW, Zhou Y *et al.* Redox tolerance test: an indicator of preoperative hepatic functional reserve in hepatoma. *Asian J Surg.* 1994;17(4):365-370

# Effects of combined use of diallyl disulfide and *N*-acetyl-cysteine on acetaminophen hepatotoxicity in $\beta$ -naphthoflavone pretreated mice

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**Subject headings** diallyl disulfide; *N*-acetyl-cysteine; acetaminophen; beta-naphthoflavone; liver/drug effects; glutathione; 7-alkoxycoumarin O-dealkylase

## Abstract

**AIMS** To assess the protective effect of diallyl disulfide (DADS) and its combined use with *N*-acetyl-cysteine (NAC) on acetaminophen (APAP) hepatotoxicity in C57BL/6N (B6) mice pretreated with  $\beta$ -naphthoflavone (BNF).

**METHODS** B6 mice were divided into six groups and all compounds used were injected intraperitoneally. Except for control and APAP group (receiving APAP only), the other groups received an injection of APAP (350mg/kg) 48 hours after BNF (200mg/kg) and either of DADS (200mg/kg), or NAC (500mg/kg) or both DADS and NAC. DADS was given 2 hours before APAP and NAC was injected with APAP. The mean survival time was recorded and livers were examined histologically. Hepatic glutathione (GSH) levels and plasma ALT were also determined at different time points. To evaluate the effect of DADS or NAC on hepatic P450 induction by BNF, liver microsomes were prepared and 7-ethoxyresorufin O-dealkylase (ERD) activity was determined using spectrofluorometrical methods. In vitro effect of DADS or NAC on ERD activity was assayed by directly incubating microsomal suspension with DADS or NAC of different concentrations.

**RESULTS** APAP was not toxic to mice without BNF pretreatment, but caused severe liver necrosis and death of all BNF-treated mice in 4 hours. A sharp depletion of GSH (approximately 62% of its initial content at 2 hours and 67% at 4 hours) and a linear elevation of ALT levels (536.8 $\pm$ 29.5 Sigma units at 2 hours and 1302.5 $\pm$ 74.9 at 4 hours) were observed. DADS and NAC given indi-

vidually produced mild protection, resulting in prolonged survival, a slower decline of GSH level and a less steeper elevation of ALT level. All mice died eventually. Co-administration of DADS and NAC completely protected mice. GSH level in this group lowered by about 35% and 30% at 2 and 4 hours, and ALT was 126 $\pm$ 18 and 157.5 $\pm$ 36.6 Sigma units at 2 and 4 hours. ERD activity in BNF-treated mice was about 5 times that of the constitutive level determined in normal mice. Neither DADS nor NAC inhibited P450 1A1/1A2 induction as determined by their effect on the induction of ERD activity. In vitro assay indicates that DADS, but not NAC, was a potent inhibitor of ERD activity (IC<sub>50</sub>=4.6 $\mu$ M).

**CONCLUSIONS** A combined use of both DADS and NAC produced full protection in BNF treated mice against APAP hepatotoxicity. The mechanism is that DADS inhibits P450 1A1/1A2 activity, but not induction, which substantially reduces production of NAPQI, while NAC enhances liver detoxifying capability via serving as a precursor of GSH and stimulating GSH synthesis.

## INTRODUCTION

Acetaminophen (APAP) has been widely used for decades, especially as an over the counter analgesic in North America. It has been long known that an overdosed APAP can cause severe damages to the liver or even death of the experimental animals and individuals who have ingested large quantities of APAP accidentally or in an attempt to commit suicide. In the liver, APAP is bioactivated and converted by cytochrome P450 to its metabolite, *N*-acetyl-p-benzoquinoneimine (NAPQI), which has been shown to be toxic to animals in vivo<sup>[1]</sup> and to cultured hepatocytes<sup>[2,3]</sup>. Although the precise mechanism of APAP hepatotoxicity is not well understood, a number of studies have suggested that NAPQI exerts a cytotoxic effect through its covalent binding to cytosolic or microsomal proteins and membrane components<sup>[4-6]</sup>, inhibition of mitochondrial respiration, depletion of ATP<sup>[7,8]</sup>, etc.

For bioactivation of APAP, several P450 isoforms are implicated, including P450 1A1, 2A1, 2B1, 2C11, 2E1, 3A1 and 3A2<sup>[9,10]</sup>. These enzymes are inducible by cigarette smoking, alcohol consumption, drugs, and some chemical compounds

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Received 1997-12-30

used daily at home for cleaning or as a pesticide. Elevation of P450 activity by these inducers can markedly enhance APAP hepatotoxicity in humans<sup>[11]</sup> and experimental animals<sup>[12]</sup>. For instance, alcohol is an inducer of P450 2E1 and therefore alcoholics are more susceptible to and at higher risk for development of APAP hepatotoxicity<sup>[11]</sup>. More attention should be paid to these risk factors because these individuals may develop hepatotoxicity at the therapeutic doses. In recent years, several compounds have been tested for their protective effects on overdose APAP. However, information about the possible treatment for APAP toxicity in P450-elevated animal models or human subjects is very limited. Diallyl sulfide (DAS) is a P450 inhibitor specifically for 2E1. Recently, a study showed that DAS prevented APAP hepatotoxicity in normal rats<sup>[13]</sup>. The current work investigated the role of diallyl disulfide (DADS) and its combined use with *N*-acetyl-cysteine (NAC) in protecting APAP-caused liver damage in mice in which P450 levels were induced by  $\beta$ -naphthoflavone (BNF) pretreatment. To our knowledge, this is the first investigation on the inhibitory effect of DADS on hepatic P450 1A1/1A2 induced by BNF in mice and the protective effect of the use of DADS and NAC in combination on APAP caused-hepatotoxicity.

## MATERIALS AND METHODS

### *Effect of DADS and NAC on APAP hepatotoxicity*

**Animals and animal treatment.** C57B/6N (B6) mice (4 - 6 weeks of age, 18 g - 20 g in body weight) were purchased from Harlan Sprague Dawley (Indianapolis, USA). The mice were divided into six groups (10 mice each) and treatment for each group is shown in Table 1. All compounds used were purchased from Sigma, USA (except indicated otherwise) and injected intraperitoneally. BNF was injected at dose of 200mg/kg body weight (in corn oil) 48 hours prior to administration of APAP (350 mg/kg body weight in saline) as described previously<sup>[14]</sup>. DADS (200mg/kg body weight, purchased from Aldrich, USA) was diluted in 0.2ml corn oil and given 2 hours before APAP challenge. NAC of 500mg/kg body weight was injected at the same time when APAP was given. For control, corn oil and saline were used in replace of BNF and APAP, respectively. The animals were then kept in warm environment under close observation.

**Table 1 Treatment of animals in different groups**

	APAP	BNF+APAP	DADS	NAC	DADS+NAC
BNF		200	200	200	200
DADS			200		200
APAP	350	350	350	350	350
NAC				500	500

Values were doses used (mg/kg body weight)

**Evaluation of APAP hepatotoxicity.** After APAP challenge, the survival time for each group was recorded, calculated and expressed as mean $\pm$ SD. APAP-induced hepatotoxicity in each group was also evaluated histologically and enzymatically. For histology, immediately after death of the animal or sacrifice of the mice surviving longer than 48 hours, the liver was removed, fixed in 10% neutral buffered formalin, washed with phosphate buffer saline (PBS), dehydrated in an increasing concentration of ethanol, and finally embedded in paraffin. Sections of 6  $\mu$ m in thickness were stained with hematoxylin and eosin and examined under light microscopy. The extent of liver necrosis was evaluated semi-quantitatively using a scale of 0-4 according to the scoring system introduced by Mitchell *et al*<sup>[15]</sup>. Score 0, indicates no evidence of necrosis; score 1, less than 6%; score 2, 6%-25%; score 3, 26%-50%; and score 4, more than 50% of the liver cells are necrotic.

Blood samples were collected at 2, 4 and 6 hours after APAP administration and plasma ALT levels were determined using Sigma GPT Kit (following the manufacturer's instruction). The values for each group were expressed as mean  $\pm$ SD. ALT less than 20 Sigma units is considered normal and a range of 20-35 units is a border line according to the manufacturer's instruction.

### *Effects of DADS and NAC on hepatic GSH levels*

Another set of groups of B6 mice was used in order to determine the effects of DADS and NAC on hepatic GSH levels. After the same treatment as shown in Table 1, the mice were sacrificed at 2, 4, 6, 8 and 24 hours after APAP administration. The liver was homogenized in 4 volumes of 0.1M PBS and the homogenate was mixed with an equal volume of 4% sulfosalicylic acid. After centrifugation, 0.5ml supernatant was added to 4.5ml 0.1mM bis-(3-carboxy-4-nitrophenyl) disulfide in 0.1M PBS (pH 8.0). The mixture was incubated at 25°C for 60 minutes in the dark. GSH concentration was determined at absorbance 412nm using a Shimadzu UV-Visible Recording Spectrophotometer UV-160.

### *Effect of DADS and NAC on elevated P450 1A1/1A2 activity induced by BNF*

To explore the mechanism of DADS protection against APAP hepatotoxicity, we further investigated the effect of this compound on hepatic P450 1A1/1A2 activity induced by BNF. Five groups of B6 mice were used and treated as control, BNF-APAP, DADS, NAC, and DADS+NAC groups as summarized in Table 1, except that APAP was omitted in all of these groups and NAC was given 47 hours after BNF. Forty-eight hours after BNF, the mice were killed and livers were immediately removed for determination of activity of hepatic microsomal 7-ethoxyresorufin (ER) O-dealkylase by the method

introduced by Burke *et al*<sup>[16]</sup>. Briefly, livers were homogenized in 0.1M Tris buffer containing 0.25M sucrose (pH 7.5). The supernatant fractions from the centrifuged liver homogenate ( $13\,500 \times g$  for 20min) were recentrifuged at  $105\,000 \times g$  for 60min. The pellets were washed and resuspended in 0.1M PBS and used for assay immediately. The reaction mixture contained 2ml PBS, 20 $\mu$ l microsomal suspension, and 10 $\mu$ l ethoxyresorufin (50 $\mu$ M). A baseline of fluorescence was recorded at an excitation wavelength of 510nm and an emission wavelength of 586nm using a Shimadzu fluorospectrometer RF-540. After addition of 200 $\mu$ l NADPH to the reaction mixture, the increase in fluorescence was recorded in air at room temperature. The specific activity of the enzyme was calculated by comparing with a standard curve.

Assessment of direct effects of DADS or NAC on induced P450 1A1/1A2 activity was conducted by incubating microsomal suspension prepared from BNF-treated mouse liver with different concentrations of DADS or NAC, respectively. Incubation was carried out at room temperature for 30min and followed by the measurement of 7-ethoxyresorufin dealkylase activity as described above.

### Statistical analysis

Statistical significance was analyzed using statistical software StatView 4.1 (Jandel Scientific Inc., USA). One-way ANOVA and Student *t* test were used for difference among multiple values or between two values, respectively.

## RESULTS

### Effects DADS and NAC on APAP hepatotoxicity in BNF-pretreated B6 mice

APAP group showed no sign of APAP toxicity. However, BNF+APAP mice suffered from severe toxicity and all died eventually. The difference was statistically significant between the APAP group and BNF+APAP group in terms of mortality and mean survival time ( $P < 0.001$ ). The use of either DADS or NAC alone prolonged the mean survival time ( $P < 0.01$ ) but did not affect the mortality rate when comparing DADS group and NAC group with BNF+APAP group. The use of DADS and NAC in combination completely protected the animals, and the mortality rate was reduced to 0 ( $P < 0.001$ ), (Table 2).

Histological evaluation of the liver necrosis revealed that mild damage (Score 1) was found in 10% of mice receiving APAP only, and 90% of this group appeared normal morphologically. However,

extremely severe hepatic necrosis was observed in BNF+APAP group, with Mitchell's score 4 for 9 mice and 3 for 1. The necrosis mainly occurred in the lobular center and in some cases, the normal lobular structures were destroyed. Although the use of either DADS or NAC (Group 4 and 5) could significantly prolong the mean survival time, Mitchell's scores of these mice were still recorded as high as 3 for 9 mice of DADS group and 8 mice of NAC group, respectively. Seven animals of DADS+NAC group were recorded histologically as score 1 (mild damage), and three as score 2. There was a statistical significance between DADS+NAC group and BNF+APAP group ( $P < 0.01$ ).

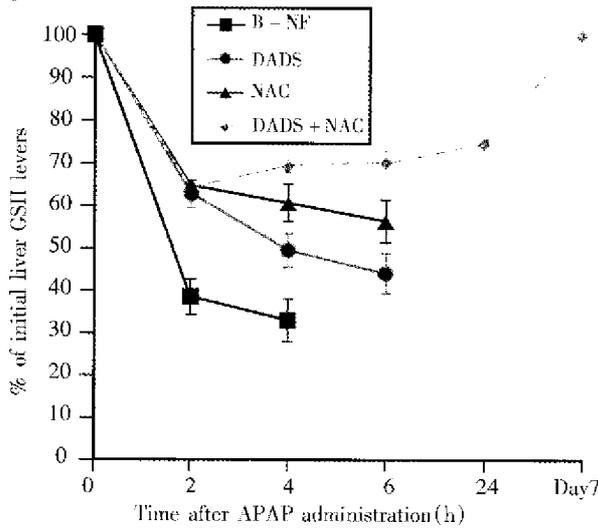
As shown in Figure 1, hepatic GSH in BNF+APAP group sharply declined by 60% of its initial concentration in first two hours after APAP and continued to drop until all mice died at 4 hours. In DADS, NAC and DADS+NAC groups, GSH levels also dropped by about 35% in first two hours, though significantly higher than that of BNF+APAP group ( $P < 0.05$ ). At 4 and 6 hours, GSH in DADS+NAC group maintained at a significantly higher level ( $P < 0.01$ , as compared with DADS or NAC group) and all mice survived whereas liver GSH in either DADS or NAC group continued to decline and all died about 7 hours after APAP. Plasma ALT levels of these groups are illustrated in Figure 2. ALT in BNF + APAP mice increased almost linearly, and DADS or NAC group showed moderate elevation while ALT in DADS+NAC group went up only mildly. Statistical significance ( $P < 0.05$ ) was found between BNF+APAP group and either DADS or NAC group and DADS + NAC group, but not between DADS and NAC groups ( $P > 0.05$ ).

### Effects of DADS on hepatic P450 1A1/1A2 activity induced by BNF

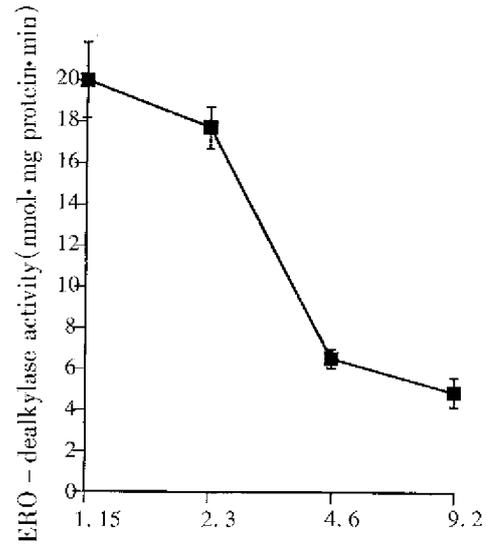
In vivo study on effects of DADS, NAC or DADS and NAC used in combination on hepatic P450 1A1/1A2 are shown in Figure 3. BNF can significantly induce P450 1A1/1A2, as determined by ERD activity, and compared with the constitutive activity in the control mice ( $P < 0.01$ ). There was no significant difference in ERD activity between BNF, DADS, NAC, and DADS + NAC groups ( $P > 0.05$ ). However, in vitro study by direct incubation of microsomal suspension prepared from BNF-treated mouse liver demonstrated that DADS can significantly inhibit P450 1A1/1A2 activities induced by BNF, IC<sub>50</sub> being approximately 4.6 $\mu$ M of DADS, whereas NAC showed no direct inhibitory effect on the enzyme activity (Figure 4 a and b).

**Table 2** Effects of DADS and NAC on mortality and mean survival of mice treated with APAP

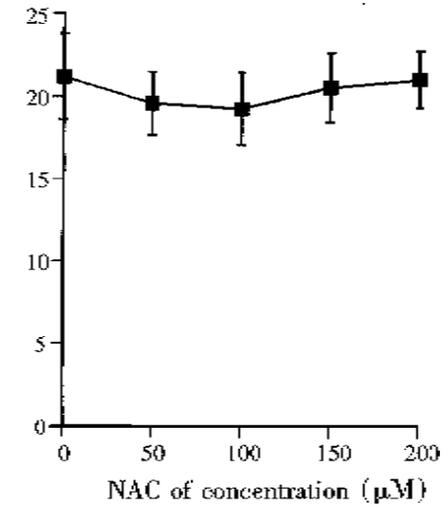
	Control	APAP >48	BNF+APAP 3.17 $\pm$ 0.18	DADS 6.29 $\pm$ 0.49	NAC 7.0 $\pm$ 0.40	DADS+NAC >48
Mean survival time(h)						
Mortality (%)	0	0	100	100	100	0



**Figure 1** Effect of DADS and NAC on liver GSH level.



**Figure 2** DADS and NAC on plasma ALT levels.



**Figure 3** Effects of DADS and NAC on BNF-induced CYP-1A1/1A2 activity (O-ER activity).

1. No BNF (constitutive activity)      2. BNF (induced activity)  
 3. BNF+DADS                                4. BNF+NAC  
 5. BNF+DADS+NAC  
 Enzyme activity assayed in absence (A) and presence (B) of anti-CYP 1A1/1A2 antibodies.

**DISCUSSION**

Studies on APAP hepatotoxicity remain a very active area since much is still uncertain despite continuous efforts worldwide. We have previously demonstrated the inducibility of P450 1A1/1A2 by BNF in B6 mouse hepatocytes *in vivo*<sup>[14]</sup>. The current study showed that BNF-pretreated mice developed severe liver necrosis and all mice died in a few hours after challenged with APAP. However, when APAP of the same dosage was applied to mice without BNF pretreatment, the animals tolerated it well. These findings indicate that P450 induction by BNF in hepatocytes markedly enhanced APAP hepatotoxicity and, therefore, is a critical step in the development of liver necrosis. P450 1A1/1A2 is probably the major isoform involved in this case, which is in agreement with the study by Snawder, *et al*<sup>[12]</sup>.

In treatment of APAP poisoning, compounds which inhibit P450 activity and drugs which increase

hepatic GSH pooling (i.e. enhancing liver detoxifying capability) are expected to protect animals against APAP cytotoxicity since NAPQI is converted from APAP by P450 enzymes and normally and mainly detoxified by conjugation with GSH. The reaction increases the solubility of NAPQI and facilitates its elimination through the kidneys. DADS is attractive because of its nature as an extract derived from garlic<sup>[13]</sup>. Recently, Hu<sup>[13]</sup> reported that DAS (another compound derived from garlic) given intra-gastrically at a dose of 200mg/kg body weight reduced the mortality from 40% to 0% in rats challenged with APAP at dose of 750mg/kg body weight. The protective action of DAS was thought to be related to its inhibitory effects on the hepatic P450 activity, especially for P450 2E1<sup>[16,17]</sup>. In the current study, we observed that DADS given intraperitoneally prolonged the survival time of B6 mice in which P450 activity had been induced by BNF. However, this regimen did not improve the histological evaluation and the total mortality rate, although hepatic GSH content and plasma ALT levels showed signs of improvement. NAC has been clinically used as the mainstay of APAP poisoning. Nevertheless, NAC used individually in this study did not provide satisfactory protection. The combination regimen we tested produced a full protection evidenced by a significant reduction in severity of liver necrosis and the mortality rate reduced to zero. Significant improvement in plasma ALT and liver GSH was also observed. Therefore, this regimen may have a clinical potential and may be the choice of treatment for APAP intoxication in subjects with elevated P450 enzyme activity.

The mechanism of protection by DADS against APAP hepatotoxicity is more likely to be associated with its inhibitory action on hepatic P450 1A1/1A2 activity since *in vitro* enzyme assay demonstrated that DADS is a potent P450 1A1/1A2 inhibitor, although it does not affect induction of this enzyme by BNF<sup>[18]</sup>. DADS was markedly suppressed, but was unable to completely eliminate the elevated hepatic P450 activity, and the non-suppressed portion of P450 enzyme can still produce an amount of NAPQI sufficient to cause liver necrosis and animal death. On the other hand, NAC does not directly detoxify NAPQI but serves as a precursor of GSH and stimulates hepatic GSH pooling. Hence, effectiveness of measures in enhancing liver detoxifying capability depends upon how potent these prodrugs are and how quickly they can be converted into forms that can be used to detoxify NAPQI. By combination regimen, DADS reduces generation of NAPQI by inhibiting the key enzyme and the NAPQI generated by non-suppressed P450 can be substantially trapped and conjugated by increased GSH due to administra-

tion of NAC. Therefore, the combination regimen acts on two steps in the metabolic pathway of APAP to provide the full protection.

Interestingly, the hepatic GSH depletion and plasma ALT elevation in either DADS or NAC group were much less severe than those of BNF group but the death of animals was not avoided. It is likely that adequate maintenance and/or rapid restoration of liver GSH by combination regimen contributes, at least in part, to the full protection. Moreover, plasma ALT level, a commonly used liver damage marker, may not be parallel with the extent of hepatic necrosis and may not truly reflect the severity of intoxication if DADS or NAC had been given. Therefore, plasma ALT level may not be used as a solely reliable parameter to assess the severity of liver damage and to predict the prognosis.

**ACKNOWLEDGMENT** We would like to thank Dr. M. Kurpakus for the use of the laboratory facilities and to Dr. D. Shi for his assistance in statistics.

## REFERENCES

- 1 Miner DJ, Kissinger PT. Evidence for the involvement of N-acetyl-p-quinoneimine in acetaminophen metabolism. *Biochem Pharmacol*, 1979;28:3285-3290
- 2 Hurman AW, Kyle ME, Serroni A, Farber JL. The killing of cultured hepatocytes by N acetyl-p-benzoquinoneimine (NAPQI) as a model of the cytotoxicity of acetaminophen. *Biochem Pharmacol*, 1991;41(8):1111-1117
- 3 Miller MR, Wentz E, Blair JB, Pack D, Hinton DE. Acetaminophen toxicity in cultured trout liver cells. *Experiment Molecul Pathol*, 1993;58:114-126
- 4 Roberts WD, Bucci TJ, Benson RW, Warbritton AR, McRae TA, Pumford NR et al. Immunohistochemical localization and quantification of the 3-(cystein-S-yl) acetaminophen protein adduct in acetaminophen hepatotoxicity. *Am J Pathol*, 1991;138(2):359-371
- 5 Birge RB, Bulera SJ, Bartolone JB, Ginsberg GL, Cohen SD, Khairallah EA. The arylation of microsomal membrane proteins by acetaminophen is associated with the release of a 44 kDa acetaminophen-binding mouse liver protein complex into the cytosol. *Toxic Applied Pharmacol*, 1991;109:443-454
- 6 Holtzman JL. The role of covalent binding to microsomal proteins in the hepatotoxicity of acetaminophen. *Drug Met Rev*, 1995;25:395-451
- 7 Esterline RL, Ray SD, Ji S. Reversible and irreversible inhibition of hepatic mitochondrial respiration by acetaminophen and its toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). *Biochem Pharmacol*, 1989;38:2387-2390
- 8 Donnelly PJ, Walker RM, Racz WJ. Inhibition of mitochondrial respiration *in vivo* is an early event in acetaminophen-induced hepatotoxicity. *Arch Toxicol*, 1994;68:110-118
- 9 Harvison PJ, Guengerich FP, Rashed MS, Nelson SD. Cytochrome P450 isozyme selectivity in the oxidation of acetaminophen. *Chem Res Toxicol*, 1988;1:47-52
- 10 Lee CA, Thummel KE, Kalthorn TF, Nelson SD, Slattery JT. Inhibition and activation of acetaminophen reactive metabolite formation by caffeine: roles of cytochrome P450 1A1 and 3A2. *Drug Metab Disp*, 1991;19:348-353
- 11 Pezzano M, Richard CH, Lampi E, Pelletier G, Fabre M, Rimaillho A et al. Hepatic and renal toxicity of paracetamol in chronic alcoholic subjects. *La Presse Medicale*, 1988;17:21-24
- 12 Snawder JE, Roe AL, Benson RW, Roberts DW. Loss of CYP2E1 and CYP 1A2 activity as a function of acetaminophen dose: relation to toxicity. *Biochem Biophys Res Commun*, 1994;203(1):532-539
- 13 Hu JJ, Yoo JS, Lin M, Wang EJ, Yang CS. Protective effects of diallyl sulfide on acetaminophen induced toxicity. *Food Chemical Toxicol*, 1996;34:963-969
- 14 Zhao C, Zhou YX. Induction of cytochrome P450 1A1/1A2 in mouse hepatocytes by  $\beta$ -naphthoflavone: an immunocytochemical study. *Chin J New Gastroenterol*, 1996;4(8):334-335
- 15 Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brode BB. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther*, 1973;187:185-194
- 16 Burke MD, Mayer RT. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabol Disposition*, 1974;2(6):583-588
- 17 Patten CJ, Thomas PE, Guy PL, Lee ML, Gonzalez FJ, Guengerich FP et al. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. *Chem Res Toxicol*, 1993;6:511-518
- 18 Brady JF, Li D, Ishizaki H, Yang CS. Effect of diallyl sulfide on rat liver microsomal nitrosamine metabolism and other monooxygenase activities. *Cancer Res*, 1988;48:5937-5940

# <sup>99m</sup>Tc labeled HAb18 McAb Fab fragment for radioimmunoimaging in nude mice bearing human hepatocellular carcinoma \*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; HAb18; antibodies, monoclonal; radioimmunodetection; Fab fragments; <sup>99m</sup>Tc; nude mice

## Abstract

**AIM** To establish a method of labeling anti-hepatoma McAb (HAb18) Fab fragment modifier with <sup>99m</sup>Tc.

**METHODS** HAb18 Fab was modified with 2-iminotholane and labeled with <sup>99m</sup>Tc by transchelation from <sup>99m</sup>Tc GH. Labeling yield, radiochemical purity and immunoreactivity were determined by thin layer chromatography (TLC-SG), paper chromatography (PC), gel chromatography (GC) and cell binding assay, respectively. The nude mice bearing human hepatoma were used for radioimmunoimaging (RII). **RESULTS** A radiolabeling yield of 50%-80% was obtained, and immunoreactivity (IR) was 30%-40%. Radioimaging results showed that <sup>99m</sup>Tc-HAb18 McAb Fab fragment was concentrated in the tumor 4-8 hours after injection, and the maximum concentration was seen in 12-24 hours, and the T/NT value was 5.18 and 7.48 at 6h and 8h after the injection.

**CONCLUSION** <sup>99m</sup>Tc-HAb18 McAb Fab fragment could be specifically localized in the tumor of nude mice bearing human hepatocellular carcinoma within 24 hours and this method might be effectively used for labeling McAb Fab fragment with <sup>99m</sup>Tc.

## INTRODUCTION

Many methods have been reported to radiolabel intact MAbs with <sup>99m</sup>Tc<sup>[1]</sup>. Since the long imaging time of intact McAb HAb18 (> 48 hours) is not compatible with the 6h physical  $T^{1/2}$  of <sup>99m</sup>Tc, it seems preferable to use antibody fragments for <sup>99m</sup>Tc labeling, as the fragments have smaller molecular mass, can be cleared more rapidly from the blood, and potentially increase the tumor/non-tumor ratio (T/NT). The method of labeling fragment with <sup>99m</sup>Tc is therefore one exploring aspect. In this paper, 2-iminotholane (IT) was used to modify anti-hepatoma McAb HAb18 Fab fragment, and then IT-Fab fragment modifier was labeled with <sup>99m</sup>Tc by transchelation from <sup>99m</sup>Tc-glucoheptonate (GH). Successful results were obtained when <sup>99m</sup>Tc-IT-Fab was injected into the nude mice bearing human hepatoma for radioimmunoimaging (RII).

## MATERIALS AND METHODS

### Reagents

The anti-hepatoma McAb HAb18 (10g/L, purity >95%) was prepared and supplied by the Hepatoma Targetting Drug Research Laboratory, Department of Pathology, the Fourth Military Medical University. McAb HAb18 belongs to IgG<sub>1</sub> subclass and its affinity coefficient (ka) is  $8.17 \times 10.9$  L/mol. The antigen is about 61kd and has no crossreaction with AFP, CEA, and ferritin. IT is a product of Sigma Chemical Co.; 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), Fluka Co; papain, Merck Co.; and GH and MDP kit was supplied by the Institute of Jiangsu Nuclear Medicine. TEA-B-1 solution: a mixture of 1.0mol/L TEA and TEA HCl solution (1:1 v/v), pH 8.0; TEA-B-2 solution: a mixture of KCl (50mmol/L) and MgCl<sub>2</sub> (1mmol/L) with the TEA-B-1 solution; TEA-B-SH solution: 1% 2-mercaptoethanol of TEA-B-2 solution; IT solution: IT (0.25mol/L) dissolved in TEA-B-1 solution.

### Human hepatoma nude mice model

Human hepatoma was subcutaneously implanted in the neck or the upper lumbar side of NC or BALB/c nude mice. The model was named SMMC-LTNM<sup>[2]</sup>. The mice were maintained in an aseptic condition and used for experiment when the tumor increased to 0.5 cm-1.4 cm in diameter.

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\* Supported by both "863" National Biological High-Tech Project Foundation and the State Key Laboratory Foundation of Nuclear Medicine, 863-02-12(1) and SKLFNM 9402.

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Received 1997-11-10 Revised 1998-01-03

### Instrument

Packard A5301D  $\gamma$ -counter produced by Packard Instrument Co.; Diacam/Maxdelta 3000 SPECT system by Siemens Co.; fast protein liquid chromatography system (FPLC): Waters 650 with DEAE-sepharose fast flow column; and HPLC: Bio-Rad 5000T.

### HAb18 Fab fragment

Prepared by papain digestion and purified by FPLC using DEAE-sepharose fast flow column<sup>[3]</sup>.

### Fab fragment modification

Jue's method<sup>[4]</sup> was used with some modification. Briefly, HAb18 Fab fragment (4g/L) was dialyzed overnight against TEA-B-SH solution, then mixed with IT solution (6mmol/L in concentration) at 0°C reaction for 20min. The mixture was dialyzed against the TEA-B-2 buffer at room temperature (20°C) for 3h with a buffer change each hour. After that, the mixture was run through sephadex G50 column which was absorbed with bovine serum albumin (BSA), and eluted with 0.05M PBS at a speed of 1 ml/min. The protein fraction was collected under UV-280nm monitoring, concentrated to 4.4g/L, divided into 220 $\mu$ g/vial, and lyophilized for us.

### Determination of sulfhydryl groups with DTNB

According to Ellman's method<sup>[5]</sup>, protein samples (0.5ml) were mixed with 2.5ml 0.05mol/L Tris-HCl buffer (pH 8.4), and 0.025ml 0.01mol/L DTNB solution. Half a milliliter of distilled water instead of protein sample solution was used as blank control. The increase in absorbance at 412nm was followed using a spectrophotometer (Lambda 2S). By means of the formula sulfhydryl group ( $\mu$ mol) =  $A_{412}/0.5 \times 13.6$ , the number of sulfhydryl group was calculated according to protein concentration ( $\mu$ mol).

### <sup>99m</sup>Tc labeling of IT-Fab fragment

The labeling procedure was as follows: ① 370-555MBq (1ml-1.5ml) of freshly eluted pertechnetate from a <sup>99</sup>Mo/<sup>99m</sup>Tc generator was added into an GH kit (containing 10mg sodium glucoheptonate and 1mg stannous chloride), and was placed 10min, <sup>99m</sup>Tc-GH was prepared. ② Lyophilized IT-Fab (220  $\mu$ g/vial) was dissolved in 100 $\mu$ l 0.1mol/L citrate buffer, pH 6.5, bubbled with N<sub>2</sub>. ③ 0.1ml-0.5ml <sup>99m</sup>Tc-GH solution was added to the IT-Fab vial and incubated at 37°C for over 1 hour.

### Quality control of <sup>99m</sup>Tc-IT-Fab

Radiolabeling yield and radiochemical purity were determined by paper chromatography (PC) using

No.1 Xinhua filter paper strips (saline as a solvent, <sup>99m</sup>Tc-IT-Fab and <sup>99m</sup>TcO<sub>2</sub> Rf=0-0.1, <sup>99m</sup>TcO<sub>4</sub> Rf=0.7-0.8. Acetone as a solvent, <sup>99m</sup>Tc-GH Rf=0, <sup>99m</sup>TcO<sub>4</sub> Rf=1); or instant thin layer chromatography (ITLC) using ITLC-SG paper (Gelman Co), (1% BSA pretreated, alcohol: NH<sub>3</sub>H<sub>2</sub>O:H<sub>2</sub>O = 2:1:5 as a solvent, <sup>99m</sup>Tc-IT-Fab and <sup>99m</sup>TcO<sub>4</sub> Rf = 1.0 <sup>99m</sup>TcO<sub>2</sub> Rf = 0) and gel column chromatography (GCC) with sephadex G50 (0.9 cm  $\times$  15 cm diameter), 0.05 mol/L PBS, pH 7.4, as an eluent. The protein labeling yield was calculated by routine method. Immunoreactivity assessment: in vitro immunoreactivity of <sup>99m</sup>Tc-IT-Fab was evaluated by a conventional live cell assay. Briefly, hepatoma cell suspension was prepared by mincing SMMC-LTNM tumor tissues from the mice model. It was centrifuged and washed with phosphate-buffered saline (0.1 mol/L- PBS); then 10 $\mu$ l diluted <sup>99m</sup>Tc-IT-Fab (10  $\times$  10<sup>3</sup> cpm) and 0.5ml PBS was added, and the mixture was incubated at 37°C for 2 hours. After incubation, the samples were then centrifuged and the supernatants were removed. The cells were further washed with 1ml PBS solution by centrifugation. Before and after the removal of supernatant, the activity of samples was counted in a  $\gamma$ -counter, and the percentage of binding was calculated.

### Animal biodistribution study

<sup>99m</sup>Tc-IT-Fab 18.5 MBq/0.2ml was intravenously injected into each nude mouse bearing human hepatoma xenografted model by the tail vein, and the mice were sacrificed 5 hours after the injection. The blood and major organs were removed, weighed and counted in the  $\gamma$ -counter for radioactivity, the tumor/blood ratio and the tumor/organ ratios were calculated.

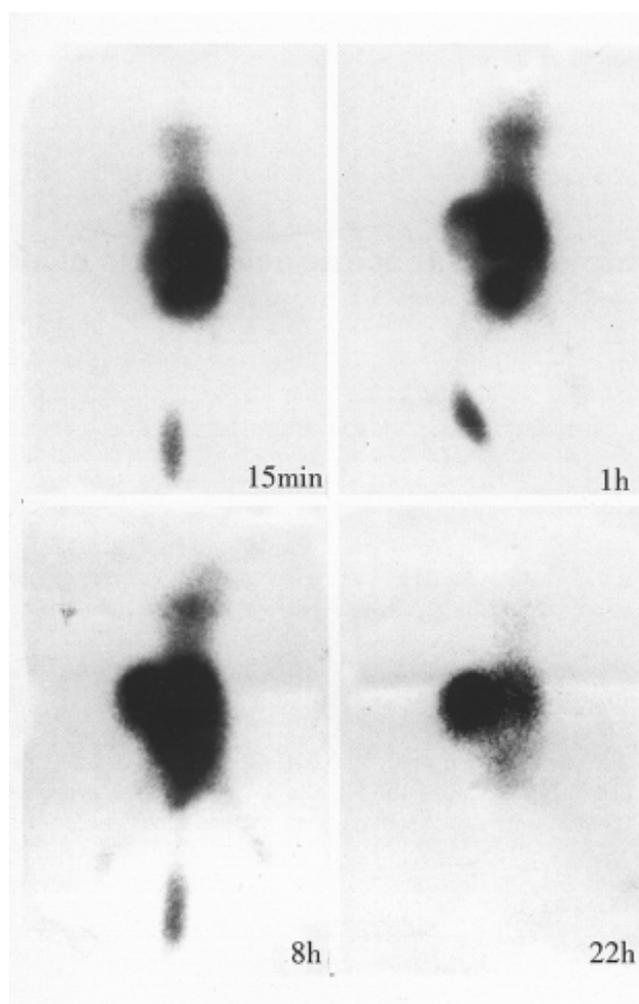
### Animal imaging study

<sup>99m</sup>Tc-IT-Fab 18.5-74 MBq/0.2ml was injected intravenously or intraperitoneally into the tumor-bearing nude mice ( $n=6$  each in half), and the same dose of <sup>99m</sup>Tc-GH was intravenously injected into the nude mice ( $n=2$ ) as control. The mice were imaged with a SPECT at 30min, 2 h, 4 h, 6 h, 12 and 22 h after administration. The T/NT ratios were calculated by ROI technique.

## RESULTS

Fab fragment yield was 30%-40%, and single peak was found by HPLC. The molecular mass weight was identified as 45kd by SDS-PAGE. The number of sulfhydryl group per molecule of IT-Fab and Fab fragment was 0.3 and 0, respectively. <sup>99m</sup>TcO<sub>2</sub> was less than 0.5%. The immunoreactivity was 30%-40% by cell binding assay. The biodistribution study showed that the T/NT ratios of tumor to the blood,

the heart, liver, lung, spleen, kidney, stomach, intestine, muscle, and brain were 0.89, 4.1, 1.1, 8.0, 5.2, 0.1, 1.6, 4.8, 4.8 and 33.1, respectively. Animal imaging showed that  $^{99m}\text{Tc}$ -IT-Fab was rapidly distributed to the whole body when injected i.v. and the radioactivity rapidly appeared in the bladder. The body distribution via intraperitoneal was 10min-30min later than that of the i.v. injection. The tumor was visible with high background at 1h-2h; the accumulation of the tumor was evident with clear kidney imaging 4h-8h after the injection, and a relatively increased tumor localization was seen with low background and decreased kidney outline 12h-22h after injection (Figure 1). The T/NT (on the back muscles) ratios by ROI were 5.18 and 7.48 at 4h and 8h. In control group,  $^{99m}\text{Tc}$ -GH rapidly appeared in the blood pool and the whole body, and accumulated rapidly in the bladder. The two kidney imagings were clear, but no tumor accumulation was seen 6h after the injection with two dim kidney outlines and low background. Radioactivity was removed in urine and the bladder image disappeared gradually.



**Figure 1** Dynamic imaging of  $^{99m}\text{Tc}$ -IT-Fab in nude mice bearing human hepatoma model (posterioranterior position).

## DISCUSSION

$^{99m}\text{Tc}$  labeling McAb  $\text{F(ab}')_2$  and Fab' fragments have been reported by some authors<sup>[1]</sup>, but Fab fragment was rarely seen. In terms of the clearance, intact IgG was the slowest,  $\text{F(ab}')_2$  fragment in the middle, and Fab' or Fab fragment the fastest. As for preparation, Fab' fragment was prepared from the reduction of  $\text{F(ab}')_2$  fragment and then was followed by purifying procedure. The key step was the difficult control of reduction condition.  $\text{F(ab}')_2$  fragment was prepared and purified from pepsin or papain digestion. Fab fragment was also prepared by papain digestion and then purification, but because of the small number of sulfhydryl group per molecule, Fab fragment was difficult to label with  $^{99m}\text{Tc}$ . We used 2-iminothiolane (a chemical compound, MW: 137) to modify Fab fragment, so the number of sulfhydryl group per molecule of IT-Fab was increased from 0 to 0.3. Park *et al*<sup>[6]</sup> reported that the number of sulfhydryl group per molecular of  $\text{F(ab}')_2$  was 4.2 and Fab 0.9. The difference might have resulted from the treatment with  $\text{SnCl}_2$  before the measurement. Rhodes<sup>[7]</sup> reviewed that the protein to be labeled need to contain free sulfhydryl (-SH) group or reactive monosulfides (-S or -S-metal), and the labeling yield was significantly and positively related to the number of sulfhydryl group. We have modified  $\text{F(ab}')_2$  with 2-iminothione in the same way (with the number of SH per molecule of 0.7) and labeled with  $^{99m}\text{Tc}$ . The labeling yield was 92% by PC, which was higher than that of  $^{99m}\text{Tc}$ -IT-Fab (50%-80%). This result proved what was mentioned above. Paik<sup>[6]</sup> proposed that there were two sites on the IgG molecule, one with a high-affinity and low-capacity and the other with a low-affinity and high-capacity. In our study, 50%-80% of labeling yield was obtained by PC, and 20%-30% by GCC, which suggested that the same phenomenon existed on the McAb fragment. Because of the low-affinity binding,  $^{99m}\text{Tc}$  was cleared on the column by Sephadex  $\text{G}_{50}$ . GH, as a renal function imaging agent, was rapidly excreted from the kidney, and was reported to have some tumor-affinity characteristics<sup>[8]</sup>. But the present results demonstrated that  $^{99m}\text{Tc}$ -GH had no affinity to hepatoma. This proved the specificity of  $^{99m}\text{Tc}$ -IT-Fab localized on the tumor. The best tumor imaging was at 12 h-22 h after the administration of  $^{99m}\text{Tc}$ -IT-Fab. The background excretion was mainly by kidney route. This was similar to the reported imaging time and excretion route of  $^{99m}\text{Tc}$ -Fab<sup>[9]</sup>. The biodistribution was mainly in the kidney (32.6% ID/g) and next in the blood, the tumor, liver, and stomach. Our results demonstrated that  $^{99m}\text{Tc}$ -IT-Fab could bespecifically localized in the tumor of nude

mice bearing human hepatocellular carcinoma within 24 hours, and this method could be effectively used for labeling McAb Fab fragment with  $^{99m}\text{Tc}$ .

## REFERENCES

- 1 Goldenberg DM, Larson SM. Radioimmunodetection in cancer identification. *J Nucl Med*, 1992;33(5):803-810
- 2 Tao WZ, Kang JS, He DH. Establishment of nude mice bearing human hepatoma model and its pathological characteristics. *J Sec Mili Med Univer* 1989;10(1): 5-8
- 3 Qiu K, Chen ZN, Liu ZG, Wang Q, He FC, Sui YF et al. Preparation of anti-human hepatoma McAb Hab18 F(ab)<sub>2</sub> and Fab fragment by papain digestion. *J Four Mili Med Univ*, 1995;16(6):414-418
- 4 Jue R, Lambert JM, Piece LR, Traut RR. Addition of sulfhydryl groups to escherichia coli ribosomes by protein modification with 2-iminothiolane. *Biochemistry*, 1978;17(25):5399-5405
- 5 Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*, 1959;82(1): 70-77
- 6 Paik CH, Phan LNB, Hong JJ, Malekeh SS, Susan CH, Richard CR et al. The labeling of high affinity sites of antibodies with  $^{99m}\text{Tc}$ . *Int J Nucl Med Biol*, 1985;12(1):3-8
- 7 Rhodes BA. Direct labeling of proteins with  $^{99m}\text{Tc}$ . *Nucl Med Biol*, 1991;18 (7):667-675
- 8 Kuang QF, Guo YZ, Lou B. The preparation of Tc-99m-Glucoheptonate sodium and the results of its animal experiments. *Chin J Nucl Med*, 1986;6(2):73-75
- 9 Massuger LAG, Claessens RAMJ, Pak KY, Boerman OC, Daddonam PE, Keonders EB et al. Tissue distribution of  $^{99m}\text{Tc}$ ,  $^{111}\text{In}$  and  $^{123}\text{I}$ -OVTL Fab' fragment in ovarian carcinoma bearing nude mice. *Nucl Med Biol*, 1991;18(1):77-82

# Serum deprivation enhances DNA synthesis of human hepatoma SMMC-7721 cells \*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; DNA, neoplasm/biosynthesis; SMMC-7721; tumor cell, cultured; cell proliferation; growth factors

## Abstract

**AIM** To determine the relationship between serum deprivation or serum levels and cell proliferation of human hepatoma SMMC-7721 cells.

**METHODS** Human hepatoma SMMC-7721 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) in 5% CO<sub>2</sub> incubator at 37°C for 24h, and culture media were replaced to serum-free or different serum FCS levels (2.5%, 5%, 10%, 20% and 25%). Six h, 12h, 18h and 24h after the culture, the cells were incorporated [<sup>3</sup>H]-TdR for 4h. At last [<sup>3</sup>H]-TdR incorporation was detected with liquid scintillation counting.

**RESULTS** DNA synthesis of SMMC-7721 cells could be sharply stimulated by short-time (6h) serum deprivation (the cpm value of <sup>3</sup>H-TdR incorporation of cells in serum-free was 39.32-fold higher than cells in 25% serum), and the incorporation of <sup>3</sup>H-TdR was negatively related to the serum levels. Longer-time serum starvation (12h, 18h and 24h) also greatly stimulated DNA synthesis, although the cpm value of <sup>3</sup>H-TdR incorporation was less than that in 6h serum deprivation. Morphology of cells cultured in different serum levels also showed significant difference.

**CONCLUSIONS** Compared with other cell lines such as BEL7404 and Swiss 3T3, human hepatoma SMMC-7721 cells had different response to the serum deprivation. Short-time serum deprivation could greatly stimulate DNA synthesis of human hepatoma SMMC-7721 cells. Precautions must be given to the changes of serum levels for the detection of growth factors and drugs using SMMC-7721 cells as a model.

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Presented at the First International Symposium on Bioanalytical Chemistry, Xi'an, China, July 26-30, 1995.

\*Supported by a grant from Science and Technology Committee of Shandong Province, \*©No. J97K01.

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Received 1997-10-28

## INTRODUCTION

Mammalian cells in culture required serum in order to proliferation<sup>[1]</sup>. Serum was a mixture of protein and other substances, among which essential hormones and growth factors can support cell proliferation<sup>[2]</sup>. In order to reduce the effects of growth factors in serum on cells, the procedure of serum deprivation was often involved to study growth regulation on cultured mammalian cells<sup>[3-5]</sup>. Prolonged serum deprivation induced fibroblastic cells such as Swiss 3T3 to enter a quiescent state (G<sub>0</sub>)<sup>[6,7]</sup>. On the contrary, mouse embryo cells<sup>[8]</sup> and HeLa S-3 cells<sup>[9]</sup> showed reversal response to serum deprivation and lower serum levels. Human hepatoma SMMC-7721 cells were established in our country<sup>[10]</sup> and were widely used for detecting the activity of growth factors and drugs. Therefore, it was very important to clarify the effects of serum deprivation and serum levels on the growth and metabolism of SMMC-7721 cells.

## MATERIALS AND METHODS

### Cell lines and culturing

Human hepatoma SMMC-7721 cells were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in our laboratory. The cells were grown as monolayers in RPMI 1640 medium supplemented with 10%-20% fetal calf serum (FCS) and incubated at 37°C in the humidified incubator with 5% CO<sub>2</sub> 95% air.

### Serum deprivation and treatment of serum concentration

Exponent growing cells in flasks were harvested by trypsinization with 0.25% trypsin and suspended in RPMI 1640 medium plus 10% FCS. Cells were plated at 1×10<sup>5</sup> cells/ml in 4 pieces of 24-well plates and incubated at 37°C in 5% CO<sub>2</sub> 95% air for 24 hours. After that, the medium was aspirated and the cells were washed with RPMI 1640 medium. The medium was replaced with RPMI 1640 plus different levels of serum (0%, 2.5%, 5%, 10%, 20% and 25%) in different treatment groups (each group having 4 wells of cells) respectively. The rates of DNA synthesis of cells cultured in different serum levels were detected at interval of 6 hours (6, 12, 18 and 24 hours).

**Measurement of DNA synthesis**

When cells were cultured in different serum levels for 6, 12, 18 and 24 hours respectively, rates of DNA synthesis were determined by pulse labeling for 4 hours in 74KBq ml<sup>-1</sup> <sup>3</sup>H-TdR. The media were aspirated and the cells were gently rinsed with phosphate buffer saline (PBS), trypsinized properly with 0.25% trypsin, collected on 49 model filter membrane and rinsed with 5% TCA (trichloroacetic acid) and 100% ethanol (three times). The membranes were dried at 80°C for 30min. The incorporation of <sup>3</sup>H-TdR was determined by liquid scintillation counting.

**Morphological observation of cells cultured in different serum levels**

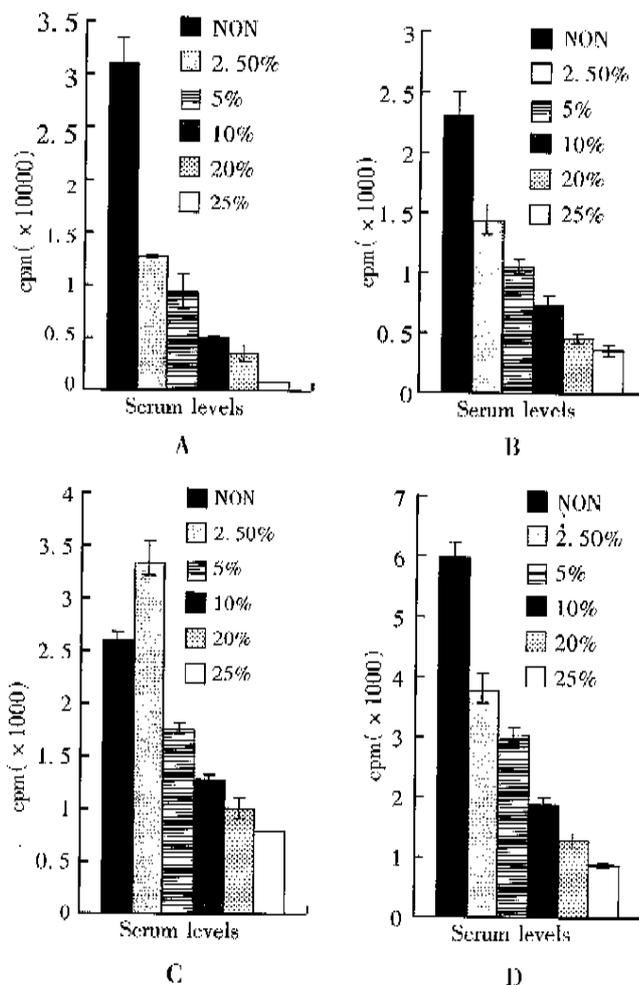
Before collected for liquid scintillation counting, the cells in different treatment groups were observed and photographed under inverted phase contrast microscope.

**Materials** RPMI 1640 medium was from Gibco, USA; trypsin was from Sigma; <sup>3</sup>H-thymidine (<sup>3</sup>H-TdR) was from the Institute of Atomic Energy of China; 24-well plates were from NUNC; and Triton X100, POPOP, PPO were from Serva.

**RESULTS**

**Effects of serum deprivation and concentration on <sup>3</sup>H-TdR incorporation**

Serum contained significant amounts of thymidine, which would affect the incorporation of their appropriate exogenous labeled forms. This was because the formulated media such as RPMI 1640 for cell culture did not usually contain unlabeled nucleotides, therefore serum provided the only source other than the labeled form. SMMC-7721 cells were pulse labeled with <sup>3</sup>H-TdR for 4 hours at 74KBq ml<sup>-1</sup> after serum deprivation and treatment of different serum concentrations for different times. The results showed decreased specific activity of the incorporation of <sup>3</sup>H-TdR with increased serum concentrations, that was, the incorporation of <sup>3</sup>H-TdR had negative relation with serum levels in medium. Cells cultured in serum-free medium for 6 hours after media replacement incorporated <sup>3</sup>H-TdR 39.32-fold higher than cells in 25% serum medium (Figure 1A). On the other hand, within 18 hours as the time of serum deprivation going down, the incorporation of <sup>3</sup>H-TdR decreased (Figure 1B, C, D). The ratio of <sup>3</sup>H-TdR incorporation between cells in serum free and in 25% serum decreased to 3.53-fold (Table 1).



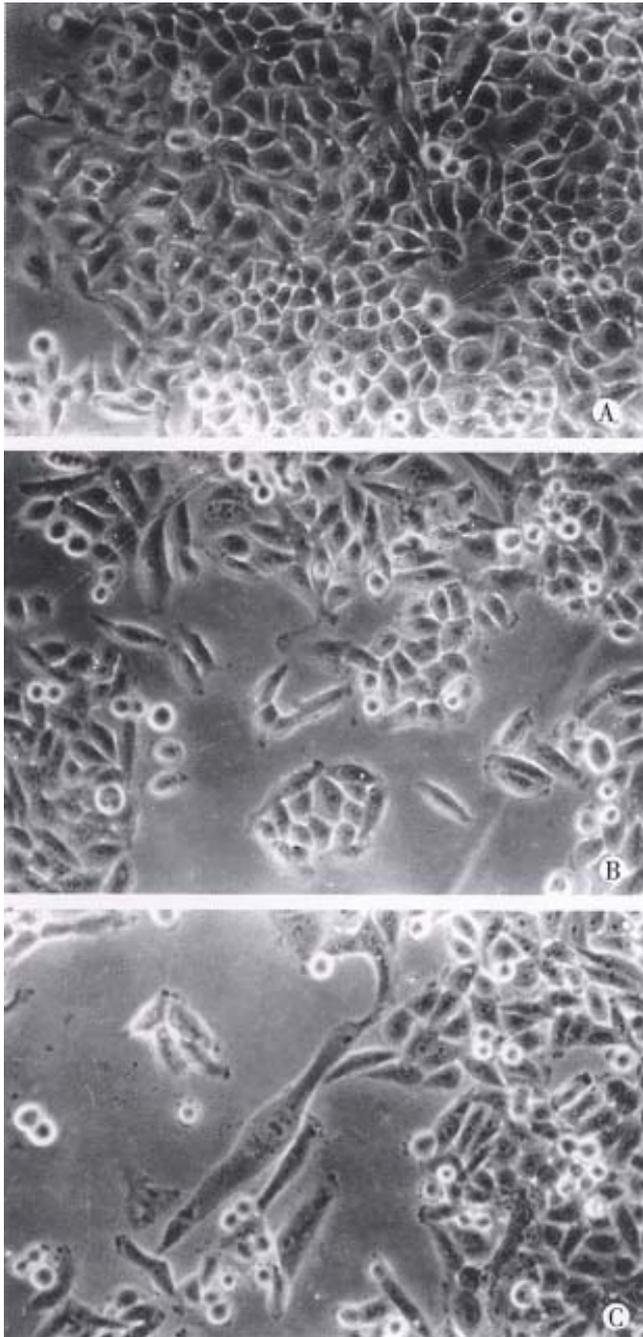
**Figure 1** Effects of serum deprivation and serum levels on the incorporation of <sup>3</sup>H-TdR. (A) The cpm value after cells were treated in different serum concentrations for 6 hours; (B) for 12 hours; (C) for 18 hours; (D) for 24 hours.

**Table 1** Ratios of the <sup>3</sup>H-TdR incorporation among cells cultured in serum-free and different serum levels

Treatments	6 h	12 h	18 h	24 h
SF/2.5% FCS	2.47	1.63	0.778	1.67
SF/5% FCS	3.53	2.26	1.51	1.98
SF/10%FCS	7.34	3.35	2.19	3.27
SF/20% FCS	11.61	5.30	2.72	4.88
SF/25% FCS	39.32	7.21	3.53	7.35

**Effects of serum deprivation and serum concentration on cellular morphology**

Cells cultured in different serum levels showed significant difference in morphology under inverted phase contrast microscope. Cells cultured in serum-free or lower serum level (2.5%) (Figure 2A) were less well spread and smaller than that in higher serum levels. Cells cultured in 5% serum and more (Figure 2B, C) were epithelial-like and well spread.



**Figure 2A** Morphology of human hepatoma SMMC-7721 cells cultured in serum-free medium for 6 hours photographed under phase contrast microscope (200×)

**B** Morphology of human hepatoma SMMC-7721 cells cultured in 5% serum medium for 6 hours photographed under phase contrast microscope (200×)

**C** Morphology of human hepatoma SMMC-7721 cells cultured in 25% serum medium for 6 hours photographed under phase contrast microscope (200×)

## DISCUSSION

Most mammalian cells were serum dependent and usually passaged in medium containing serum, and they would die if they were cultured in serum-free medium for long time. Therefore, short-time serum-free (serum deprivation) cell culture was one

of the main protocols for the study of cell growth and regulation. How cell responses to the serum deprivation has called extensive attention. The results varied greatly even reversal because of the different cells used. Brooks, Larsson<sup>[3,4]</sup> and Zetterberg<sup>[7]</sup> indicated that the proliferation of non-transformed fibroblastic cells usually depend on serum or purified growth factors in the tissue culture medium. If the serum concentration was drastically reduced, the cells ceased proliferation and entered a reversible state of quiescence ( $G_0$ ). Larsson *et al*<sup>[4]</sup> reported serum-dependent proliferating 3T3 cells prolonged their intermitotic time by 9-10 hours after exposure to serum-free medium for only 1 hour and a short exposure to serum-free medium was sufficient for cells to leave the cell cycle. Zetterberg<sup>[7]</sup> showed that in 3T3 cells, in all stages of cell cycle, serum deprivation resulted in inhibition of protein synthesis, but only in postmitotic cells in the first 3-4 hours of  $G_1$  did it produce cell-cycle arrest, a 1-hour exposure to serum-free medium was sufficient to force most  $G_1$  cells into a state of quiescence ( $G_0$ ). Loo *et al*<sup>[8]</sup> demonstrated that mouse embryo cells established and maintained in the absence of serum depend on epidermal growth factor for survival and their proliferation was reversibly inhibited by serum or platelet-free plasma. Yin *et al*<sup>[9]</sup> showed that Hela cells cultured in 10% serum medium incorporated  $^3\text{H-TdR}$  to 25% of cells cultured in 0.2% serum medium. Xu *et al*<sup>[11,12]</sup> had demonstrated that human hepatoma BEL-7404 could grow in serum-free medium and there were only less than 2%-3% apoptotic cells after serum starvation for 24 hours. Human hepatoma SMMC-7721 cell were serum-dependent proliferating cell line and widely used in the detection of growth factors and anti-cancer drugs, therefore it was very important to ascertain the effects of short-time serum deprivation and serum levels on growth and metabolism of SMMC-7721 cells, which would affect the correct assessment of the activity of growth factors and drugs. Our results suggested that short-time serum deprivation (6 hours) could stimulate the synthesis of SMMC-7721 cells and the incorporation of  $^3\text{H-TdR}$  was negatively related to the serum levels in medium. For longer (12, 18, and 24 hours) exposure to serum<sup>a2</sup>free medium, the incorporation of  $^3\text{H-TdR}$  was also negatively related to the serum level, although the ratio of  $^3\text{H-TdR}$  incorporation of cells in serum<sup>a2</sup>free medium and in 25% serum medium decreased from 39.32-fold (6 hours) to 3.53-fold (18 hours). These results were reversal to that with 3T3 cells, which could easily be overlooked, or even mistakenly attributed to the activity of growth factors and drugs. The results might be due to two main reasons, one was that DNA synthesis of SMMC-7721 cells cultured in serum-free medium was

inhibited by the lack of thymidine in RPMI 1640 medium and the cells were accumulated to the G<sub>1</sub>/S. Once <sup>3</sup>H-TdR was added to the medium, the cells began to enter S phase quasisynchronously and start DNA synthesis, the other reason was that most of malignant cells appeared less dependent on serum and could secrete some growth factors and stimulate themselves by feedback mechanism. The differences of cell morphology in serum-free and different serum levels were mainly induced by fibronectin and fetuin in serum<sup>[13]</sup>, which promoted cell attachment and spread on the surfaces of culture plates. So necessary precautions must be given to the serum level changes in the medium in detecting growth factors and drugs with human hepatoma SMMC-7721 cells, otherwise false conclusion might be implied.

## REFERENCES

- 1 Eagle H. Nutrition needs of mammalian cells in tissue culture. *Science*, 1955; 122(3168):501-504
- 2 Hayashi I, Sato G. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature*, 1976;259(5539):132-134
- 3 Brooks RF. Regulation of the fibroblast cell cycle by serum. *Nature*, 1976;260(5548):248-250
- 4 Larsson O, Dafgard E, Engstrom E, Zetterberg A. Immediate effects of serum depletion on dissociation between growth in size and cell division in proliferating 3T3 cells. *J Cell Physiol*, 1986;127(2):267-273
- 5 Zaitu H, Kimura G. Serum-dependent regulation of proliferation of cultured rat fibroblasts in G<sub>1</sub> and G<sub>2</sub> phases. *Exp Cell Res*, 1988;174(1):146-155
- 6 Campisi J, Morreo G, Pardee AB. Kinetics of G<sub>1</sub> transit following brief starvation for serum factors. *Exp Cell Res*, 1984;152(2):459-466
- 7 Zetterberg A, Laesson O. Kinetic analysis of regulatory events in G<sub>1</sub> leading to proliferation or quiescence of Swiss 3T3 cells. *Proc Natl Acad Sci USA*, 1985;82(16):5365-5369
- 8 Loo DT, Fuquary JI, Rawson CL, Barnes DW. Extended culture of mouse embryo cells without senescence: inhibition by serum. *Science*, 1987;236(4798):200-202
- 9 Yin Z, Wheatley DN. Sensitivity of 3T3 cells to low serum concentration and the associated problems of serum withdrawal. *Cell Biology*, 1994;18(1):39-46
- 10 Tong R, Zhou R, Lu F, Tao W. The establishment and primary biological characteristics of human hepatoma SMMC-7721 cell line. *J 2nd Milit Med Univ*, 1980; 1(1):5-9
- 11 Xu Y, Jiang W, Peng S, Chen Y. Antisense EGFR sequence reverses the growth properties of human liver carcinoma cell line BEL-7404 in vitro. *Cell Res*, 1993;3(1):75-83
- 12 Fu T, Liu H, Liu F, Gu J, Jiang W, Xu Y. Antisense EGFR sequence enhances apoptosis in a human hepatoma cell line BEL-7404. *Cell Res*, 1996;6(2): 145-153
- 13 Freshney RI. Culture of animal cells. New York: *Alan R Liss Inc*, 1983:74-77

# Codon 249 mutations of p53 gene in development of hepatocellular carcinoma

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; p53 gene; mutation; RNA, messenger; LOH; codon 249; immunohistochemistry; polymerase chain reaction

## Abstract

**AIM** To investigate the mechanisms of codon 249 mutation of p53 gene in the formation of hepatocellular carcinoma (HCC).

**METHODS** Codon 249 mutation accompanied by loss of heterozygosity (LOH) and its effect on translation and transcription were studied using SSCP, IHC and RT-PCR/slot hybridization.

**RESULTS** Codon 249 mutations were detected in 32.9%, LOH detected in 68.4% among the HCC patients. Mutations of codon 249 were accompanied by LOH in 90%. The positive rates of p53 protein and mRNA were 91.3% and 95.7%, in mutational group, both were significantly higher than those in the non-mutational group (91.3% vs 19.1% and 95.7% vs 40.4%, respectively, both  $P < 0.01$ ). The translation of p53 gene was strongly related to its transcription by correlation analysis ( $r = 0.8208$ ).

**CONCLUSIONS** LOH might play an important role in hepatocarcinogenesis of codon 249 mutation, which could increase both transcription and translation of p53 gene. The increased expression of p53 protein mainly depend on the increased transcription of p53 gene.

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Presented at the International Symposium on Hepatology, Beijing, China, 12-15 August, 1997.

\*Supported by the China Medical Board (CMB) of New York, Grant No. 93-582.

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Received 1998-03-15

## INTRODUCTION

HCC is one of the most common human tumors in certain areas of Africa and Asia including southern China. However, the mechanisms, especially the molecular mechanisms of hepatocarcinogenesis are still not well understood. Since codon 249 mutations of p53 gene were observed in 45%-50% of HCCs from southern Africa and Qidong, China, it has been believed that mutations of codon 249 plays an important role in hepatocarcinogenesis<sup>[1-3]</sup>. But now mutation causes HCC, is still unclear. Mutant p53 proteins with much longer half-life are often overexpressed in HCC. They can inactivate the wild proteins through dominant negative in vitro<sup>[4-7]</sup>. Losses of heterozygosity are often observed in HCC. They are favorable for mutations to enact<sup>[8-10]</sup>. Nevertheless, the effect of codon 249 mutations on transcription or translation of p53 gene and the relationship between these mutations and LOH are still unclear. Therefore, in this study, we investigated these questions using surgical specimens of HCC from southern China where the incidence of HCC is moderate to high.

## MATERIALS AND METHODS

### Materials

**Specimen** Seventy surgical specimens of HCC were collected from the First Affiliated Hospital and Cancer Hospital, Sun Yat-Sen University of Medical Sciences. Cancerous and pericancerous tissues were separated by pathologists.

**Reagents** Restriction enzyme Hae III was purchased from GIBCO BRL. p53 protein monoclonal antibody (DO-7) and LSAB system were purchased from DAKO company. Dig detection kit was from B. M. Company.

### Methods

**Examination of codon 249 mutation using RFLP analysis** Exon 7 was amplified using primers 5'-GGCGA CAGAG CGAG ATTCCA-3' (sense) and 5'-GGGTC AGCGG CAAGC AGAGG-3' (anti-sense). Twenty  $\mu$ l amplified DNA product (286bp) was digested with 2.5U- restriction enzyme Hae III. DNA fragments with and without digestion were analyzed in 2% agarose gel.

### Examination of p53 gene LOH using SSCP analysis

Exon 3-4 was amplified using primers 5'-AAATT CATGG GACTGACTTT-3' (sense) and 5'-AATGC AGGGG GATAC GGCCA GC-3' (anti-sense). PCR product was precipitated at -20°C for 1 hour by adding 2.5vol of alcohol and 1/10vol of 4M sodium acetate. Pellets were resuspended in 10µl Formamide dye mixture (95% formamide, 20mmol/L EDTA, 0.05% bromphenol blue). Samples were heated at 95°C for 5 minutes, chilled on ice and immediately loaded (5µl) on 6% acrylamide/TBE- gel. fGels were run at 40W for 4 hours at room temperature. Silver-staining was used to visualize the bands. LOH of p53 gene was ensured by comparing the band pattern from cancerous tissue and pericancerous tissue of the same patient.

#### Detection of p53 proteins using immunohistochemistry

p53 proteins in tissues were detected using immunohistochemistry (IHC). Semi-quantification was carried out in 5 groups of patients divided according to the percentage of positive cells (<1%, 1%-5%, 5%-25%, 26%-50% and >50%).

#### Detection of p53 mRNA using RT-PCR

Total cell RNA was extracted using Chomczynski's method. DNA fragments of 555bp were amplified using RT-PCR with primers 5'-TACTC CCCTG CCCTC AA-3' (sense) and 5'-GTTGG GCAGT GCTCG CT-3' (anti-sense)<sup>[11]</sup>. RT-PCR products were transferred to nitrocellulose membrane. p53 mRNA was detected by hybridization with the Dig-probes which were labeled with PCR, and then visualize the dots with Dig detection kit. The degree of dot color was classified into 5 grades.

## RESULTS

### Mutation of codon 249

Exon 7 of cancerous and pericancerous tissues from 70 cases of HCC were all amplified satisfactorily. The number of base pairs of PCR product was as the same as designed. Mutations of codon 249 were detected in 23 of these cases, with a positive rate of 32.9%. One of them showed partial mutation, which was confirmed to be an allele mutation through SSCP analysis. Typical results are shown in Figure 1.

### LOH of p53 gene

Exon 4 of p53 gene has a heterogeneous location (CGC/CCC) at condon 72. The number, however, of band pattern in this study was more than 4. It might suggest that there were two or more heterogeneous locations in DNA fragment amplified from exon 3 to 4. The increased heterogeneous locations were favorable to LOH analysis. Heterozygous form was found in 57 of 70 (81.4%) cases of HCC and LOH was detected in 39 of the 57 (68.4%) cases which carried heterozygous alleles. Heterozygous alleles were found in 20 of 23 cases with mutant

codon 249, 18 of which (90%) were mutant allele accompanied by LOH. Typical results are shown in Figure 2.

### Expression of p53 protein

p53 proteins were detected only in cancerous tissues of HCC. Positive cells 7% were found in 30 cases, with a rate of 42.9%. Typical positive stain is shown in Figure 3. The relationship between the expression of p53 proteins and codon 249 mutations is shown in Table 1.

### Relationship between expression of p53 protein and p53 mRNA

The relationship between the expression of p53 proteins and the expression of p53 mRNA among 23 cases carrying codon 249 mutation is shown in Figure 5. Grade correlation analysis demonstrated that they were strongly correlated (correlation coefficient  $r = 0.8208$ ).

### Expression of p53 mRNA

The results of p53 mRNA detection are shown in Figure 4. p53 mRNA positive rate was 58.6%. The relationship with codon 249 mutation is shown in Table 1.

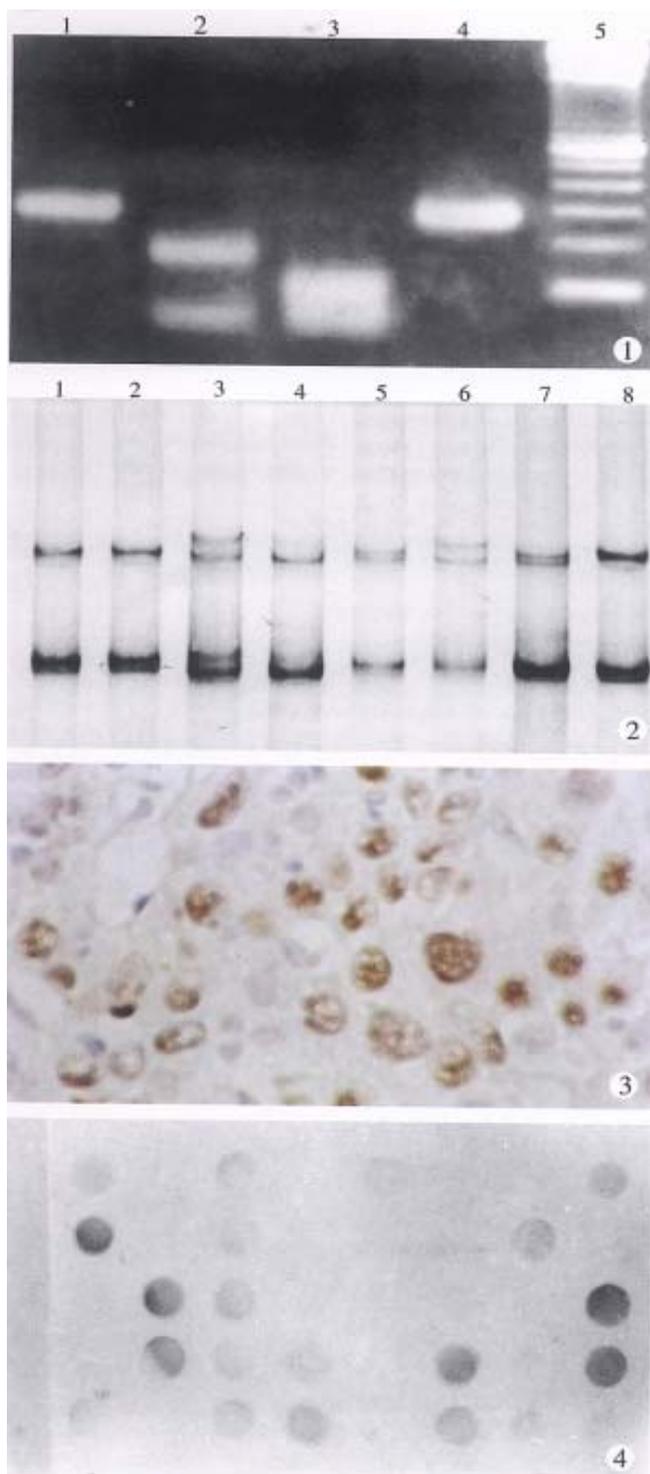
**Table 1 The relation between codon 249 mutation and expression of p53 proteins and p53 mRNA**

Mutations of codon 249	Cases p53	proteins	p53 mRNA
+	23	21 <sup>b</sup>	22 <sup>d</sup>
-	47	9	19

<sup>b</sup> $P < 0.01$ , vs codon 249 mutation negative group, <sup>d</sup> $P < 0.01$ , vs codon 249 mutation negative group.

## DISCUSSION

Tumor suppressor p53 gene often shows mutational hotspot of codon 249 in HCC, i.e., selective G to T mutation usually occurs in the third base of codon 249. This unique mutation has been reported to be involved in the carcinogenesis, differentiation and metastasis of HCC<sup>[1-6]</sup>. How this mutation causes HCC, however, is unclear. It is believed that some codon mutations of p53 gene can prolong the half-life of p53 proteins. Thus, the mutant p53 proteins often had overexpression in HCC, and inactivated its antitumor effect in combination with wild p53 proteins<sup>[4-7]</sup>. However, the effect of mutation on the transcription and translation of p53 gene is unclear. This study showed that the p53 mRNA expression in mutational group was significantly higher than that of non-mutational group. The expression of p53 proteins was strongly correlated to the expression of p53 mRNA. These results suggested that codon 249 mutation could increase p53 mRNA expression, and increased mRNA was an important factor in the overexpression of mutant p53 proteins.

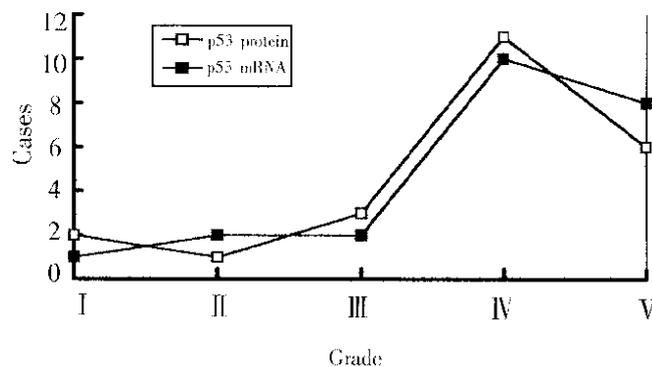


**Figure 1** Examination of codon 249 mutation using RFLP analysis. Lane 1 and lane 2 were PCR products from cancerous tissues without and with digestion of restriction enzyme Hae III. Lane 3 and lane 4 were products from pericancerous tissues with and without digestion of restriction enzyme Hae III. Lane 5 was marker of 100bp ladder from GIBco BRL.

**Figure 2** The PCR/SSCP detection of LOH, silver-staining. Lane 1 and lane 2 were products from patient 1 who was homozygote. Lane 3 to 8 were products from patients 2 to 4. They were all heterozygote. Patients 2 and 4 were LOH positive. Patient 3 has mutation of one allele.

**Figure 3** Expression of p53 proteins in HCC tissue using IHC (×400).

**Figure 4** p53 mRNA detected using RT<sup>2</sup>PCR/slot hybridization.



**Figure 5** The relation between the expression of p53 proteins and that of p53 mRNA.

When one allele mutated, it was difficult to a-mass enough cells to allow the other allele to mutate<sup>[7]</sup>. Therefore, it is important to study whether LOH exists in the other allele. Our results showed that 90% codon 249 mutations were accompanied by LOH of the other allele, suggesting that LOH of p53 gene was also important in hepatocarcinogenesis as mutations. LOH might be favorable for mutations to enact.

In conclusion, HCC will be prevented if we take some measures to avoid recurrent hepatic damage, and reduce the occurrence of LOH, and the clinical outcome of HCC will be much better if the transcription of p53 mRNA can be suppressed by chemical or gene therapy.

**REFERENCES**

- Hsu IC, Metcalf RA, Sun T, Welsh J, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. *Nature*, 1991;350(6317): 427-428
- Peng XM, Peng WW, Yao JL, Zhou YP. The relationship between hepatitis c and b virus infection and aberration of p53 gene. *Chin J Hepatol*, 1997;5(2): 124-125
- Soini Y, Chia SC, Bennett WP, Groomman JD, Wang JS, DeBenedetti VM *et al.* An aflatoxin-associated mutational hotspot at codon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. *Carcinogenesis*, 1996;17(5):1007-1012
- Hayashi H, Sugio K, Matsumata T, Adachi E, Takenaka K, Sugimachi K. The clinical significance of p53 gene mutation in hepatocellular carcinomas from Japan. *Hepatology*, 1995;22(6):1702-1707
- Qin L, Tang Z, Liu K. The relation between p53 mutations and tumor invasiveness of human hepatocellular carcinoma. *Chin J Cancer*, 1995;17(6):405-408
- Hollstein M, Sidransky D, Vogelstein B, Harris C. p53 mutations in human cancers. *Science*, 1991, 253(5015):49-53
- Ng IO, Lai EC, Chan AS, So MK. Overexpression of p53 in hepatocellular carcinomas: a clinicopathological and prognostic correlation. *J Gastroenterol Hepatol*, 1995;10(3):250-255
- Rogler CE, Chisari FV. Cellular and molecular mechanisms of hepatocarcinogenesis. *Sem Liver Dis*, 1992;12(3):265-278
- Yumoto Y, Hanafusa T, Hada H, Morita T, Ooguchi S, Shinj N *et al.* Loss of heterozygosity and analysis of mutation of p53 in hepatocellular carcinoma. *J Gastroenterol Hepatol*, 1995;10(2):179-185
- Lasko D, Cavence W. Loss of constitutional heterozygosity in human cancer. *Annu Rev Genet*, 1991;25:281-314
- Shieh YSC, Nguyen C, Vocal MV, Chu HW. Tumor-suppressor p53 gene in hepatitis C and B virus associated human hepatocellular carcinoma. *Int J Cancer*, 1993;54(4):558-562

# Endotoxins enhance hepatocarcinogenesis induced by oral intake of thioacetamide in rats \*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; endotoxins; thioacetamide; glutamyl transpeptidase/metabolism; flow cytometry; DNA, neoplasm; rats, Wistar

## Abstract

**AIM** To clarify whether endotoxin is of pathogenic importance for hepatocarcinogenesis, or the increased cancer risk results solely from the cirrhotic process.

**METHODS** The rat model of hepatoma was treated by the intake of 0.03% thioacetamide in drinking water for six months. During induction of hepatoma, rats were additionally treated with splenectomy and/or lipopolysaccharide administration. The liver nuclear DNA index and proliferation index were quantitatively analyzed by flow cytometry. Hepatic histology was examined with light and electron microscopes. Plasma endotoxin concentration and  $\gamma$ -glutamyl transpeptidase activity were measured, and hepatoma incidence was recorded.

**RESULTS** Thioacetamide induced cirrhosis and hepatoma in Wistar rats with histology or regenerative nodule, fibrosis and neoplastic foci were quite similar to the pathogenic process of human cirrhosis leading to hepatoma. In comparison with TAA controls (DNA index:  $1.15 \pm 0.21$ ), exo-endotoxin increased the DNA index by 7.8% ( $1.24 \pm 0.25$ ,  $P < 0.02$ ) and hepatoma rate by 16.7%. Splenectomy-induced enteric endotoxemia increased the DNA index by 25% ( $1.44 \pm 0.15$ ,  $P < 0.01$ ) and hepatoma rate by 33%. A summation of the effects of these two factors increased the DNA index by 36% ( $P \leq 0.01$ ) and hepatoma incidence by 50%, moreover, the level of endotoxemia showed a close relation with DNA index ( $r = 0.96$ ,  $P < 0.01$ ), as well as with the occurrence rate of hepatoma ( $r = 0.00$ ,  $P < 0.01$ ). Histological findings further verified such alterations.

**CONCLUSION** Lipopolysaccharide administration and/or splenectomy-induced enterogenic endotoxemia may enhance rat hepatocarcinogenesis induced by oral intake of thioacetamide.

## INTRODUCTION

Endotoxemia is often associated with hepatic injury and cirrhosis<sup>[1]</sup>. Patients with hepatic cirrhosis or hepatocarcinoma often present a 3 to 8-fold high systemic endotoxemia above normal<sup>[2]</sup>. If cirrhosis is established, there is a 200-fold increased risk for development of hepatocarcinoma<sup>[3]</sup>. It is unknown whether endotoxin is of importance in hepatocarcinogenesis. Recognized etiologic or predisposing factors include chronic liver disease caused by hepatitis B, C and D; hepatotoxins such as aflatoxin and alcohol; and cirrhosis of any cause<sup>[4]</sup>. The pathogenetic mechanisms involved in human hepatocarcinogenesis remain largely unsolved.

Numerous animal models of cirrhosis have been developed<sup>[5,6]</sup>. Continuous administration of thioacetamide (TAA) in drinking water to rats produced cirrhosis in 4 months and hepatocarcinoma in 6 months<sup>[7]</sup>. The histology of the TAA model was more similar to human cirrhosis than that of the CCl<sub>4</sub>-treated rats<sup>[8]</sup>. Wistar rats were used in the TAA model.

Using a TAA-induced rat model of cirrhosis leading to hepatocarcinoma, we tried to prove the hypothesis that lipopolysaccharide (LPS) administration and/or splenectomy-induced enterogenic endotoxemia may play an important role in the pathogenesis of liver tumors. The present study was designed to investigate the histologic changes of hepatocarcinoma in the TAA models treated with LPS and/or splenectomy; DNA aneuploidy and incidence of hepatocarcinoma in various groups; and the relationship between endotoxemia and hepatocarcinogenesis.

## MATERIALS AND METHODS

### Animals and treatment

Female Wistar rats (Experimental Animals Center of Shanxi Medical University) weighing  $125 \text{ g} \pm 9 \text{ g}$ , were housed in wire-bottom cages under a 12-hour light/dark cycle and fed with a balanced pellet diet

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Presented at the International Symposium on Hepatology, August 12-16, 1997, Beijing, China.

\*Supported by the Funds of Shanxi Provincial Commission of Science and Technology, No. 96025.

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Received 1997-09-01 Revised 1997-09-30

ad libitum. Animals were randomly assigned to five groups: sham group ( $n = 5$ ) as an untreated control receiving tap water ad libitum; TAA control group ( $n = 6$ ), given 0.03% w/v thioacetamide (TAA, purity >99%, Shanghai Central Chemical Factory) in drinking water; TAA+ST group ( $n = 6$ ) splenectomized (ST) a week before the experiment; during the last two months, 0.8mg% lipopolysaccharide (LPS, from *-Escherichia coli*-stereotype 055:B5, Sigma) added to drinking water containing 0.03% TAA for both TAA+LPS group ( $n = 6$ ); and TAA+ST+LPS group ( $n = 6$ ). Without splenectomy, animals underwent midline laparotomy and spleen manipulation as a sham surgery. All operations were performed under light ether anesthesia and sterile conditions. At the termination of a 6-month course, rats were anesthetized with ethyl ether and blood from abdominal aorta was collected in heparinized tubes for measurement of plasmic endotoxin concentration and glutamyl transpeptidase (GGT) activity.

#### **Analysis of endotoxin and $\gamma$ -glutamyl transpeptidase activity in plasma**

Endotoxin in plasma was determined by means of a chromogenic limulus amoebocyte lysate (LAL) assay<sup>[9]</sup>. Limulus kit (Shanghai Yihua Clinical Medical Biotech Co.) was used for endotoxin-specific quantitative measurement in this study. Syringes, needles and glasswares were made pyrogen-free with dry heat (200°C for 4 hours). The plasma was pre-treated with perchloric acid (PCA) for removal of the possible inhibitors to LAL assay. Briefly, 0.1ml of 0.18mol/L NaOH was added to a 0.1ml of test plasma and incubated at 37°C for 5min. Then 0.1ml of 0.32mol/L PCA was added for an additional 10min incubation. The precipitate formed was dissolved by addition of 0.2ml of 0.18mol/L NaOH with vigorous mixing. The solution was centrifuged at 2000rpm for 10min. Then 0.05ml of the supernatant diluted with 0.05ml of 0.4mol/L Tris-HCl (pH 8.0) was added to 0.1ml of limulus amoebocyte lysate (LAL) in pyrogen-free cuvette, and incubated in a thermostatic bath at 37°C for exactly 30min. The synthetic chromogenic substrate 0.05ml, *n*-tert-butoxycarbonyl-*L*-leucyl-*L*-glycyl-*L*-arginine-*p*-nitroaniline (Boc-Leu-Gly-Arg-PNA) was added to the lysate and incubated for 5min. The reaction was stopped with 0.5% sodium nitrite. The amount of PNA released from the substrate was detected after diazo-coupling by adding 0.5ml of 0.05% ammonium sulfate and 0.5ml of 0.05% *n*-*l*-naphthylethylenediamine dihydrochloride. Absorbance was read at 545nm on 721A spectrophotometer, using distilled water as the 0 absorbance value. A standard curve was plotted with the known endotoxin concentration for each run.

Simultaneously, saline replacing plasma was as reagent control.

$\gamma$ -glutamyl transpeptidase (GGT) activity in plasma was detected according to the method as described by Xu KC<sup>[10]</sup>. In brief, 0.1ml of plasma was added to a 0.5ml of 10mmol/L substrate,  $\gamma$ -L-glutamyl- $\alpha$ -naphthylamide (Shanghai Third Reagent Factory), and incubated at 37°C for 120min. The amount of  $\alpha$ -naphthylamide released from the substrate was detected after adding 10ml of 0.2% sulfanilic acid and 0.004% sodium nitrite. The absorbance was read at 500nm on a 721A spectrophotometer, using sample control of adding plasma after 120min incubation as the 0 absorbance value. A standard curve was plotted with the known  $\alpha$ -naphthylamide concentration for each run.

#### **Light and electron microscopy**

One half of the left lobe of the liver was resected, fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (HE) for light microscopy. For electron microscopic study, the selected areas with hepatocarcinoma were cut into small pieces and fixed in 2% glutaraldehyde for 2 hours. After postfixation in 1% osmium tetroxide, the fragments were dehydrated in a graded alcohol series and embedded in Polybed. They were trimmed into ultrathin section and stained with uranyl acetate and lead citrate, and observed under an electron microscope at 100KV.

#### **Flow cytometry**

According to Zuo LF<sup>[11]</sup>, with slight modification. The specimens for flow cytometry were prepared immediately after removal of liver. About 2g tissue was mechanically minced in 5ml of 0.05% collagenase (type IV, Sigma) and then through a 200 mesh filter. After washing with 0.1mol/L phosphate buffer solution (PBS), the well-dispersed hepatocytes were resuspended and fixed in 70% ethanol overnight, the cells were stained with propidium iodide reagents (propidium iodide 50mg/L, RNase 20mg/L, and Triton-X-100 1%) at 4°C for 30min. Chicken erythrocytes were used as internal controls. Flow cytometry was performed on a Fluorescence Activated Cell Sorter (FACS) 420 cytometer (USA). Data were analyzed on a compatible computer with HP-300 consort 30. A total of 10<sup>4</sup> cells were examined for each sample, DNA index (DI) expressed as a relative DNA content, was calculated by dividing mean channels of G0/1 peak in the treated rat by that of sham group. The coefficient of variation (CV) of G0/1 peak of the DNA ploidy population was expressed by normalized standard deviation (CV=standard deviation/mean peak position  $\times$  100). CV was always less than 5%. For the

DNA ploid criteria, it was classified as DNA-diploid if  $DI=1.0\pm 2CV$ , and DNA-aneuploid if  $DI\neq 1.0\pm 2CV$ . Cell cycle parameters were analyzed by DNA cell cycle analysis software Sum of Broadened Rectangles Model, and the cell proliferation index (PI) was expressed as the percentage of cells in S plus G2M phases.

### Statistical analysis

All results were expressed as  $\bar{x} \pm s$ . Data were analyzed by Student's *t* test and multiple regression. *P* values  $<0.05$  were considered statistically significant.

## RESULTS

The oral intake of TAA in drinking water induced cirrhosis and, in some rats with hepatocarcinoma with null mortality. TAA-treated animals showed a progressive decline in body weight, particularly in the first month (data not shown). On the contrary, the ratio of liver weight to body weight were obviously higher in all TAA treated animals ( $P<0.01$ ), compared with the sham group. The hepatocyte proliferation index was fairly high in the TAA+LPS or TAA+ST groups, and the highest in the TAA+ST+LPS group ( $P<0.05$ ) compared with the TAA-model control (Table 1).

**Table 1 Hepatocyte proliferation and liver weights**

Groups	<i>n</i>	PI	Ratio of liver weight to 100g body weight
Sham	5	0.25±0.032	4.32±2.6
TAA control	6	0.33±0.047	8.38±2.0 <sup>b</sup>
TAA+LPS	6	0.35±0.045	7.35±1.3 <sup>b</sup>
TAA+ST	6	0.35±0.077	7.40±1.5 <sup>b</sup>
TAA+ST+LPS	6	0.45±0.039 <sup>a</sup>	7.28±1.5 <sup>b</sup>

<sup>a</sup> $P<0.05$ , vs TAA control, TAA+LPS and TAA+ST groups respectively. <sup>b</sup> $P<0.01$ , vs sham group. PI, proliferation index. Rats were treated as described in Materials and Methods.

### Endotoxemia level and GGT activity in systemic circulation

The TAA-treated rats with splenectomy or administration of 0.8mg% LPS (approximately corresponding to 50 µg/100 g body weight daily) in drinking water during the last two months of the study showed increased endotoxemia level ( $P<0.05$ ), compared with the sham group. The blood endotoxin level in the TAA+ST+LPS group further mounted up ( $P<0.05$ ), compared with the sham group or the TAA control group. However GGT activity was markedly increased in the blood of the TAA-treated rats with splenectomy plus LPS administration ( $P<0.05$ ), compared with solely TAA-treated group (Table 2).

**Table 2 Endotoxemia levels and GGT activities in plasma**

Groups	<i>n</i>	Endotoxin (EU/100ml)	GGT (U/100ml)
Sham	5	95±15	17±7
TAA control	6	108±12	226±33 <sup>d</sup>
TAA+LPS	6	118±12 <sup>d</sup>	236±44 <sup>d</sup>
TAA+ST	6	130±31 <sup>d</sup>	266±127 <sup>d</sup>
TAA+ST+LPS	6	130±18 <sup>ad</sup>	442±99 <sup>abcd</sup>

<sup>a</sup> $P<0.05$  vs TAA control; <sup>b</sup> $P<0.05$ , vs TAA+LPS group; <sup>c</sup> $P<0.05$ , vs TAA+ST group; <sup>d</sup> $P<0.05$ , vs sham group. Rats were treated as described in Materials and Methods.

### DNA index and hepatoma rate

The livers of rats in the TAA control group showed many superficial yellowish macronodules and one case of visible hepatocarcinoma out of six rats. With LPS administration and/or splenectomy, numerous micronodules appeared in livers and more cases of noticeable tumors. Moreover much more hepatocarcinomas were observed under the light microscope. DNA index as an indicator of the severity of malignant lesions were stepped up in the TAA models additionally treated with LPS and/or splenectomy. Therefore, they have significant differences between groups ( $P<0.02$ ), particularly in the splenectomized TAA-treated rats with oral LPS administration (Table 3).

**Table 3 DNA ploidy and incidence of hepatoma in various groups**

Groups	<i>n</i>	DNA index	Rate of DNA aneuploid%	Incidence of hepatoma			
				Visible Under microscopy		Under microscopy	
				(%)	<i>n</i>	(%)	<i>n</i>
Sham	5	1.0	0	0	0 (0/5)	0	0 (0/5)
TAA control	6	1.15±0.21	50	17	(1/6)	50	(3/6)
TAA+LPS	6	1.24±0.25 <sup>a</sup>	50	33	(2/6)	50	(3/6)
TAA+ST	6	1.44±0.15 <sup>ab</sup>	100	50	(3/6)	67	(4/6)
TAA+ST+LPS	6	1.56±0.07 <sup>abc</sup>	100	67	(4/6)	67	(4/6)

<sup>a</sup> $P<0.02$ , vs TAA control group; <sup>b</sup> $P<0.02$ , vs TAA + LPS group; <sup>c</sup> $P<0.02$ , vs TAA + ST group. DNA index=mean channels of G0/1 peak in the treated rat ÷ mean channels of G0/1 peaks in the normal controls. 10<sup>4</sup> cells of a rat liver were examined by flow cytometry. Rats were treated as described in Materials and Methods.

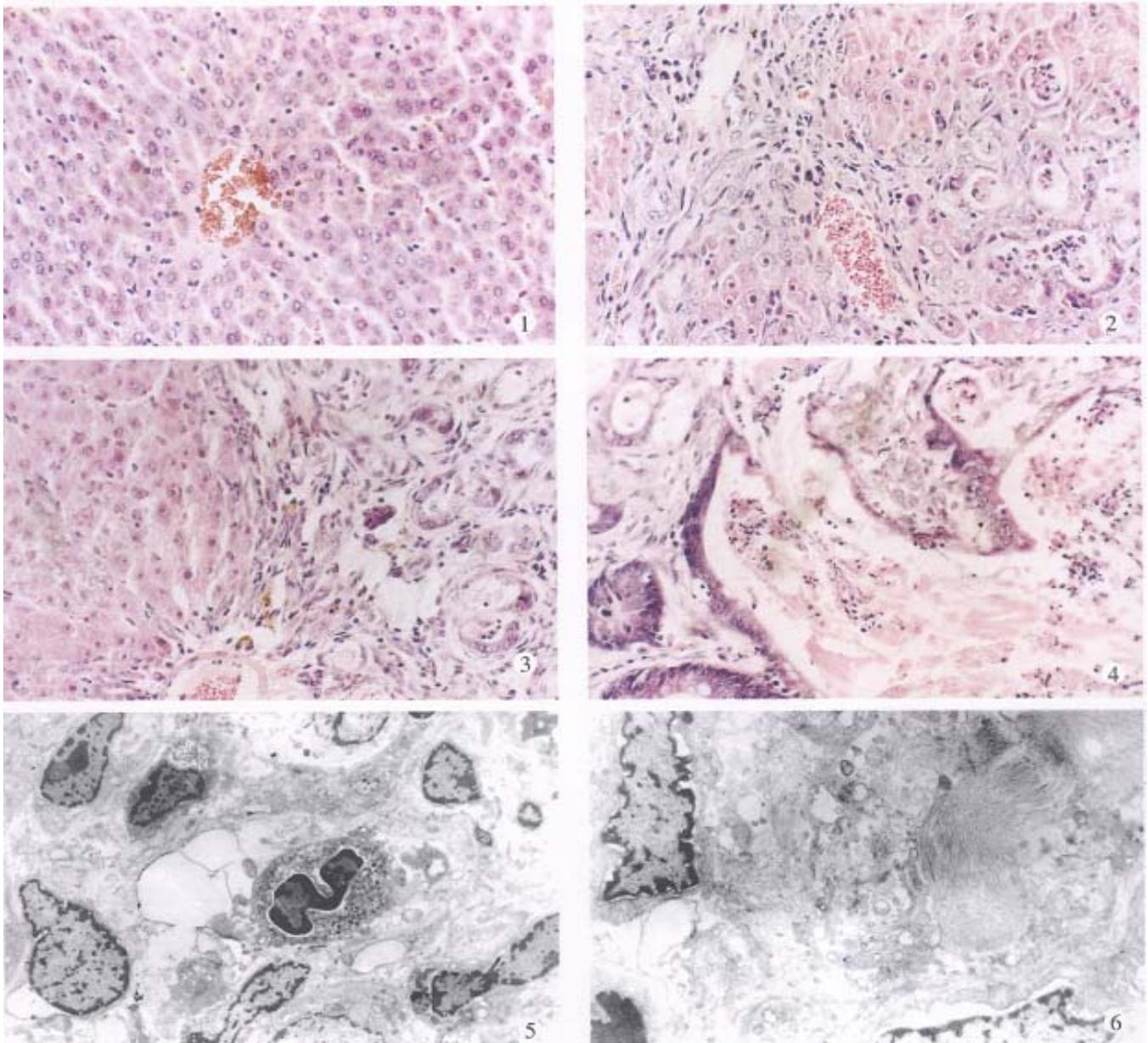
### Histology

Under the light microscope, no noticeable changes were observed in untreated control rats (Figure 1). The livers of the TAA models with or without LPS and/or splenectomy had numerous micronodules of various sizes in all lobes. The nodules were separated by markedly proliferated collagen fiber, forming pseudolobules. Proliferation of spindle cells, probably fibroblasts, was recognized around such hyperplastic nodules (Figure 2). In the transforming

hepatocytes, atypical hyperplasia with nuclear heterochromatism and basophilic cytoplasm were seen mainly in perivenular and in periportal areas. Nuclear atypism was also seen in the hepatocytes. Moreover, disarrangement of the hepatic cords became apparent and formed considerable pseudoglandular (acinar) structures which were transformed from original liver parenchymal cells (Figure 3). Highly malignant lesions were observed in the TAA

+ST+LPS group, in which atypism was more apparent and formed a “mucoïd lake” with a float of small atypical cells (Figure 4).

Electron microscopic investigation clearly showed that the disarrangement of the altered hepatocytes and nuclear atypism were very apparent, together with less cytoplasm containing very few organelles (Figure 5). Collagen fibrils were markedly present around the neoplastic cells (Figure 6).



**Figure 1** Section of liver from an untreated control (sham) rat. HE×20.

**Figure 2** Section of liver from a TAA control rat treated as described in Materials and Methods. The nodules show the transforming hepatocytes and the hepatocarcinoma. The spindle fibroblasts are recognized around such hyperplastic nodules. HE×20.

**Figure 3** Section of liver from a rat in TAA+ST group. Pseudoglandular structures and spindle cells are observed in perivenular areas. HE×20.

**Figure 4** Section of liver from a rat in TAA+ST+LPS group. The disorganized and atypical hepatocytes formed a “mucoïd lake” with floating of small cells. HE×20.

**Figure 5** Ultrastructure of hepatocellular carcinoma from a rat in TAA+ST+LPS group. Uranyl acetate and lead citrate staining ×400.

**Figure 6** Ultrastructure of cirrhosis from a rat in TAA+ST+LPS group. The bundles of collagen fibrils were present with atypical hepatocytes. Uranyl acetate and lead citrate staining ×800.

## DISCUSSION

TAA-induced cirrhosis and hepatocarcinoma has been observed in Wistar rats. The results of the present study demonstrated for the first time that endotoxin may play an important role in the development of hepatocarcinoma in rats induced by continuous administration of 0.03% TAA in drinking water.

TAA is hepatotoxic owing to effects on DNA, RNA, protein synthesis and GGT activity, through which it induces cirrhosis and hepatocarcinoma<sup>[1,8]</sup>. After 6 months of chronic TAA administration, the liver exhibited an external appearance of cirrhosis characterized by the presence of numerous yellowish nodules and whitish hepatocarcinoma. The microscopic study showed that micronodular cirrhosis with hepatocarcinoma resembles human cirrhosis with hepatocarcinoma in both its biological and morphological aspects in close agreement with other studies<sup>[5-7]</sup>. Table 3 shows that TAA increases nuclear DNA content by 15% in rat liver, producing 50% cases with aneuploidy and 50% cases of hepatocarcinoma seen under microscopy was a suitable model for evaluating the effects of endotoxin on hepatocarcinogenesis.

DNA index representing a ratio of the DNA content in the treated rat to the mean DNA content of the diploid normal rats indicate precise malignancy of hepatocarcinoma, together with aneuploidy rates in various groups, which were highly correlative to the incidence of hepatocarcinoma (the former  $r = 0.98$ ,  $P < 0.01$ ; the latter  $r = 0.96$ ,  $P < 0.01$ ). Moreover, the histologic studies with light and electron microscopes well confirmed the interesting findings. In the hepatocyte, clonal perpetuation of accumulated DNA aneuploid affecting growth regulatory genes eventually leads to a continuation of severe progressive abnormalities characterized by dysplasia, formation of adenoma and, finally, cancer<sup>[13]</sup>. In addition, plasma GGT activity, an enzyme of fetal hepatocytes, was a sign of poor differentiation and correlated with cancerous change ( $r = 0.93$ ,  $P < 0.05$ ) and the degree of endotoxemia ( $r = 0.88$ ,  $P < 0.05$ ).

Like Kupffer cells, splenic macrophages have a potential for endotoxin clearance by way of phagocytosis. In this study, rats undergoing splenectomy showed reduced endotoxin clearance and increased enterogenic endotoxin levels in systemic circulation by 37% ( $P < 0.05$ ). And rats with cirrhosis after LPS administration at a dose of 50 $\mu$ g/100g body weight daily for two months increased their endotoxemia by 24% ( $P < 0.05$ ), compared with the normal controls. When the rat endotoxemia increased, inhibition of hepatic DNA synthetic enzymes by endotoxin<sup>[12]</sup> resulted in a decrease of liver weights and formation of micronodules in comparison with the TAA control rats with

macronodules. In addition, splenic macrophages have an ability to generate tumor necrosis factor- $\alpha$  and play an important role in immunosurveillance<sup>[13,14]</sup>. Therefore the down-regulation of Kupffer cell activity after splenectomy may have deleterious effects on host defenses<sup>[15]</sup>.

This study shows that endotoxemia from orally administered LPS, during the last two months, and/or from splenectomy before commencement of the experiment, significantly enhanced hepatocarcinogenesis ( $P < 0.02$ ), based on the alterations of the DNA index. It seemed that the TAA model with splenectomy combining LPS administration was more deleterious to the development of hepatocarcinoma in rats than the TAA model treated with ST or LPS solely. In addition, it was noticed that the blood endotoxin levels in rats were significantly correlated with malignancy ( $r = 0.96$ ,  $P < 0.01$ ) and the incidence ( $r = 0.99$ ,  $P < 0.01$ ) of hepatocarcinoma. If so, it may be of importance in the pathogenetic process of hepatocarcinoma and in clinical therapeutic strategy. However, patients with cirrhosis presenting relatively high endotoxemia<sup>[2]</sup> may have higher risk for development of hepatocarcinoma epidemiologically<sup>[3]</sup>, but what are the possible mechanisms involved in the pathogenesis and whether this result may be extrapolated into humans await further studies.

## REFERENCES

- Han DW. A study of pathogenic mechanism of hepatic failure-hypothesis of intestinal endotoxemia. *Chin J Hepatol*, 1995;3(3):46-49
- Lumsden AB, Henderson JM, Kutner MH. Endotoxin levels measured by a chromogenic assay in portal, hepatic and peripheral venous blood. *Hepatology*, 1988; 8(2):232-236
- Niederer C, Fisher R, Sonnenberg A, Stremmel W, Tramprishch HJ, Strohmeyer G. Survival and causes of death in cirrhotic and noncirrhotic patients with primary hemochromatosis. *N Engl J Med*, 1985;313(4):1256-1262
- Han DW. Etiology of liver diseases. In: Han DW, ed. *Hepatic pathophysiology*. Vol 2. Taiyuan: Shanxi United Press of Universities, 1991
- Mullen KD, McCullough MAJ. Problem with animal models of chronic liver disease: suggestions for improvement in standardization. *Hepatology*, 1989;9(2):500-503
- Noda S, Musumi S, Moriyama M, Yukiko K, Ohta M, Sugano T et al. Population of hepatic macrophages and response of perfused liver to platelet-activating factor during production of Thioacetamide induced cirrhosis in rats. *Hepatology*, 1996;24(2):412-418
- Moreira E, Fontana L, Peviago JL, Medina FS, Gil A. Changes in fatty acid composition of plasma, liver microsomes, and erythrocytes in liver cirrhosis induced by oral intake of Thioacetamide in rats. *Hepatology*, 1995;21(1): 199-206
- Porter WR. TAA-induced hepatic necrosis. *J Pharmacol Exp Ther*, 1979;208(1):386
- Inada K, Endo S, Takahashi K, Suzuki M, Narita T, Yoshida T et al. Establishment of a new perchloric acid treatment method to allow determination of the total endotoxin content in human plasma by the limulus test and clinical application. *Microbiol Immunol*, 1991;35(4):303-314
- Xu KC, Meng XY, Wu JW. Diagnostic value of serum glutamyl transferase isoenzyme for hepatocellular carcinoma: a 10-year study. *Am J Gastroenterol*, 1992;87(8):91
- Zuo LF, Guo JW, Liu JH. Flow cytometry DNA ploidy analysis in 208 cases of precancerous lesions. *Chin J Physic Med*, 1992;14(1):31-34
- Ikuyo T, Rieko N, Shosuke K. Effect of endotoxin on rat liver regeneration after partial hepatectomy. *Biochem International*, 1992;27(4):1047-1050
- Dragan YP and Pitot HC. The role of stages of initiation and promotion in phenotypic diversity during hepatocarcinogenesis in the rat. *Carcinogenesis*, 1992;13(5):379-750
- Battafarano RJ, Burd RS, Kurrelmeier KM, Rtz CA, Dunn DL. Inhibition of splenic macrophage tumor necrosis factor $\alpha$  secretion in vivo by anti-polysaccharide monoclonal antibodies. *Arch Surg*, 1994;129(2):179-186
- Shohachi S, Satoaoshi N, Atsushi S, Takanori S, Hiroyuki K, Hiroyuki M et al. Role of Kupffer cells and the spleen in modulation of endotoxin-induced liver injury after partial hepatectomy. *Hepatology*, 1996;24(1):219-225

# Stage II surgical resection of hepatocellular carcinoma after TAE: a report of 38 cases \*

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**Subject headings** carcinoma, hepatocellular/therapy; liver neoplasm/therapy; carcinoma, hepatocellular/surgery; liver neoplasms/surgery; embolization, therapeutic; hepatectomy

## Abstract

**AIM** To evaluate the curative effect of stage II surgical resection of hepatocellular carcinoma after TAE.

**METHODS** Thirty-eight patients with unresectable hepatocellular carcinoma were treated by transcatheter arterial embolization (TAE). When the sizes of tumors were markedly reduced after TAE, stage II surgical resections were performed.

**RESULTS** Before TAE, the diameters of tumors were  $12.84 \text{ cm} \pm 4.87 \text{ cm}$  ( $\bar{x} \pm s$ ), but reduced to  $5.12 \text{ cm} \pm 1.82 \text{ cm}$  ( $\bar{x} \pm s$ ) after TAE ( $P < 0.001$ ). Pathologic examination of the resected specimens revealed obvious necrosis in most cases. After surgery, 26 patients were alive, with the longest survival of 96 months, twelve died and 10 had tumor recurrence.

**CONCLUSION** Patients in moderate and advanced stages of hepatocellular carcinoma after TAE should be treated surgically, but the indication must be controlled strictly.

## INTRODUCTION

Transcatheter arterial embolization (TAE) has been recognized as a new effective method for the treatment of moderate and advanced hepatocellular carcinoma<sup>[1-10]</sup>. Some patients are able to gain a chance of stage II surgical resection after TAE. But, what are the indications of stage II surgical resection? How about the curative effect? What are the influential factors? Up to date, there has been no report about these questions. A total of 1288 patients with advanced hepatocellular carcinoma were treated by TAE in our hospital from February 1987 to December 1992, 248 of them were followed up and 38 gained stage II surgical resection. The results are reported below in an attempt to explore the above mentioned questions.

## MATERIALS AND METHODS

This group consisted of 38 patients with hepatocellular carcinoma diagnosed pathologically. They were treated by TAE via catheterization of hepatic artery on account of no indication for surgery. TAE procedures were carried out alike as previously reported<sup>[1,6,9,10]</sup>. The duration from initial TAE treatment to stage II surgical resection in this group was 4 weeks to 16 months, averaging 6.38 months. TAEs were done 1 to 9 times for each patient with an average of 3.6 times and at intervals of 50-60 days. According to hepatic arteriographic findings, 28 of 38 cases were classified as huge mass type or huge mass plus nodular type (the diameter of tumor  $\geq 10\text{cm}$ ), 10 as nodular type (the diameter of tumor 5cm-9cm) and none as diffuse type.

## RESULTS

### *Change of tumor size after TAE and pathologic examination*

Before TAE, the diameters of tumors were  $12.84 \text{ cm} \pm 4.87 \text{ cm}$  ( $\bar{x} \pm s$ ) and after TAE decreased to  $5.12 \text{ cm} \pm 1.82 \text{ cm}$  ( $\bar{x} \pm s$ ), ( $P < 0.001$ ) (Figures 1-6). Tumors were removed in 36 cases, but not in the remaining 2 because of spreading to diaphragm and lesser epiploon, absolute ethanol was injected directly into the tumors with ligation of feeding hepatic artery. Liver tumor capsules were intact in 19 of 36 resected specimens observed with naked eyes, the capsules were not intact in 14, and none in 3. Under light microscope, there was complete necrosis of tumor cells in 12 cases, obvious necrosis

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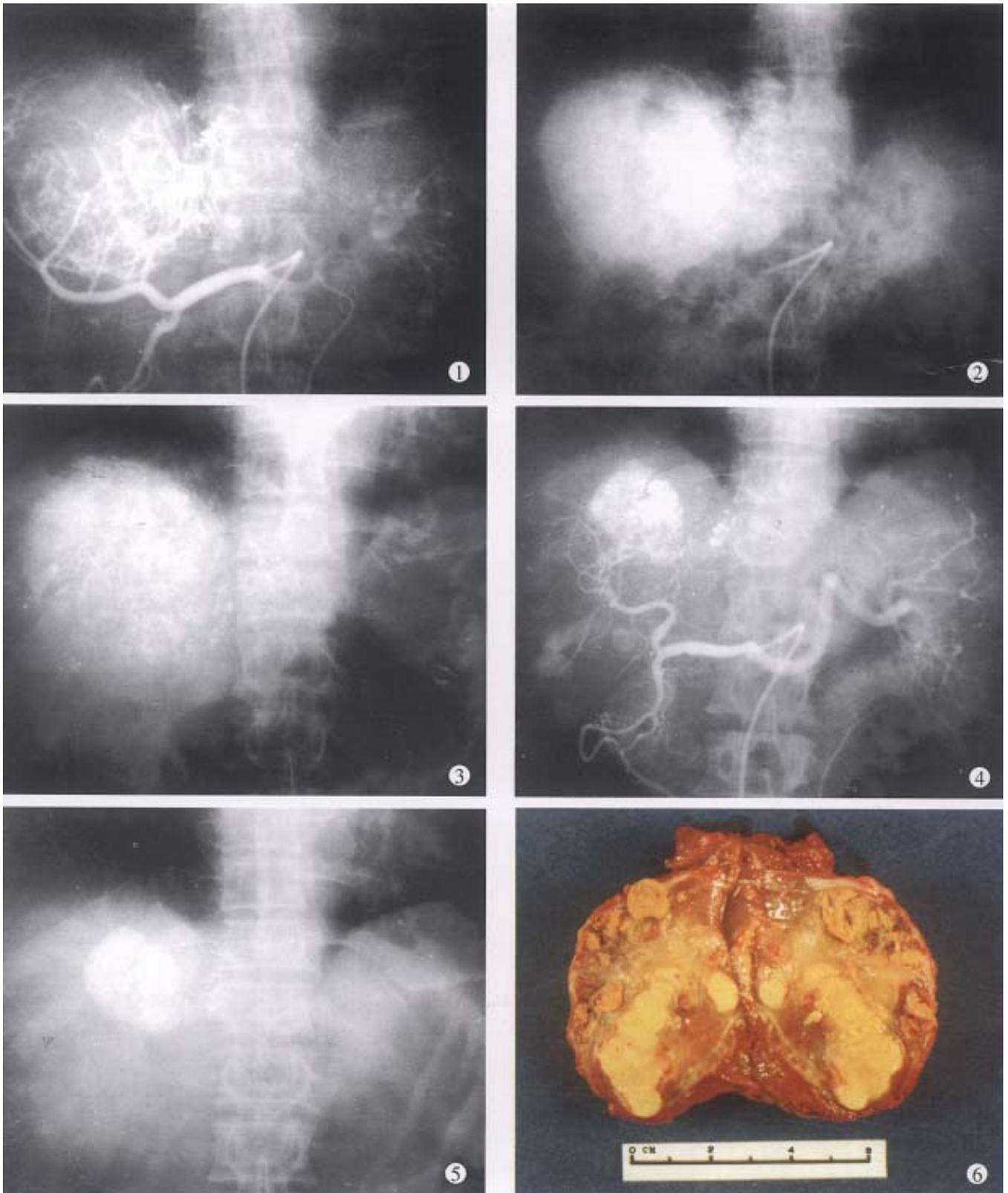
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\*Presented at the 1st Asian-Pacific Congress of Cardiovascular & Interventional Radiology, Seoul, 24-27 May, 1993

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Received 1997-09-02



**Figure 1** The hepatic arteriography shows a large tumor with hypervasculars in the right lobe of liver.

**Figure 2** Abdominal plain film immediately after TAE shows accumulation of lipiodol within the tumor.

**Figure 3** A parenchymal phase of the hepatic arteriography. Tumor mass staining is well visualized.

**Figure 4** The hepatic angiography after three times of TAE shows occluded branches of right hepatic artery with some collateral vessels and remarkable tumor reduction.

**Figure 5** Abdominal plain film after four times of TAE shows deposition of lipiodol within the tumor. The diameter of tumor was reduced to 4cm.

**Figure 6** After four times of TAE, the tumor was resected. The specimen showed necrosis of tumor and fibrotic capsule.

in most parts of the tumor with a few living tumor cells in 20 cases, and partial necrosis with parts of tumor cells actively growing in 4 cases. There were inflammatory reaction and different degrees of fibrotic proliferation around the necrotic tissues. During operation, mild liver cirrhosis was seen in 11 cases while moderate and serious ones in 21 and 4 cases respectively, and none in 2.

#### ***The survival of patients after stage II surgical resection***

So far, 26 of 38 patients have been alive, 22 of 26 being healthy. The average survival was 24.6 months, and the longest survival period has reached 96 months. Tumor recurred in 4 cases, 2 had bone metastasis one month after surgery, one was found to have multiple intrahepatic tumor nodules and one had elevated AFP values with right liver recurrent tumor of 4.5cm×5.2cm in diameter, occurring 3 months and 19 months after surgery, respectively. Twelve patients died, 2 died separately from coronary arterial embolization and massive upper digestive tract bleeding one week after surgery, 8 died from recurrence of liver tumor, and the other 2 died of serious liver cirrhosis and liver function failure 4 and 16 months after surgery. The time between surgery and death was 4-28 months, with an average of 11.5 months (excluding 2 deaths one week after surgery).

#### ***Recurrence condition of liver tumors after surgery***

Tumor recurred after surgery in 10 patients, 8 of the 10 recurrences were within liver, whereas 2 had bone metastasis. So far, 8 patients have died. Postoperative tumor recurrence time was 1 to 21 months with an average of 7.2 months. Cancerous emboli were found within the main trunks of portal vein or its large branches in the 10 patients, and 1 of them had also cancerous emboli in the right hepatic vein.

### **DISCUSSION**

#### ***TAE effectiveness depends on the thoroughness of embolization***

In our 38 patients, the sizes of liver tumors were markedly reduced after TAE. Pathologic examination of the resected specimens revealed obvious necrosis in most parts of tumor masses in nearly all the cases and complete necrosis in a few ones<sup>[6,9,10]</sup>. All these indicated the good therapeutic effects of TAE for hepatocellular tumor. The reason for the better results obtained in this group by TAE was the thorough embolization of tumor vessels and feeding arteries, as shown by the accumulation of large amount of lipiodol in all or most parts of tumor mass on CT scanning. Besides, most of the tumors in this group were of huge mass type or single nodule type.

Furthermore, the lipiodol emulsion containing anticancer drugs obstructed the blood supplying to the tumors, resulting in ischemic necrosis<sup>[2,5,8]</sup> on one hand, and on the other hand, the lipiodol emulsion continued to release anticancer drugs for killing tumor cells which were already in an ischemic and more vulnerable state<sup>[6,9,10]</sup>. Therefore, the thoroughness of embolization for liver tumors directly influenced the effect. As far as the patients' liver function would be tolerated, the embolization procedure should be undertaken to fully fill the tumor mass with lipiodol emulsion during each TAE, especially for the first time<sup>[9,10]</sup>.

#### ***Necessity of stage II surgical resection***

The surgical specimens from the 38 cases revealed that most parts of the liver tumors were necrotic, but a few living cancerous cells still remained, which might cause tumor recurrence or metastasis later<sup>[6,9,10]</sup>. On the other hand, after several times of TAE procedure, the main tumor feeding arteries had been obstructed and more collateral vessels thus formed, causing difficulties in super-selective catheterization via the collateral vessels and continuous TAE. The majority of patients were only treated by arterial infusion chemotherapy, which was usually ineffective in controlling tumor growth. Therefore, the liver tumor which had been markedly reduced in size after TAE should be removed further by stage II surgery. Thus, the prognosis would be much improved.

#### ***Prevention and reduction of tumor recurrence after surgery***

In this group, ten patients had liver tumor recurrence on an average of 7.2 months after stage II surgery (10/38, or 26.3%). There were three types of recurrences: ① 10 patients showed all cancerous emboli in the main trunks of portal veins and their bigger branches, with 1 case also having cancerous emboli in the right hepatic vein; ② in some cases, several tumor nodules were still present besides the main tumor mass; and ③ in a few cases, merely palliative surgical resections were performed because the tumor sizes found during operation were still too large. Therefore, the indication for stage II surgical resection after TAE should be controlled strictly. The contraindications are as follows: hepatic arteriogram and CT scanning reveal main tumor mass with several satellite tumor nodes which can not be removed completely by surgery; the diameter of tumor is over 5 cm, which is only resected palliatively; cancerous emboli within the main trunk of portal vein or its large branches or large hepatic venous branches; distant metastasis; and serious liver cirrhosis.

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## REFERENCES

- 1 Yamada R, Sato M, Kawabata M, Nakatsuka H, Nakamura K, Takashima S. Hepatic artery embolization in 120 patients with unresectable hepatoma. *Radiology*, 1983;148(2):397-401
- 2 Nakakuma K, Tashiro S, Hiraoka T, Uemura K, Konno T, Miyauchi Y. Studies on anticancer treatment with an oily anticancer drug injected into the ligated feeding hepatic artery for liver cancer. *Cancer*, 1983;52(12):2193-2200
- 3 Ohishi H, Uchida H, Yoshimura H, Ohue J, Katsuragi M, Matsuo N *et al*. Hepatocellular carcinoma detected by iodized oil. Use of anticancer agents. *Radiology*, 1985;154(1):25-29
- 4 Nakakuma K, Tashiro S, Hiraoka T, Ogata K, Ootsuka K. Hepatocellular carcinoma and metastatic cancer detected by iodized oil. *Radiology*, 1985;154(1):15-17
- 5 Miller DL, Timothy J, O'Leary, Girton M, RT. Distribution of iodized oil within the liver after hepatic arterial injection. *Radiology*, 1987;162(3):849-852
- 6 Kenematsu T, Furuta T, Takenaka K, Matsumata T, Yoshida Y, Nishizaki T *et al*. A 5 year experience of lipiodolization: selective regional chemotherapy for 200 patients with hepatocellular carcinoma. *Hepatology*, 1989;10(1):98-102
- 7 Uchida H, Ohishi H, Matsuo N, Nishimine K, Ohue S, Nishimura Y *et al*. Transcatheter hepatic segmental arterial embolization using lipiodol mixed with an anticancer drug and gelfoam particles for hepatocellular carcinoma. *Cardiovasc Intervent Radiol*, 1990;13(3):140-145
- 8 Yamada R, Kishi K, Sonomura T. Transcatheter arterial embolization in unresectable hepatocellular carcinoma. *Cardiovasc Intervent Radiol*, 1990;13(2):135-137
- 9 Lin G, Wang JH, Gu ZM, Yan ZP, Wang XL. Hepatic arterial infusion chemotherapy and embolization in the treatment of moderate and advanced stages of hepatic carcinoma: therapeutic effects and some influential factors. *Chin J Radiol*, 1992;26(5):311-315
- 10 Wang JH, Lin G, Yan ZP, Wang XL. Hepatic arterial infusion chemotherapy plus embolization for unresectable liver cancer: a report of 40 patients. *Chin J Oncol*, 1992;14(4):276-278

# Regulatory effects of lipopolysaccharide in murine macrophage proliferation

FAN Kai

**Subject headings** lipopolysaccharide; macrophage; granulocyte-macrophage colony-stimulating factor; transforming growth factor beta; interferon- $\gamma$ ; polymerase chain reaction; cytokines; RNA, messenger

## Abstract

**AIMS** To study the regulatory effects of bacterial lipopolysaccharide (LPS) in murine macrophage proliferation.

**METHODS** Using murine peritoneal exudate macrophage (PEM) and macrophage cell line J<sub>774</sub>A.1 as targets, LPS effects on M-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulated macrophage colony-forming cells (CFU-M) were detected. 125I-GM-CSF receptor binding assay was used to examine LPS regulation on GM-CSF receptor expression. RT-PCR was employed to test TGF- $\beta_1$  inhibition on IFN- $\gamma$  mRNA expression on macrophage induced by LPS.

**RESULTS** Without direct effect on macrophage proliferation, LPS could inhibit the macrophage proliferation stimulated by GM-CSF. However, with the concomitant existence of GM-CSF and TGF- $\beta_1$ , the LPS inhibitory effect was eliminated. RT-PCR analysis indicated that the strongest macrophage growth inhibitory factor IFN- $\gamma$  mRNA expression in macrophage induced by LPS was remarkably suppressed by TGF- $\beta_1$ . 125I-GM-CSF receptor binding assay showed that LPS could enhance GM-CSF receptor expression likewise as TGF- $\beta_1$ .

**CONCLUSIONS** LPS is involved in the network of macrophage proliferative regulation by multiple cytokines, displaying inhibitory and stimulatory effects based on the coexisting cytokines.

## INTRODUCTION

Lipopolysaccharide (LPS), main component of Gram<sup>+</sup>negative bacterial endotoxin, is involved in immune reaction by intriguing macrophage and B lymphocyte. It has been demonstrated that interferon- $\gamma$  (IFN- $\gamma$ ) is the strongest inhibitor of macrophage colony formation<sup>[1,2,6]</sup>. Therefore, LPS is thought to inhibit macrophage growth. Recent reports indicated that LPS at low concentration (< 0.1  $\mu$ g/L) had stimulatory effects in some hematopoietic cells<sup>[3,4]</sup>. So, the effects of LPS in cell growth regulation is multifunctional. Tissue macrophages migrated from peripheral monocytes are the main targets of LPS and participate in immune response by LPS activation. Besides, macrophages are known to be able to form colony under M-CSF and GM-CSF stimulations. In this study, LPS effects in macrophage proliferation was investigated.

## MATERIALS AND METHODS

### Animal and cell

Murine peritoneal exudate macrophages (PEM) in 6-8-week old CsH/HeJ mice were induced by thioglycollate medium J<sub>774</sub>A.1 was obtained from ATCC.

### Reagent

rMuG-M-CSF was obtained from Immunex Co, rHum-M-CSF as gift from Cetus Co, rHuTGF- $\beta_1$  from NCI, and LPS was purchased from List Co, and RT-PCR Kit and primers from Cetus Co.

### GM-CSF and M-CSF iodination

Bolton-Hunter method was used for GM-CSF iodination and chlormate T method for M-CSF iodination. Receptor binding assay was undertaken according to Reference 5.

### Macrophage colony formation (CFU-M)

PEM 1000/ml in a medium containing 10% FCS (Hyclone Co.) was cultured in 24-well plates at 5% CO<sub>2</sub> atmosphere and 37°C temperature. rMuGM-CSF (0.5  $\mu$ g/L), LPS (0.01  $\mu$ g/L - 100  $\mu$ g/L) or rHuTGF- $\beta_1$  (0.5  $\mu$ g/L) were added at different intervals. After 7-9 days, CFU-M (cell number > 50) was formed and calculated by a Coulter counter.

### RT-PCR

Reverse-transcription polymerase chain reaction (RT-PCR) was applied to examine cytokine mRNA expression. Experimental detail was referred to

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**Received** 1997-09-12 **Revised** 1998-01-22

Reference 5. The IFN- $\gamma$  and  $\beta$ -actin primers are as follows:

IFN- $\gamma$  5'-primer(71):TGAACGCTACACACTGCTTCTTGG  
3'-primer(530):CGACTCCCTTTTCCGCTTCCTAG

$\beta$ -actin 5'-primer(25):GTGGGCCGCTCTAGGCACCAA  
3'-primer(564):CTCTTTGATGTCACGCACGATTC

**RESULTS**

**LPS effects in murine macrophage growth**

PEM was potential to form CFU-M in vitro under optimal GM-CSF stimulation. Figure 1 shows that LPS at a concentration of lower than 1.0 $\mu$ g/L considerably inhibited macrophage growth and this inhibition was typically dose-dependent. However, with concomitant addition of rHuTGF- $\beta_1$  (0.5 $\mu$ g/L), LPS inhibition was reversed to be stimulatory while LPS and TGF- $\beta_1$  had no direct effects in CFU-M (Figure 2). Our previous paper reported that TGF- $\beta_1$  enhancement on GM-CSF stimulation was associated with the increase of GM-CSF receptor number<sup>[6]</sup>. Accordingly, LPS effect in GM-CSF receptor regulation was further examined.

**LPS effects in GM<sup>2</sup>CSF receptor number**

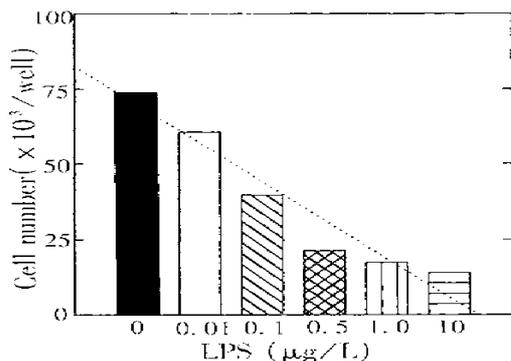
LPS could remarkably decrease M-CSF receptor number within a short time (37 $^{\circ}$ C, 1h), but without effect on GM-CSF. After LPS treatment in PEM was prolonged to 24 hours, GM-CSF receptor number was increased by over 80%, being time-dose dependent (Figure 3).

**LPS effects in murine macrophage cell line**

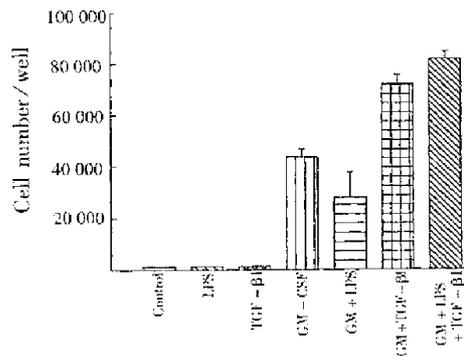
Although murine macrophage cell line J<sub>774</sub>A.1 autonomous growth was inhibited by LPS, its membrane GM-CSF receptor number was enhanced (Figure 4). These results confirmed the upregulation of LPS in GM-CSF receptor expression.

**The inhibition of IFN- $\gamma$  mRNA expression by TGF- $\beta_1$**

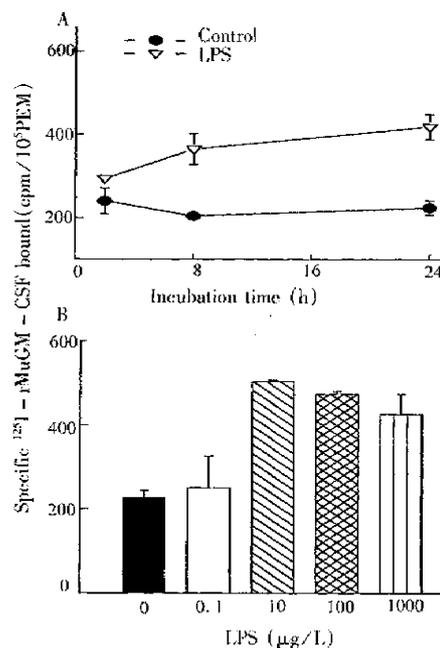
IFN- $\gamma$  mRNA expression in PEM was examined with (RT-PCR) (Figure 5). Consequently, IFN- $\gamma$  mRNA was expressed after LPS induction in PEM for 2 hours, which was totally suppressed by TGF- $\beta_1$  treatment for 2 hours.



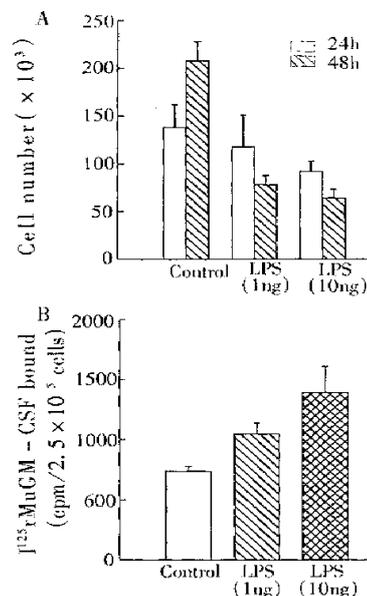
**Figure 1** Dose-dependence of LPS inhibitory effects in macrophage growth.



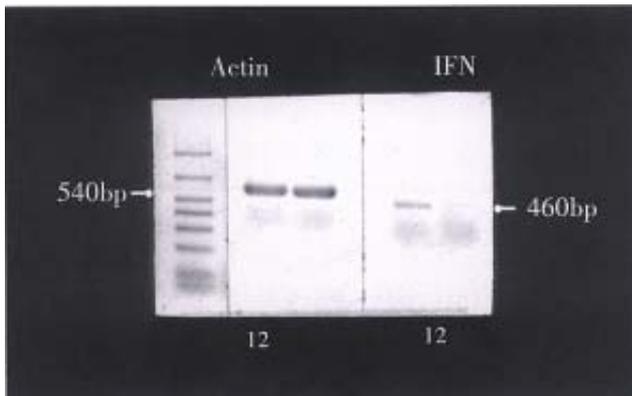
**Figure 2** Effects of LPS, TGF- $\beta_1$  and GM-CSF in macrophage growth in co-stimulation.



**Figure 3** Enhancement of GM-CSF receptor number by LPS. A: Time-dose of LPS at 5.0 $\mu$ g/L concentration; B: Effects of different doses of LPS on GM-CSF receptor number (37 $^{\circ}$ C, 24h).



**Figure 4** A: LPS inhibition in J<sub>774</sub>.1 autonomous growth. B: GM-CSF receptor number increased by LPS in J<sub>774</sub>.1.



**Figure 5** TGF- $\beta_1$  inhibition on IFN- $\gamma$  mRNA expression. Lane 1: LPS (1.0 $\mu$ g/L) induction for 2 hours. Lane 2: TGF- $\beta_1$  (1.0  $\mu$ g/L) treatment for 2 hours.

## DISCUSSION

Macrophages play an important role in a series of physiological and pathogenic processes, such as anti-infection, anti-tumor, immune response and inflammation. Macrophage growth regulation is modulated by multiple cytokines. Among them only GM-CSF and M-CSF can stimulate macrophage colony formation. Others (IL-4, TNF- $\alpha$ , L-3, IFN- $\gamma$ , TGF- $\beta_1$ ) influence macrophage growth by regulating M-CSF or GM-CSF receptor number and expression<sup>[4-7]</sup>. Bacterial lipopolysaccharide mainly activates macrophage and plays a certain role in its proliferation. LPS at in vivo concentration of 0.1 $\mu$ g/L-10 $\mu$ g/L, can inhibit GM-CSF stimulated macrophage growth but without direct effect. Interestingly, the addition of TGF- $\beta_1$  turns the

LPS effect from inhibitory to stimulatory. Receptor binding assay indicates that LPS and TGF- $\beta_1$  can both enhance GM-CSF receptor number, probably because of effects of the three modulators (GM-CSF, LPS and TGF- $\beta_1$ ) in macrophage growth. As IFN- $\gamma$  is induced by LPS and is the strongest autocrine inhibitor of macrophage growth. TGF- $\beta_1$  may suppress IFN- $\gamma$  expression in macrophage by RT-PCR technique. According to our results, a regulating network of LPS in macrophage proliferation is presumed. LPS is capable of increasing GM-CSF receptor number, but IFN- $\gamma$  displays stronger inhibition on macrophage growth. With the coexistence of TGF- $\beta_1$ , IFN- $\gamma$  expression is remarkably suppressed and GM-CSF receptor number is further enhanced. As a result, the concomitant presence of LPS, TGF- $\beta_1$  and GM-CSF stimulates macrophage proliferation. Our findings could lead to a conclusion that it is necessary to further reveal the complexity, complementarity and continuity of three or more cytokines in macrophage proliferation.

## REFERENCES

- 1 Rennick D, Yang G, Gemmell L, Lee F. Control of hemopoiesis by a bone marrow stromal cell clone: lipopolysaccharide and interleukin-1 inducible products of colony stimulating factor. *Blood*, 1987;69(2):682-691
- 2 Ding AH, Nathan CF. Trace level of bacterial lipopolysaccharide prevents interferon- $\alpha$  or tumor necrosis factor alpha from enhancing mouse peritoneal macrophage respiration burst capacity. *J Immunol*, 1987;139(6):1971-1977
- 3 Moore RN, Steel PS, Mannel DN, Mergenhagen SE. Role of lipopolysaccharide in regulating colony stimulating factor-dependent macrophage proliferation in vitro. *Infect Immun*, 1980;30(3):797-804
- 4 Yui S, Yamazaki M. Induction of macrophage growth by Lipids. *J Immunol*, 1986;136(2):334-339
- 5 Fan K, Ruan Q, Sensenbrenner L, Chen B. Upregulation of granulocyte-macrophage colony stimulating factor (GM-CSF) receptors in murine peritoneal exudate macrophages by both GM-CSF and IL-3. *J Immunol*, 1992;149(1):96-102
- 6 Chen BD, Chou TH, Ratanatharathorn V. Expression of gamma interferon receptor in murine bone-marrow derived macrophages associated with macrophage differentiation: evidence of gamma-interferon receptors in the regulation of macrophage proliferation. *J Cell Physiol*, 1987;133(2):313
- 7 Takway JP, DeFranco AL. Pertussin toxin inhibition of B cell and macrophage response to bacterial lipopolysaccharide. *Science*, 1986;234(6):743

# Evaluation of various solutions for small bowel graft preservation \*

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**Subject headings** intestine, small/transplantation; organ preservation; adenosine triphosphate; intestine, small/transplantation; Na (+)-K(+)-exchanging ATPase; adenine nucleotides

## Abstract

**AIM** To elucidate the effect of various solutions for small bowel graft preservation in pigs under hypothermic storage.

**METHODS** The swine segmental small bowel graft was autotransplanted after it was preserved with lactated Ringer's (LR), Euro-Collins (EC), hyperosmolarity citrate adenine (HC-A) and WMO-1 solutions for 10, 18 and 24 hours, respectively. The recipient survival rate, morphological structure, graft mucosal energy substances and Na + K<sup>+</sup> ATPase activity were studied, and graft absorption was estimated with D-xylose absorption test.

**RESULTS** The morphological study of the grafts preserved with LR or HC-A solution for 10 hours or with EC and WMO-1 solution for 18 hours was normal 6 days after operation. Mucosal ATP, total adenine nucleotides (TAN) contents and Na<sup>+</sup>-K<sup>+</sup>ATPase activity of the graft preserved with EC or WMO solution were higher than that of the graft preserved with LR or HC-A solution. Serum level of D-xylose was higher in EC and WMO-1 groups than in LR and HC-A groups when the graft was preserved for 24 hours.

**CONCLUSIONS** EC and WMO-1 solutions can preserve the swine small bowel up to 18 hours, which are superior to LR and HC-A solutions.

## INTRODUCTION

One of the problems accompanying successful clinical small bowel transplantation is the graft preservation, for the intestinal mucosa is probably the most sensitive tissue to ischemia in the body. The effective time of cold storage for small bowel graft (< 8 hours) is much shorter than that of other organs, and optimal preservation solution is not yet clearly defined for small bowel grafts. In this study, the swine small bowel was preserved with lactated Ringer's (LR), hyperosmolarity citrate adenine (HC-A), Euro-Collins (EC) and WMO-1 solutions to elucidate which is appropriate for swine small bowel preservation.

## MATERIALS AND METHODS

### Study design

Outbred pigs weighing 18.5kg-22.5kg were used. The pigs were fasted for 24 hours before operation, and general anesthesia was achieved with intravenous sodium pentobarbital.

Heterotopic segmental small bowel autotransplantation was performed as described previously<sup>[1]</sup>. The small bowel was sectioned before the ileocecal valve and mid-small intestine. The superior mesenteric artery of the graft was perfused with 400ml-500ml LR, EC, HC-A or WMO-1 solutions, and stored in the same solution at 4°C for 10, 18 and 24 hours, respectively. The perfusion pressure was 10.8kPa-11.8kPa, and perfusion time was 20 minutes. The lumen was perfused with metronidazole solution at 4h after preservation, and the small bowel graft was autotransplanted. Both ends of the grafts were brought out as stomas (Thiry-Vella loops). Intestinal continuity was restored with two end-to-end anastomoses. The experimental groups and preservation time are shown in Table 1.

### Recipient survival rate

The recipient survival rate on the 21st postoperative day (POD) was observed.

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Received 1997-10-28

**Table 1 Experimental groups and preservation time**

Groups	Preservation time		
	10 h	18 h	24 h
LR	5	5	6
EC	5	7	6
HC-A	6	5	5
WMO-1	5	6	5

### Mucosal morphology

Biopsy specimens obtained from the graft stomas, fixed in 10% neutral formalin and embedded in paraffin, and sections were cut at 4  $\mu\text{m}$  thickness, and stained with haematoxylin and eosin. All specimens were observed under light microscopy.

### Mucosal energy substances

Small bowel graft energy phosphates (ATP, ADP and AMP) were determined by high performance liquid chromatography (HPLC) as described previously<sup>[2]</sup>. The mucosa (weighing about 0.5g) was separated from the seromuscular wall and stored in liquid nitrogen. Atkinson energy charge (AEC) was calculated,  $AEC = (ATP + 0.5ADP) / (ATP + ADP + AMP)$ , total adenine nucleotides (TAN)  $ATP + ADP + AMP$ .

### Mucosal $\text{Na}^+ - \text{K}^+$ ATPase activity

Small intestinal mucosal  $\text{Na}^+ - \text{K}^+$  ATPase activity was determined using Zemelman's method<sup>[3]</sup>. The mucosa was separated and stored by the above-mentioned method.

### Absorptive function

Absorptive function of the graft was estimated with D-xylose absorption test. Five g D-xylose (dissolved in 100ml normal salt solution) was irrigated into the graft from the proximal stoma, and serum D-xylose level was determined by Eberts' method<sup>[4]</sup>.

### Statistical analysis

All results were expressed as  $\bar{x} \pm s$ , statistical variances were made by Student's *t* test or  $\chi^2$  analysis, differences were considered significant if  $P < 0.05$ .

## RESULTS

### Recipient survival rate

Six animals died because of failure in vascular anastomosis. Five animals were observed in each group. The survival rate on the 21st POD was similar in the graft preserved with each of the four solutions either for 10 or 18 hours. When the graft was preserved for 24 hours, the 21st POD survival rate was lower in LR group than in EC and WMO-1 groups ( $P < 0.05$ , Table 2).

### Morphological study

Epithelial necrosis and edema occurred by all the solutions. On the 3rd or the 4th POD, the grafts became normal in all groups histologically when the graft was preserved with each of four solutions for 10 hours 6 or 7 days after operation. When the graft was preserved for 18 or 24 hours, more severe tissue reperfusion injury was seen after revascularization in LR and HC-A groups than in EC and WMO-1 groups. On the 21st POD mucosa histological structure of the grafts preserved with EC and WMO-1 solutions for 18 hours were normal. Necrosis was pre-

sent in the graft preserved with LR and HC-A solutions. When the graft was preserved with each of the four solutions for 24 hours, the mucosal histological structure can not recover to normal.

### Mucosal energy substances

The concentration of phosphorylated adenine nucleotides was similar in the graft preserved with the four solutions for 10 hours. Cold storage significantly reduced the contents of ATP and TAN (Figures 1 and 2). Tissue contents of ATP and TAN were significantly higher in the graft preserved with EC and WHO-1 solutions after 24 hours of cold storage. The energy change as a parameter for the freely available energy in the cell was significantly higher in EC and WMO-1 groups ( $0.66 \text{ nmol/g} \pm 0.03 \text{ nmol/g wet weight}$ ,  $0.63 \text{ nmol/g} \pm 0.03 \text{ nmol/g wet weight}$ ) than in LR and HC-A groups ( $0.55 \text{ nmol/g} \pm 0.04 \text{ nmol/g wet weight}$ ,  $0.58 \text{ nmol/g} \pm 0.03 \text{ nmol/g wet weight}$ ).

### Mucosal $\text{Na}^+ - \text{K}^+$ ATPase activity

Mucosal  $\text{Na}^+ - \text{K}^+$  ATPase activity remained unchanged during cold storage despite storage time and preservation solution.  $\text{Na}^+ - \text{K}^+$  ATPase activity was decreased after 30 minutes of reperfusion (Table 3) in all the grafts. Mucosal  $\text{Na}^+ - \text{K}^+$  ATPase activity was significantly lower in LR and HC groups than in EC and WMO-1 groups after 24 hours of cold storage.

**Table 2 Recipient survival rate on the 21st POD (%)**

Groups	Preservation time		
	10 h	18 h	24 h
LR	100	60	0 <sup>a</sup>
EC	100	100	60
HC-A	100	80	20
WMO-1	100	100	60

<sup>a</sup> $P < 0.05$ , compared with EC and WMO-1 groups

**Table 3 Mucosal  $\text{Na}^+ - \text{K}^+$  ATPase activity after 30 min of reperfusion ( $\mu\text{mol Pi/mg protein} \cdot \text{h}$ ,  $\bar{x} \pm s$ )**

	Control	10 h	18 h	24 h
LR	15.15 $\pm$ 3.71	8.29 $\pm$ 2.89	5.79 $\pm$ 1.57	1.15 $\pm$ 0.47
EC	16.16 $\pm$ 2.59	9.23 $\pm$ 3.47	6.89 $\pm$ 2.62	4.27 $\pm$ 1.34 <sup>a</sup>
HC-A	14.74 $\pm$ 1.56	9.79 $\pm$ 2.15	5.79 $\pm$ 1.69	2.92 $\pm$ 1.39
WMO-1	13.49 $\pm$ 3.92	9.34 $\pm$ 1.29	6.03 $\pm$ 1.27	5.06 $\pm$ 1.27 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , compared with LR and HC-A groups

### Absorptive function

After 10 and 18 hours of cold storage, serum level of D-xylose was similar in all groups while after 24 hours it was higher in EC and WMO-1 groups than in LR and HC-A groups (Figure 3).

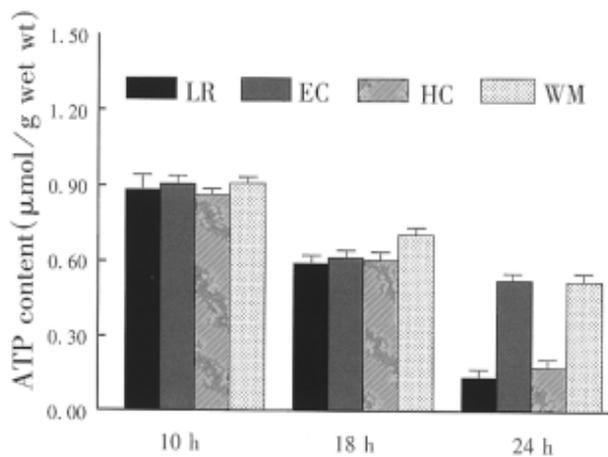


Figure 1 Mucosal concentration of ATP.

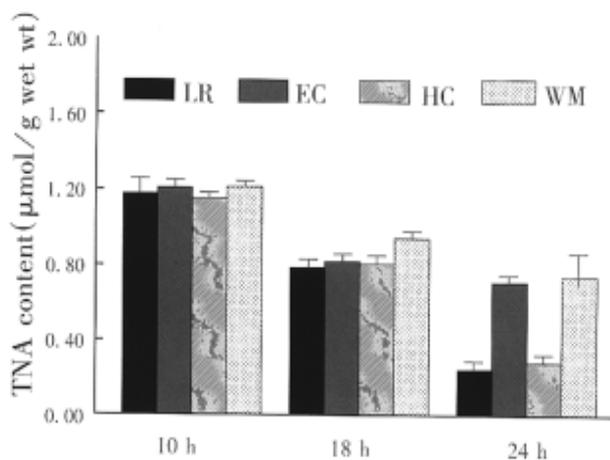


Figure 2 Mucosal concentration of TNA.

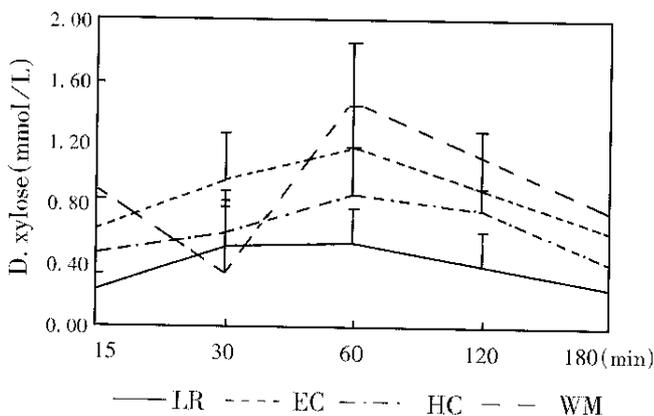


Figure 3 Results of D-xylose absorption test after 24 hours of cold storage.

### DISCUSSION

Simple cold storage is the most practical method in organ preservation. Several solutions have been used in organ preservation, however, the optimal one is not clearly defined for small bowel grafts. A recently developed organ preservation solution, UW (University of Wisconsin) has provided 72 hours preservation for pancreas and kidney, and 30 hours or longer for the liver, but provided only 8-10 hours preservation for small bowel in human<sup>[5]</sup>.

LR solution, an extracellular fluid, is widely used in experimental small bowel preservation. Luther *et al*<sup>[6]</sup> and Zhang *et al*<sup>[7]</sup> reported that LR solution is superior to UW and EC solutions for small bowel storage in the rat model. In our studies, no animal receiving the graft preserved for 24 hours survived more than 21 days. The recipient survival rate in EC and WMO-1 groups was 60%, respectively. Mucosal contents of ATP and TAN, and Na<sup>+</sup>-K<sup>+</sup> ATPase activity in EC and WMO-1 groups were higher than in LR and HC-A groups after 24 hours of cold storage. Our results indicated that EC and WMO-1 solutions are superior to LR solution for small bowel preservation in the pig model.

HC-A solution, a modified Ross solution, contains adenine, precursor for ATP production, and its osmolarity was reduced from 400 to 380mOsm/L. The solution has provided 48 hours or longer preservation for the cadaveric kidneys. In our study, mucosal contents of ATP and TAN and Na<sup>+</sup>-K<sup>+</sup> ATPase activity in HC-A preserved small bowel was not higher than in EC and WMO-1 preserved one, so adenine added into the HC-A solution could neither improve the ATP production nor inhibit the tissue ATP degradation.

WMO-1 solution, containing ATP and Ca<sup>2+</sup> could provide 48 hours preservation for the liver in the rat model. It is believed that ATP in WMO-1 solution could increase the energy state of tissue and prevent ATP degradation and the calcium could stabilize the membranes. Our study indicated that mucosal contents of ATP and TAN were not higher in WMO-1 preserved small bowel than in EC-preserved one. Mucosal Na<sup>+</sup>-K<sup>+</sup> ATPase activity was similar in the two groups, therefore, ATP added into WMO-1 solution could not prevent ATP degradation in tissues. It was not introduced in latest-developed organ preservation solution, such as UW. In the healthy cells, cytosolic calcium is at very low level (10<sup>-7</sup>mmol/L), and hypothermic preservation may disrupt the cell membrane, resulting in influx of calcium into the cell. Elevated intracellular calcium was also shown to potentiate cell damage<sup>[8]</sup>, therefore the effects of ATP and Ca<sup>2+</sup> in the WMO-1 solution should be studied.

As shown previously, the EC solution is a standard organ preservation solution and has been widely used in liver, kidney, pancreas and small bowel

transplantations<sup>[5]</sup>. In this study, EC solution is superior to HC-A and LR solutions, and mucosal contents of ATP and TAN and Na<sup>+</sup>-K<sup>+</sup> ATPase activity were higher in EC group than in LR and HC-A groups and were similar to that of WMO-1 group.

Recently, a direct correlation between ATP content during cold preservation and outcome in animal and clinical organ transplantation has been reported<sup>[9]</sup>. Na<sup>+</sup>-K<sup>+</sup> ATPase is an important membrane-bound enzyme that acts by active extrusion of Na<sup>+</sup> from the intracellular compartment to counterbalance the osmotic effect of impermeable intracellular macromolecules. We investigated the effect of four solutions in recipient survival rate, mucosal energy substances, Na<sup>+</sup>-K<sup>+</sup> ATPase activity and absorption of the graft. The results showed that Na<sup>+</sup>-K<sup>+</sup> ATPase activity maintained unchanged during cold storage and reduced significantly after reperfusion but the contents of ATP and TAN decreased during cold storage. There was a direct relationship between contents of ATP and TAN after cold storage and Na<sup>+</sup>-K<sup>+</sup> ATPase activity after reperfusion and recipient outcome after transplantation. EC and WMO-1 solutions were superior to LR and HC-A solutions for small bowel graft.

In conclusion, the swine small bowel can be

successfully preserved in good condition for 18 hours using simple cold storage in EC and WMO-1 solutions as assessed by the recipient survival rate, mucosal energy substances, Na<sup>+</sup>-K<sup>+</sup> ATPase activity, and D-xylose absorption test and the EC and WMO-1 solutions are superior to LR and HC-A solutions for preservation of small bowel in the pig model.

## REFERENCES

- 1 Li N, Li JS, Liao CX, Li YS, Wu XH. Segmental small bowel allotransplantation in pigs. *Chin J Organ Transplant*, 1993;14(1):2-4
- 2 Schweinsberg PD, Loo TL. Simultaneous analysis of ATP, ADP, AMP and other purines in human erythrocyte by high-performance liquid chromatography. *J Chrom*, 1980;18(1):103-107
- 3 Zelman BV, Alker A, Chu SW. Expression and development regulation of Na<sup>+</sup>-K<sup>+</sup> adenine triphosphates in the small intestine. *J Clin Invest*, 1992;90(3):1016-1022
- 4 Eberts JL, Sample KHB, Cilick MR, Ellis GM. A simplified colorimetric micro-method for xylose in serum or urine with pholoroglucind. *Clin Chem*, 1979;25(8):1440-1443
- 5 Tolodo-pereyra LH. Small bowel preservation, evolution and ideas, and current concepts. *Transplant Proc*, 1992;24(3):1083-1084
- 6 Luther B, Lehman C, David H, Klinnert J. Preservation of isolated intestinal segment using the University of Wisconsin solution. *Transplant Proc*, 1991;23(5):2459
- 7 Zhang S, Koluda Y, Neato Em. Biochemical evidence on mucosal damage of intestinal graft during cold preservation in University of Wisconsin, Euro-collins and lactated Ringer's solution. *Transplant Proc*, 1994;24(6):1087-1088
- 8 Clavien PA, Harvey PRC, Strasberg SM. Preservation and reperfusion injury in liver allografts. *Transplantation*, 1992;53(5):957-978
- 9 Harvey PRC, In S, Mckeown CMB, Petranka CN, Ilson RG, Strasberg SM. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. *Transplantation*, 1988;45(6):1016-1020

# Experimental research on production and uptake sites of TNF $\alpha$ in rats with acute hemorrhagic necrotic pancreatitis \*

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**Subject headings** tumor necrosis factor/blood; endotoxins/blood; pancreatitis/blood; acute diseases; disease models, animal; enzyme-linked immunosorbent assay; pancreas/pathology; necrosis

## Abstract

**AIM** To determine the site of production and uptake of tumor necrotic factor alpha (TNF $\alpha$ ), and evaluate the relationship between serum TNF $\alpha$  and plasma endotoxin (ET) in rats with acute hemorrhagic necrotic pancreatitis (AHNP).

**METHODS** Sprague Dawley rats were divided into AHNP group and control group ( $n = 12$ ). AHNP model was induced by retrograde injection of 5% sodium taurocholate via pancreatic bile duct. The blood samples were obtained through portal vein 2 and 6 hours after the operation.

**RESULTS** The contents of TNF $\alpha$  in portal vein were increased rapidly in the development of AHNP. They were lower in hepatic vein ( $280.59 \pm 20.02$ ) and femoral artery ( $310.82 \pm 7.97$ ) than in portal vein ( $354.91 \pm 25.50$ ) ( $P < 0.05$ ), and higher in femoral artery than in hepatic vein 6 hours after the operation ( $P < 0.05$ ). TNF $\alpha$  level in plasma was increased significantly when ET level in portal vein showed no increase.

**CONCLUSION** Pancreas, spleen, liver, intestinal tract and lung are the main organs to produce TNF $\alpha$ , and liver is also an important site for TNF $\alpha$  uptake in the development of AHNP. Plasma endotoxin is not a trigger for TNF $\alpha$  release in rats with AHNP.

## INTRODUCTION

Recent studies have indicated that during the pathogenesis of acute hemorrhagic necrotic pancreatitis (AHNP), tumor necrotic factor alpha (TNF $\alpha$ ) and endotoxemia play important roles in the progression of pancreatic inflammation and the damage of exopancreatic vital organs. The purpose of this paper is to determine the production and uptake sites of TNF $\alpha$ , and the relationship between TNF $\alpha$  and plasma endotoxin (ET) in rats with AHNP, thus providing a direct theoretical basis for the treatment of AHNP by blocking the production of TNF $\alpha$  and clearing the secreted TNF $\alpha$  in plasma.

## MATERIALS AND METHODS

### Animals and grouping

Twenty-four Sprague-Dawley (SD) rats weighing 295g-320g were divided randomly into AHNP group and control group (12 animals for each group).

### Experimental design

SD rats were fasted for 24 hours, and were anesthetized by intraperitoneal injection of sodium pentobarbital (30mg/kg). Normal saline (0.9%) was injected into each rat through a PE-50 placed catheter in femoral vein. Sodium taurocholate (5% 1.5ml/kg) were infused retrogradely to AHNP rats through pancreatic bile duct under a microsurgical procedure, after 5min - 10min, pancreatic edema and dotted bleeding occurred. Isovolumic of 0.9% normal solution was infused to the pancreatic bile duct of control rats using the same method, and no abnormality was noticed 2 and 6 hours after the operation. Blood samples from hepatic vein, portal vein and femoral artery were harvested, at these time points, hemorrhage, necrosis of pancreas and hydremic ascites (5ml-10ml) were found in AHNP-rats, while no abnormality was seen in the control rats.

### Determination of serum TNF $\alpha$ .

TNF $\alpha$  in portal vein, hepatic vein and femoral artery were determined with ELISA (provided by the Department of Immunology, the Third Military Medical University). ET in portal vein was assayed by a quantitative azostromatic coloration limulus test (kit provided by the Shanghai Medical Analysis Institute).

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\*Project supported by the China Postdoctoral Sciences Foundation, No.C.P.S.F. 1996.2#.

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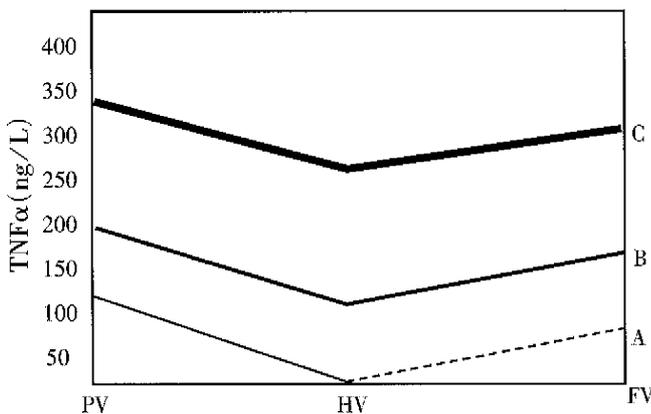
Received 1997-12-05 Revised 1997-12-30

## RESULTS

Change of serum TNF $\alpha$  levels (Table 1 and Figure 1)**Table 1** TNF $\alpha$  level ( $\bar{x}\pm s_{\bar{x}}$ , pg/L) of rats 2h and 6h after operation

Groups	Time(h)	PV	HV	RHU(%)	FA
Control	2	54.83 $\pm$ 3.24			
	6	52.14 $\pm$ 1.87			
AHNP	2	182.48 $\pm$ 9.90	119.85 $\pm$ 5.64	38.63	127.32 $\pm$ 7.15
	6	354.91 $\pm$ 25.50	280.59 $\pm$ 20.02	20.95	310.82 $\pm$ 7.97

PV: portal vein; HV: hepatic vein; RHU: rate of hepatic uptake; FA: femoral artery.



**Figure 1** Changes of TNF $\alpha$  level in portal vein, hepatic vein and femoral artery 2 and 6 h after operation. A: TNF $\alpha$  level of control group 6 h after operation; B, C: TNF $\alpha$  level of AHNP group 2 and 6 h after operation.

There was significant difference in TNF $\alpha$  levels among the samples from portal vein, hepatic vein and femoral artery ( $P<0.05$ ) 2 and 6 hours after operation, and the difference was also significant as compared with the control group ( $P<0.05$ ). Two hours after operation, TNF $\alpha$  level in hepatic vein and femoral artery was lower than in portal vein ( $P<0.05$ ), but no difference was observed between that in femoral artery and hepatic vein. Six hours after operation, TNF $\alpha$  level in femoral artery was obviously higher than in hepatic vein ( $P<0.05$ ), but remained lower than in portal vein ( $P<0.05$ ). There was significant difference in the rate of hepatic uptake of TNF $\alpha$  between AHNP group and control group ( $P<0.05$ ).

## Change of plasma ET level (Table 2)

**Table 2** Change of plasma ET level ( $\bar{x}\pm s_{\bar{x}}$ , EU/ml)

Groups	Time(h)	Plasma ET
Control	2	0.022 $\pm$ 0.007
	6	0.033 $\pm$ 0.006
AHNP	2	0.028 $\pm$ 0.002
	6	0.340 $\pm$ 0.038

Two and 6 hours after operation, there was significant difference in plasma ET level in portal vein of AHNP rats. When compared with the control group, significant difference of plasma ET level in portal vein of AHNP rats was observed at 6 hours, but not at 2 hours after operation.

## DISCUSSION

TNF $\alpha$  is an important inflammatory mediator during the development of AHNP. It can damage the pancreas and ex-pancreatic vital organ through the following mechanism: ① Enhancing the adhesion of PMN to endothelium. Recent research shows that there are some adhesive molecules or receptors, such as ICAM-1, ELAM-1 and VCAM-1 on the surface endothelia. TNF $\alpha$  can stimulate the expression of receptors for adhesive molecules on the surface of endothelium, leading to the adhesion of unstimulated PMN to endothelia and the migration of the PMN to inflammatory loci through vascular barrier. The interaction of PMN with target tissue cells and the releasing of oxygen free radicals (OFR) and neutral proteinase could cause the damage of adjacent tissues. ② The cytotoxic effect of TNF $\alpha$ . TNF $\alpha$  can bind with the specific receptors on the cellular membrane and facilitate the production of OFR, which damages the cell membrane and DNA, and cleaves DNA into fragments by endonuclease, leading to the death of cells. ③ TNF $\alpha$  can activate the inflammatory cells, stimulate the release of PMN from bone marrow, and promote the degranulation, the production of OFR and the releasing of lysosome enzyme, alkali proteinase, etc. from eosinocytes. The persistent increase of plasma TNF $\alpha$  may promote the endocytosis, degranulation and the releasing of OFR and leukocyte enzymes (lysosome enzyme, plastic enzyme, etc.), resulting in the continuous hemorrhage, necrosis of pancreas and the development of MOF.

The exact mechanism of production, the production and absorption site of TNF $\alpha$  during the progression of AHNP remained unknown<sup>[2]</sup>. The stimulation of inflammatory cells by pancreatic enzyme was believed to be related with the production of TNF $\alpha$ . It is indicated that the disturbance of microcirculation of pancreas may lead to release of trypsinogen activated peptides<sup>[3]</sup>, suggesting that the disturbance of microcirculation of pancreas may be the primary factor responsible for production of TNF $\alpha$ . Our results revealed that, at the early stage of AHNP, the TNF $\alpha$  level in portal vein was elevated obviously, which was significantly higher than in hepatic vein and femoral artery. There was no difference in TNF $\alpha$  level between hepatic vein and femoral artery. So it is concluded that at the early stage of AHNP, TNF $\alpha$  in portal vein may originate from pancreas, spleen and gastrointestinal tract, and the liver may clear some of TNF $\alpha$  originated from portal vein. With the aggravation of AHNP,

TNF $\alpha$  in portal vein increased rapidly, and so did that in femoral artery, both being higher than in hepatic vein, but still lower than in portal vein. Based on these results, we believe that lung may be the organ of secondary production site of TNF $\alpha$ . TNF $\alpha$  in hepatic vein mainly originate from pancreas, spleen and gastrointestinal tract, and part of TNF $\alpha$  in hepatic vein might come from the liver. Although the liver could clear some TNF $\alpha$ , its potential is somewhat limited. As the hepatic damage gets worse, its clearance to TNF $\alpha$  decreases.

TNF $\alpha$  is a soluble polypeptide with an MW of 17Ku, under the condition of severe infection, macrophagocytes, T cells and NK cells would produce TNF $\alpha$  due to the action of Gram negative bacteria and lipopolysaccharide. It is believed that at early stage of AHNP, the production of TNF $\alpha$  is not associated with ET<sup>[4]</sup>. Our results showed that plasma TNF $\alpha$  level elevated significantly when there was no increase of ET level in portal vein, indicating that ET was not the primary factor for the produc-

tion of TNF $\alpha$  in AHNP rats, but TNF $\alpha$  level elevated much more significantly with the increase of ET. Recent studies also showed that, in late-stage AHNP, ET was contributed to the substantially increased production of TNF $\alpha$ . The synergetic effect of TNF $\alpha$  and ET is an important cause of high morbidity and mortality of AHNP. Therefore, control of infection, inhibition of the TNF $\alpha$  production, and removal of secreted TNF $\alpha$  are of great importance for the treatment of AHNP.

## REFERENCES

- 1 Hughes CB, Grewal HP, Gaber LP, Kotb M, El-din M, Mann M *et al*. Anti-TNF $\alpha$  therapy improves survival and ameliorates the pathophysiologic sequelae in acute pancreatitis in the rat. *Am J Surg*, 1994;171(1):274-278
- 2 Grewal HP, Mohy EL, Din A, Kotb M. Induction of tumor necrosis factor in severe acute pancreatitis and its subsequent reduction after hepatic passage. *Surgery*, 1994;115(1):213-221
- 3 Mothofer K, Castillo CFD, Frich TW, Foitzik T, Bassi DG, Lewandrowski KB, *et al*. Increased intrapancreatic trypsinogen active ischemia induced experimental pancreatitis. *Ann Surg*, 1995;221(4):364-371
- 4 Hughes CL, Gaber LW, Kotb M, Mohey el din AB, Pabst M, Gaber O. Induction of acute pancreatitis in germ free rats: evidence of a primary role for tumor necrosis factor alpha. *Surgery*, 1995;117(1):201-205

# A clinical evaluation of serological diagnosis for pancreatic cancer \*

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**Subject headings** pancreatic neoplasms/diagnosis; tumor markers, biological; antigens, neoplasm/analysis; CA-19-9 antigen/analysis; pancreatopeptidase/analysis; carcinoembryonic antigen/analysis; alpha 1-antitrypsin/analysis; enzyme-linked immunosorbent assay; radioimmunoassay

## Abstract

**AIM** To assess the diagnostic values of tumor markers for pancreatic cancer.

**METHODS** Pancreatic cancer-associated antigen from colonic mucosa (PCAAC), pancreas-specific antigen (PaA), pancreatic oncofetal antigen (POA) and minimolecular pancreatic antigen (mPOA) were detected by double antibodies Sandwich ELISA; CA19-9, elastase 1 (E1), human pancreatic elastase 1 (HPE1) and carcinoembryonic antigen (CEA) by radioimmunoassay (RIA); general activities of ribo-nuclease (RNase) and its isoenzymes (RNase I and RNase II) by biochemistry and PAEG; glycyproline dipeptidyl aminopeptidase (GPDA) by biochemistry and  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) by rocket immunoelectrophoresis (rocket-IE).

**RESULTS** The detection of serum POA, mPOA, PaA, PCAAC, CA19-9, RNase and RNase I was able to differentiate pancreatic cancer from the benign disorders and non-pancreatic malignancies with a sensitivity from 66.75% to 80.0% and a specificity from 88.5% to 96.69%. POA, mPOA, PCAAC, HPE1, E1 and GPDA were related to the pancreatic cancer at the head which demonstrated higher sensitivity from 63.64% to 85.71%. The detection of serum HPE1 was especially helpful for the diagnosis of pancreatic cancer with smaller diameters. The determination of 3 or 4 kinds of tumor markers simultaneously would increase the detection rate of pancreatic cancer, which will be an important procedure for the diagnosis of this malignancy.<sup>2,3</sup>

**CONCLUSION** A single test of tumor markers is helpful to detect pancreatic cancer clinically, but the determination of 3 or 4 kinds of tumor markers simultaneously would significantly increase the detection rate of pancreatic cancer, which will be an important procedure for the diagnosis of this malignancy.

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\*Project supported by "8th 5-year" Major Scientific Research Foundation of Chinese PLA.

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Received 1997-12-05 Revised 1997-12-30

## INTRODUCTION

The incidence of pancreatic cancer is increasing yearly, but its early diagnosis remains very difficult, for the symptoms are faint, vague and nonspecific. Therefore, it is important to seek a tumor marker with satisfactory specificity and/or sensitivity for the diagnosis of the cancer clinically. This paper reports our ten years' research on tumor markers to evaluate the clinical application of the serum detection of tumor markers for the diagnosis of pancreatic cancer.

## MATERIALS AND METHODS

### Samples

We detected 13 tumor markers in samples from 763 cases including 143 pancreatic cancer, 125 liver or peri-ampullar carcinoma and 120 gastrointestinal carcinoma, which were identified by surgical operation and/or pathological examination. One hundred and five cases of acute and chronic pancreatitis were observed. The acute cases were identified by clinical approach and chronic ones by X-ray photograph, surgical operation and pathological examination. Blood samples from 270 healthy fasting donors were examined likewise and served as control. Fasting blood samples were obtained through venous puncture in the morning and the serum was preserved at  $-20^{\circ}\text{C}$  for assay.

### Methods

Pancreatic cancer-associated antigen from colon mucosa (PCAAC), pancreas-specific antigen (PaA), pancreatic oncofetal antigen (POA) and minimolecular pancreatic antigen (mPOA) was detected by double antibodies Sandwich ELISA<sup>[1-4]</sup>; CA19-9, elastase 1 (E1), human pancreatic elastase 1 (HPE1) and carcinoembryonic antigen (CEA) by radioimmunoassay (RIA); general activities of ribonuclease (RNase) and its isoenzymes (RNase I and RNase II) by biochemistry and PAEG<sup>[5]</sup>; glycyproline dipeptidyl aminopeptidase (GPDA) by biochemistry according to Kojima's report<sup>[6]</sup> and  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) by rocket immunoelectrophoresis (rocket-IE).

### Statistical analysis

The data of distribution percentage, *t* test, FLSD test, stepwise regression and discriminatory analysis of the tumor markers were processed on a micro-computer with a medical program system, SPMR, designed by the department of mathematics of our college.

**RESULTS**

**Single assay of the tumor markers**

Circulating levels of the tumor markers in patients with pancreatic cancer as well as those with benign pancreatic diseases, non-pancreatic malignancies and normal control subjects are shown in Tables 1 and 2. All the tumor markers except for RNase 2 and CEA were elevated in the sera of patients with pancreatic cancer and exhibited a sensitivity ranging from 45% to 80.8% and a specificity from 66.67% to 97.14%.

**Tumor markers related to location, size and differentiation of pancreatic cancer**

As shown in Table 3, the detection of POA, mPOA, PCAAc, E1, HPE1 and GPDA demonstrated that the positive percentage ranged from 63.64% to 85.73% in the patients with pancreatic cancer at the head, which was significantly higher than that at the body or tail and entire. The detection of PaA and CA19-9 could be better for the cancer at tail and/or body. It is worth-mentioning that higher positivity of the detection with HPE1 was found in the patients with the tumor smaller than 5.0 cm. The elevation of serum mPOA, PCAAc, PaA, HPE1, E1 and GPDA was closely related to well-differentiated cancer.

**Simultaneous detection of tumor markers**

Although single assay of tumor markers distinguishes pancreatic cancer from the other groups, the conjoint detection could improve the diagnostic positivity and specificity for the cancer clinically. As shown in Table 4, four tumor markers, including PCAAc, mPOA, PaA and GPDA were selected for this purpose, and the results showed a positivity of 40.6%-80.8% for the diagnosis of pancreatic cancer by single test, 75%-96.77% by conjoint detection with any two markers and 100% by any three markers.

**Table 1 Sensitivity and specificity of a single test of tumor markers for pancreatic cancer**

Groups	Sensitivity (%)	Specificity (%)
POA	67.80	88.50
mPOA	74.36	95.6
PCAAc	68.70	96.49
PaA	78.79	89.40
HPE1	66.67	83.25
E1	62.50	66.67
CA19-9	70.97	90.63
GPDA	42.12	68.42
RNase	80.80	80.00
RNase I	45.00	94.28
RNase II	25.00	97.14
α1AT	50.00	74.28
CEA	25.00	85.71

**Table 2 Results of single test of tumor markers**

Groups	Normal control	Pancreatic cancer	Non-pancreatic malignancy	Pancreatitis
POA(U/ml)	84.80±13.96(1/40)	252.5±164.33(19/28)	136.37±93.63(9/62)	101.98±57.88(1/15)
mPOA(mg/L)	4.88±1.96(2/40)	18.45±10.57(29/39)	6.24±3.82(5/62)	4.69±1.52(0/17)
PCAAc(mg/L)	12.59±6.34(1/40)	57.25±82.93(23/33)	12.64±5.14(3/49)	13.53±9.91(0/14)
PaA(μg/L)	13.05±3.84(3/64)	61.54±45.59(26/33)	17.62±10.31(5/44)	21.35±11.83(3/20)
HPE1(μg/L)	23.80±3.40(1/82)	83.30±73.40(62/93)	48.93±54.63(26/63)	124.90±168.30(25/35)
E1(ng/dl)	276.60±85.36(0/10)	1116.00±1281.00(10/16)	1321.00±2842.00(9/13)	
CA19-9(U/L)	7.90±9.74(2/81)	165.40±128.03(19/31)	139.88±121.72(6/15)	
GPDA(U/L)	54.84±10.80(0/60)	101.02±75.84(14/33)	64.89±72.64(27/69)	50.94±15.50(2/25)
RNase(U/ml)	58.19±11.09(1/30)	92.22±19.66(16/20)	65.88±12.32(5/20)	67.95±9.77(2/15)
RNase I(U/ml)	9.53±13.43(1/30)	39.09±39.31(9/20)	9.23±8.98(1/20)	11.87±11.30(1/15)
RNase II(U/ml)	50.75±13.36(0/30)	52.78±29.68(5/20)	56.89±16.82(0/20)	60.17±19.55(1/15)
α1AT(mg/L)	30.51±6.35(1/30)	49.86±18.47(10/20)	38.65±16.30(6/20)	39.23±10.02(3/15)
CEA(mg/L)	11.15±2.95(1/20)	16.82±10.26(5/20)	15.73±11.98(5/20)	10.22±3.42(0/15)

**Table 3 The relationship between the tests of tumor markers and the location, size or differentiation of pancreatic cancer**

Groups	Location			Size			Differentiation	
	Head	Body or tail	Entire	<5.0 cm	5 cm-9.0 cm	>9.0cm	Well & moderate	Poor & undifferentiated
POA	9/11(81.82)	3/6(50.00)	1/3(33.33)	4/8(50.00)	8/10(80.00)	1/2(50.00)	10/10(100.00)	9/13(69.23)
mPOA	22/27(81.48)	5/9(55.56)	2/3(66.67)	5/5(100.00)	14/19(73.68)	10/15(66.67)	10/10(100.00)	9/13(69.23)
PCAAc	17/22(77.27)	4/9(44.44)	1/2(50.55)	3/4(75.00)	9/13(69.23)	3/6(50.00)	5/5(100.00)	2/4(50.00)
PaA	16/22(72.73)	10/11(90.91)	2/2(100.00)	4/4(100.00)	12/15(80.00)	6/10(60.00)	8/8(100.00)	4/5(80.00)
E1	14/17(82.35)	2/6(33.33)	2/3(66.67)	6/8(75.00)	4/10(40.00)	1/2(50.00)	3/6(50.00)	2/4(50.00)
HPE1	35/41(85.37)	20/35(57.14)	7/17(41.18)	40/51(78.43)	15/25(60.00)	7/17(41.18)	43/60(71.67)	19/33(57.58)
GPDA	14/22(63.64)	0/9(0.00)	0/2(0.00)	8/17(47.06)	3/7(42.86)	3/7(42.86)	4/7(57.14)	1/4(25.00)
RNase	31/41(75.61)	24/35(68.23)	15/17(88.23)	20/29(68.96)	19/25(76.00)	15/17(88.24)	46/60(76.67)	24/33(72.72)
CA19-9	10/17(58.82)	6/8(75.00)	6/6(100.00)	7/15(46.67)	9/12(75.00)	4/4(100.00)	8/9(88.89)	8/8(100.00)

**Table 4 Results of simultaneous detection of four tumor markers**

Groups	Pancreatic cancer (n=30)	Non-pancreatic malignancy (n=43)	Benign diseases (n=21)
All the tests negative	0(0.00)	17(39.54)	18(85.71)
Single test positive	4(12.90)	21(48.83)	3(14.29)
Double tests positive	6(19.36)	5(11.63)	0(0.00)
Three tests positive	16(51.61)	0(0.00)	0(0.00)
All the tests positive	5(16.13)	0(0.00)	0(0.00)

## DISCUSSION

### Single assay significance of tumor markers for pancreatic cancer diagnosis

A single assay of tumor markers was helpful in distinguishing patients with pancreatic cancer from normal controls and those with non-pancreatic malignancy. By stepwise discriminatory analysis, seven out of all 13 tumor markers including CA19-9, POA, mPOA, PaA, PCAAc, RNase and RNase I were associated well with the diagnosis of pancreatic cancer with the sensitivities ranging from 45% to 80.8% and the specificity from 80% to 96.49%. Although the detection of HPE1 and E1 had the sensitivities of 66.67% and 62.5% to the disease, these were regarded as helpful diagnostic indices for the poor differentiation value. A single test of GPDA,  $\alpha$ 1AT, CEA and RNase II was not significant to the diagnosis of the cancer for their unsatisfied sensitivity and/or specificity.

Although the mechanism of the tumor markers expressed in blood was not clear, the problem of false positive rate was noticed in clinic; especially to some carcinoma adjacent to pancreatic cancer such as primary hepatic carcinoma, cholangioampulla carcinoma, etc.<sup>[7,8]</sup>. In our study, the false positive rates ranging from 8.33% to 21.74% was found in patients with primary hepatic carcinoma or cholangioampulla carcinoma with a single test of POA, mPOA, PCAAc, PaA and RNase I, and 36.92% to 87.09% with CA19-9, HPE1, E1 and GPDA. These suggest that a single test of tumor markers has limitations for making a definite diagnosis of pancreatic cancer unless it is combined with other detection, especially the image analysis.

### Correlation of tumor markers to location, size or differentiation of cancer

The higher positive rates in detecting POA, PCAAc, HPE1, E1 or GPDA were found in patients with pancreatic head cancer, with the sensitivities of 63.64%-85.71%. It is interesting that GPDA were only elevated in the sera of patients with the cancer at pancreatic head but not at body or/and tail. The mechanism of elevated GPDA might be related to the obstruction of bile duct. Unfortunately, none of the 13 tumor markers were significantly related to the cancer at body or/and tail.

According to our studies, the sensitivities of CA19-9, RNase, GPDA and POA detection were gradually increased with the development of the mass, which were 46.67%-50% in the patients with the tumor size less than 5.0cm. Although ele-

vated serum mPOA and PCAAc were associated with the patients with the tumor size less than 5.0cm, the methods per se for correct diagnosis of pancreatic cancer in the early stage still need further studies. It is worth-mentioning that the detection of serum HPE1 was significantly related to the tumors less than 5.0cm. In this study, significantly higher serum HPE1 was observed in 22 of 51 cases with the tumor size less than 3.0cm, among them the concentration was  $118.1 \mu\text{g/L} \pm 102.7 \mu\text{g/L}$  and the positive rate was 78.46%. Furthermore, 4 of 6 cases with the tumor size less than 2.0cm also had elevated serum HPE1. This suggests that serum HPE1 might be a reference tumor marker helpful for the diagnosis of patients in the early stage.

### Correlation of tumor markers with differentiation of cancer

The serum variation of some tumor markers such as mPOA, PCAAc, PaA, HPE1 and GPDA were related to the differentiation degree of the tumors. Our results showed that positive rate and elevation range in highly and moderately differentiated pancreatic cancer were higher than low and non-differentiated one, which suggested that these tumor markers can be regarded as differentiating antigen for assessment of the transformation of the disease.

### Significance of combined assay of tumor markers for pancreatic cancer

Combined detection of tumor markers would increase and improve the detection rate and the diagnostic accuracy of the malignancy. According to our data, the sensitivity to the diagnosis of the disease was 25%-80.0% with single test of the 13 tumor markers, 75%-96.77% with two tests, and 100% with over 3 tests. The combined tests with simultaneous positive results of 2 out of the 13 tumor markers could be used to rule out pancreatic benign disease. Non-pancreatic malignancies never showed positive results of over three tumor markers simultaneously. Our results suggest that determination of 3 or 4 tumor markers conjointly would increase the detection rate of pancreatic cancer, especially for screening among large populations. It might become an important procedure for the diagnosis of this malignancy.

## REFERENCES

- Guo XZ, Yu SY, Guo P, Dai XJ, Liu WW. The value of measurement of serum pancreatic oncofetal antigen in the diagnosis of pancreatic carcinoma. *Chin J Dig*. 1989;9(2):88-91
- Zhao XY, Yu SY, Guo P, Bai L. Preliminary study of the diagnosis of pancreatic cancer with a pancreatic associated antigen. *J Med Coll PLA*. 1995;10(3):162-165
- Zhao XY, Yu SY, Bai L, Guo P. Diagnosis of pancreatic cancer with simultaneous detection of a pancreatic cancer-associated antigen and a pancreas-specific antigen. *Chin J Intern Med*. 1990;29(10):612-615
- Dai XJ, Yu SY, Guo P, Guo XZ. A preliminary study on serum pancreatic elastase I for the diagnosis of pancreatic cancer. *Chin J Dig*. 1989;9(5):299-300
- Wang YM, Yu SY, Guo P. A preliminary study on serum ribonuclease and isoenzyme for the diagnosis of pancreatic cancer. *Chin J Dig*. 1988;8(3):134-137
- Kojima J, Kanatani M, Nakamura N, Kashiwagi T, Tohjoh F, Akiyama M. Serum and liver glycylprinine dipeptidyl amino peptidase activity in rats with experimental hepatic cancer. *Chin Acta*. 1980;107(1-2):105-110
- Tian T, Appert H, Myles J, Howard JM. Prognosis value of serum CA19-9 levels in pancreatic adenocarcinoma. *Ann Surg*. 1992;215(4):350-355
- Saito S, Taguchi K, Nishimuri N, Watanabe A, Ogoshi K, Niva M et al. Clinical usefulness of computer associated diagnosis using combination assay of tumor markers for pancreatic carcinoma. *Cancer*. 1993;72(2):381-388

# ERCP and CT diagnosis of pancreas divisum and its relation to etiology of chronic pancreatitis \*

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**Subject headings** pancreatitis/etiology; pancreas divisum/radiography; cholangiopancreatography, endoscopic retrograde; pancreatitis/diagnosis; chronic diseases; amylases/blood; pancreatic ducts/radiography

## Abstract

**AIM** To inquire into the ERCP and CT features of pancreas divisum (PD) and its role in the etiology of chronic pancreatitis.

**METHODS** Fourteen patients with PD were analyzed in regard to the findings in ERCP and CT, the activities of serum amylase and the incidence of pancreatitis. Dorsal ductography via minor papilla cannulation was performed in six of them.

**RESULTS** The length of dorsal and ventral pancreatic duct was  $16.56\text{cm} \pm 2.52\text{cm}$  and  $5.55\text{cm} \pm 1.46\text{cm}$ . Most of the patients had dilatation of dorsal (10/14) and ventral (8/14) duct and the stenosis of dorsal duct terminal (10/14). Delayed clearance of contrast in dorsal duct was found in 8 patients. The size and contour of the pancreas were normal in all the patients at conventional CT. Pancreatitis was identified in 13 patients.

**CONCLUSION** Dorsal ductography was necessary in the diagnosis of PD. Conventional CT play little role in the diagnosis of PD. Patients with PD run a higher risk of pancreatitis due to the stenosis of the minor papilla.

## INTRODUCTION

Pancreas divisum (PD) is a congenital pancreatic ductal anomaly in which the dorsal and ventral pancreatic ducts fail to fuse (complete PD, CPD) or the rudimentary ducts communicate through a fine secondary or tertiary branch (incomplete PD, IPD) during the organogenesis<sup>[1-4]</sup>. It is rare in Asia<sup>[1]</sup>. There has been a heated controversy between those whose epidemiologic studies showed increased prevalence of pancreas divisum among patients with idiopathic pancreatitis and those who found no significant difference in their control population<sup>[1]</sup>. We analysed the ERCP and CT manifestations of PD in 14 patients and inquired into its possible role in the etiology of chronic pancreatitis.

## MATERIALS AND METHODS

Between 1983 and 1994, endoscopic retrograde cholangiopancreatography (ERCP) was performed in 3554 patients suspected to have pancreaticobiliary diseases. Among them, the diagnosis of IPD was made in 8 patients when a typical ventral pancreatic duct was first observed, with further filling, a dominant dorsal ductal system was filled via a fine secondary or tertiary branch (Figure 1). On the other hand, dorsal ductography via minor papilla cannulation was attempted in 10 cases when the "main pancreatic duct" was short and tapered, without communicating with pancreatic body and tail (Figure 2). An ideal dorsal ductogram was obtained in 6 patients, and the body and tail segmental duct was filled only via the minor papilla (Figure 3). CPD was confirmed in these patients.

The clearance time of the contrast in the dorsal and ventral duct was recorded in all patients. If the contrast was present at 12min after the duct had been filled in the prone position, the patient was considered to have a prolonged drainage.

This series consisted of 4 men and 10 women. The mean age was  $46.1 \text{ years} \pm 12.2 \text{ years}$  (24-69). Six with IPD and five with CPD complained of chronic upper abdominal pain, the others had jaundice, which was caused by biliary stones or biliary duct carcinoma confirmed by CT or pathologic examinations.

CT examination was made in all the patients, with a Shimadzu 3000TX or Somatom DR3 scanner. Collimation of 5 mm and 5 mm or 10 mm scan spacing were used.

Serum amylase activity was assayed using blue

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\*Project supported by the National Natural Science Foundation of China, No.39370225.

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**Received** 1997-11-10 **Revised** 1998-01-04

starch method in all patients before ERCP was performed.

We studied the ERCP and CT features of pancreas and the activity of serum amylase and analysed the relationship among the sphincter motility, the dilated degree and drainage time of the ducts and the incidence of pancreatitis.

**RESULTS**

*Ventral and dorsal ductograms*

**Dorsal duct.** The mean length was 16.56cm±2.52 cm. The moderate dilatation and irregular contour were seen in 10 cases (associated with protein emboli in 2) and the others had a normal shape.

**Ventral duct.** The length was 5.55 cm±1.46 cm and dilatation existed in 8 patients (associated with protein emboli in 2 patients). No anomalous changes were found in the remaining 6 cases.

**Communicating duct.** Its shape was similar to that of pancreatic ductal secondary or tertiary branch and the contour was regular (Figure 1). The united point of the branch with the dorsal duct was situated in the pancreatic neck segment and the angle was 42.7° ±19.2° .

**The trend and drainage time.** The results are shown in Table 1.

**Table 1 The trend and drainage time of dorsal (ventral) duct**

Group	Trend			Drainage time	
	Ascend	'S' like	Descent	>12min	<12min
IPD	3(8)	2(0)	3(0)	4(1)	4(7)
CPD	3(5)	2(0)	1(1)	4(0)	2(6)

*Sphincter motility of dorsal duct*

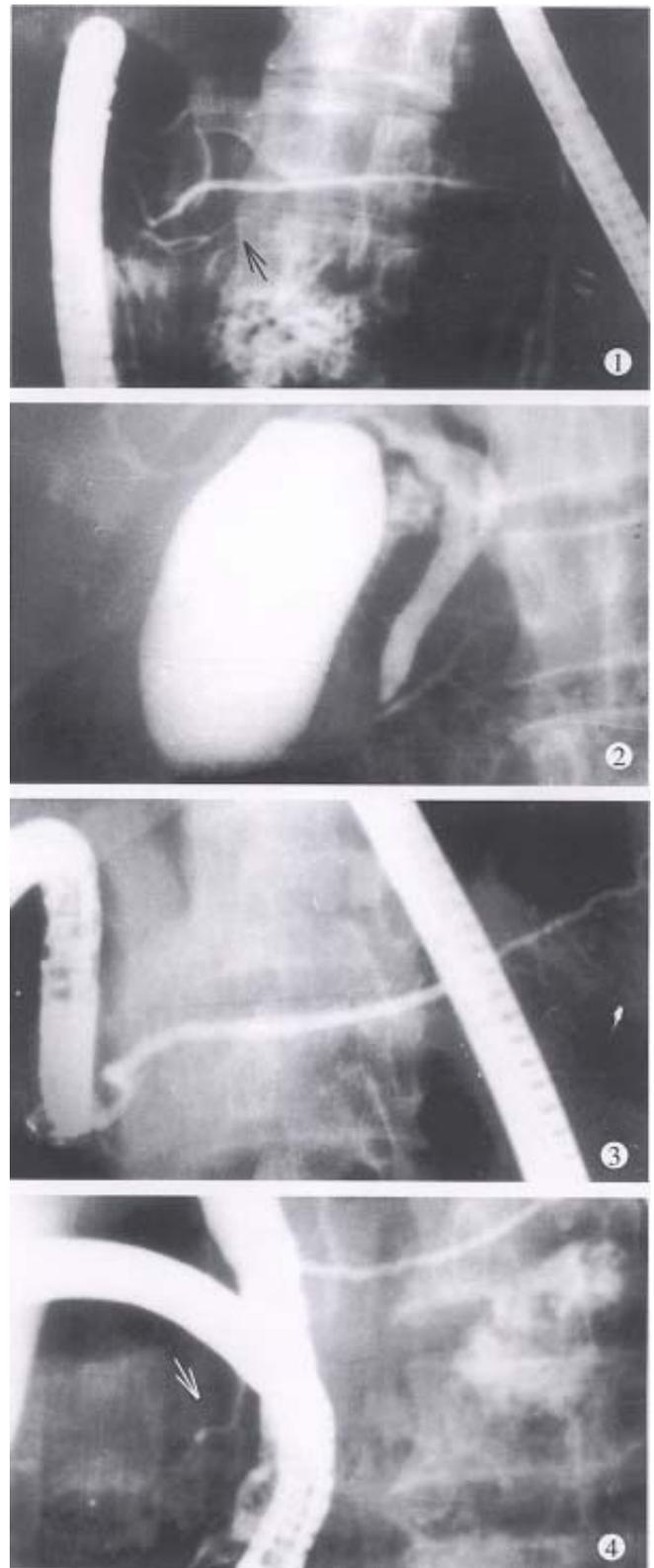
The rigidity and constriction of the ductal sphincter segment were present in nine of ten patients with dorsal ductal dilatation and one of four with normal shape (Figure 4).

*CT manifestations*

All patients had a normal pancreatic size and contour. Among them, the dorsal ductal dilatation was found in one patient with IPD and two with CPD who also had apparent dilatation at ERCP.

*Serum amylase activities*

The activities were significantly increased in two of IPDs and three of CPDs (585IU/L-1553IU/L) and were normal (60 IU/L - 180 IU/L) in the others.



**Figure 1** The ERCP manifestations of IPD (↑: communicating branch).

**Figure 2** The ventral ductograms of CPD at conventional ERCP. The duct was short, tapered and arborized into a fine side branches without communicating with the body and tail of the pancreas.

**Figure 3** The dorsal ductograms of the above case via the minor papilla cannulation. The duct was dominant and trended from the pancreatic head to tail.

**Figure 4** A permanent stenosis of the dorsal ductal terminal was present at the ductograms via the minor papilla cannulation (↑).

### ***Pancreatitis and biliary tract diseases***

Pancreatitis was found in seven of IPDs and six of CPDs according to the diagnostic standards of Guilin Symposium in 1987. The bile duct stones were found in 1 of IPDs and 3 of CPDs. Two patients had carcinoma of the biliary duct.

### **DISCUSSION**

Because the conventional ERCP fails to show the whole pancreatic ductal system. Dorsal pancreatic ductography should be applied to those patients who are suspected to have PD. Minor papilla cannulation can be achieved using the catheters with tapered or 25-gauge needle tips. If the minor papilla or its orifice is not distinctly seen, secretin can be administered at a dose of 1 IU/kg body weight to stimulate the pancreatic juice to flow out.

At conventional ERCP, when a characteristic ventral duct was seen, and with further filling, it was communicated with a dominant dorsal duct system through a fine secondary or tertiary branch, IPD could be diagnosed. If no communicating branch was found and the dorsal duct was not filled or clear secretions flowed briskly from the minor papilla instead of the major papilla in response to secretin administered, IPD or CPD could be suspected and dorsal ductography via the minor papilla cannulation should be performed. Under this condition, if the dorsal duct was dominant and trended from pancreatic head to tail, the diagnosis of CPD could be made.

The dorsal and ventral ducts might have some other abnormal changes, including dilatation, distortion, contour irregularities and protein emboli. Anomalous pancreaticobiliary ductal union was found in 2 patients in this study. It implied that the two kinds of pancreatic duct abnormalities were likely to have a common origin in the embryogenesis.

No characteristic manifestations were found in

conventional CT. It suggested the limitation of conventional CT to the diagnosis of PD. Lindstrom *et al*<sup>[2]</sup> investigated the pancreatic size and contour in 60 patients undergoing the thin-section CT and confirmed that the cranio-caudal length of the pancreatic head in CPD was longer by about 40% than that in normal subjects. He thought that this might indicate the presence of PD, but a local mass must be ruled out. Zeman *et al*<sup>[3]</sup> claimed that a characteristic oblique fat cleft in the pancreas head separating the ventral from dorsal pancreas could be seen in 25% of patients at thin section CT.

In the patients with PD, most of the pancreatic juices excreted via the relatively strictured minor papilla. Though the juices could fully drain in the basal stage of secretion, it could be hindered in the peak stage or when the minor papilla was injured by the mechanical, chemical and biological factors. As a result, the attack of pancreatitis was not surprised. In this study, the incidence of pancreatitis in the patients with IPD (87.5%) and CPD (100%) was significantly higher than that in the reference patients with other pancreaticobiliary diseases (15.3%) which was previously reported by us<sup>[4]</sup>. It indicated that PD was likely to have an etiologic effect on pancreatitis. Besides, a permanent stenosis of the dorsal ductal terminal and the prolongation of the ductal drainage time were present in most of the patients, especially in the patients associated with pancreatic duct dilatation. Therefore there might be a relationship between the attack of pancreatitis and the limitation of the pancreatic juices drainage.

### **REFERENCES**

- 1 Warsaw AL, Simeone JF, Scapironi RH, Warsaw NF. Evaluation and treatment of the dominant dorsal duct syndrome (pancreas divisum redefined). *Am J Surg*, 1990;159(1):59-66
- 2 Lindstrom E, Ihse I. Computed tomography findings in pancreas divisum. *Acta Radiol Diagn*, 1989;30(6):609-613
- 3 Zeman RK, McVay LV, Silverman PM, Cattau EL, Benjamin SB, Fleischer DF *et al*. Pancreas divisum: thin section CT. *Radiology*, 1988;169(2):395-398
- 4 Wu BH, Lu WF. The association between anomalous pancreaticobiliary duct union and pancreaticobiliary diseases. *Chin J Radiol*, 1993;27(12):840-845

# Pathogenetic factors affecting gastroesophageal reflux in patients with esophagitis and concomitant duodenal ulcer: a multivariate analysis

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**Subject headings** esophagitis/etiology; gastric acid/secretion; duodenal ulcer/etiology; gastroesophageal reflux/etiology; hydrogen-ion concentration; multivariate analysis

## Abstract

**AIM** To assess the relationship between gastric acid output (GAO) and both pattern of gastroesophageal reflux (GER) and esophageal lesions, and to evaluate the role of GAO and other potential pathogenetic factors in the development of esophagitis.

**METHODS** Gastric acid secretory testing and 24-h intraesophageal pH monitoring were performed in 31 patients with esophagitis and concomitant duodenal ulcer (E+DU) and compared with those of 72 patients with esophagitis (E) alone.

**RESULTS** The GAO in patients with E+DU was significantly higher than in patients with E ( $P < 0.05$ ). There was no significant difference between the two groups of patients as to endoscopic findings and parameters of GER ( $P > 0.05$ ). A multiple regression analysis with stepwise deletion showed that the pre-sence of hiatal hernia (HH), GER in upright position and age appeared to correlate significantly with the presence of esophagitis.

**CONCLUSIONS** No parallel relationship between GAO and severity of GER or esophageal lesions exists in patients with E+DU, and that GAO is not a major pathogenetic factor in GER disease.

## INTRODUCTION

It is generally agreed that gastroesophageal reflux (GER) may be multifactorial in its pathogenesis. Over the past decade, many investigators have focused their attention on the role of lower esophageal sphincter (LES), hiatal hernia (HH), esophageal mucosal sensitivity, and esophageal or gastric emptying in the development of GER disease. Some studies have shown that LES, hiatal hernia, esophageal emptying and esophageal mucosal sensitivity are, to a certain extent, involved in the pathogenesis of GER<sup>[1-10]</sup>. However, although gastric acid is believed to be an important factor in the development of GER disease, it is still unclear whether the severity of GER and esophageal lesions is necessarily related to increased gastric acid output. Moreover, little is known which factors are crucial in the pathogenesis of GER disease. Therefore, the aims of this study are: to assess the relationship between gastric acid output (GAO) and both pattern of GER and severity of esophageal lesions in patients with esophagitis and concomitant duodenal ulcer (E+DU), in comparison with patients with esophagitis only (E); and to evaluate the role of GAO and other potential pathogenetic factors in the development of reflux esophagitis by multiple regression analysis with stepwise deletion.

## PATIENTS AND METHODS

### Patients

Two groups of patients were enrolled in this study. Of these patients, 31 (27 men, 4 women; mean age of 44 years) had E+DU and 72 (53 men, 19 women; mean age of 42 years) had esophagitis only. All patients had symptoms of GER disease, i. e., chronic heartburn and regurgitation, and/or upper abdominal pain, for a median duration of 55.4 months (range from 3 to 120 months). None of the patients had taken H<sub>2</sub>-receptor blockers or H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitors for 2 weeks before 24-h pH-monitoring and gastric acid secretory testing.

### Methods

All patients underwent upper GI endoscopy, followed by 24h intraesophageal pH monitoring and acid secretory testing over a 2-week period. Endoscopy was performed by the same gastroenterologist in all patients. The degree of esophagitis was

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assessed endoscopically by using the criteria of Savary and Miller<sup>[11]</sup> (grades I to IV). Of these patients, only few presented grades II and III esophagitis, and therefore grades I and II, and III and IV, respectively, were grouped together.

The 24-h intraesophageal pH monitoring was carried out by a routine method used in our laboratory<sup>[12,13]</sup>. A glass pH-electrode with an incorporated potassium chloride reference (Ingold electrode, No. 440) was introduced via the nasoesophageal route and positioned with the tip 5 cm above the gastroesophageal junction, identified with the pH<sup>92</sup>meter. Esophageal pH values were recorded with a solid-state recorder (Autronicord CM 18).

Analysis of pH recording was made on a computer with a dedicated program. The parameters recorded included the frequency and duration of GER in upright position (day-time) and supine position (night-time), and GER frequency exceeding 5 minutes. GER was defined as abnormal if total reflux duration amounted to 7% during the 24-h monitoring<sup>[14]</sup>.

Basal acid secretory analysis was performed in the absence of any antisecretory medication for 2 weeks before the study, in accordance with Raufman *et al*<sup>[15]</sup>. In brief, after a nasogastric tube was introduced into and positioned in the gastric antrum, the gastric contents were emptied by aspiration. Four consecutive 15-min samples of gastric secretion were obtained by continuous aspiration, and the samples were titrated with 0.01 N NaOH to pH 7.0. Fasting basal acid output (BAO) was expressed as milliequivalents of acid per hour (mEq/h). After a BAO was obtained, the patients underwent stimulated acid secretory testing. Six µg/kg of pentagastrin were injected intramuscularly. Sum of the four highest consecutive 15-min samples was represented as a maximal acid output (MAO), and that of the two highest consecutive 15-min period within 2 h of receiving the stimulant was represented as a peak acid output (PAO). At the same time, basal gastrin level in the serum was measured. Normal values for BAO, MAO, PAO and basal gastrin in our laboratory are as follows: BAO, 0.37 mEq/h ± 0.27 mEq/h; MAO, 2.05 mEq/h ± 1.07 mEq/h; PAO, 3.36 mEq/h ± 1.19 mEq/h; and basal gastrin, 37.8 ng/L ± 2.84ng/L.

Chisquare test and Student's *t* test were used to evaluate the data of GI endoscopy and gastric acid secretion, respectively. Statistical evaluation was made using the Mann-Whitney *U* test for the parameters of 24 h intraesophageal pH monitoring. Multiple regression analysis with stepwise deletion was used to evaluate role of some potential pathogenetic factors in the development of reflux esophagitis, which were rated in order of importance. The methods and steps are as follows:

a. The formula of multiple regression:

$$y = b_0 + b_1X_1 + b_2X_2 + \dots + b_kX_k$$

where *y* and *b*<sub>0</sub> represent a dependent variable and a constant factor, *X*<sub>1</sub>, *X*<sub>2</sub>, ..., *X*<sub>*k*</sub> represent independent variables, and *b*<sub>1</sub>, *b*<sub>2</sub>, ..., *b*<sub>*k*</sub> are the standard partial regression coefficient of independent variables<sup>[16]</sup>.

b. Dependent variable and independent variables. In this study, the dependent and independent variables were selected on the basis of the hypothesis that the pathogenesis of the reflux esophagitis was multifactorial, and GER, gastric acid, hiatal hernia, etc. may be all involved, to a certain extent, in the pathogenesis of esophagitis. Accordingly, esophagitis was defined as the dependent variable, and its numerical values assigned to different grades of esophagitis were: 1 (Grade I or II) and 2 (Grade III or IV). Ten variables such as age, HH, GER, gastric acid, etc. were defined as the independent variables. Table 1 gives in detail these independent variables and their definition.

**Table 1 Independent variables**

Variables	Items	Definition
X1	Age	Years
X2	Hiatal hernia	0(no) 1(yes)
X3	Smoking cigarette	0(<1.2 cig./week) 1(<10 cig./day) 2(>10 cig./day)
X4	Alcohol consumption	0(<once/month) 1(once/week) 2(>once a week)
X5	GER in upright position	Percentage time
X6	GER in supine position	Percentage time
X7	BAO	mEq/h
X8	MAO	mEq/h
X9	PAO	mEq/h
X10	Gastrin	µg/L

c. Multiple regression with stepwise deletion. This analysis was done by means of a statistical package (Statpak 3.1, Northwest Analytical, Inc. Portland, Oregon, U.S.A.). Coefficients of all independent variables were calculated by the multiple regression equation. The variables with very small or negative coefficients were dropped because they were shown to give very weak joint contribution to the dependent variable *y*. The independent variables with weaker contribution to dependent variable were further deleted from small to large value by backward regression analysis. After above procedures were repeated, the independent variables with weaker contribution to the dependent variable were removed in a step-by-step fashion. In the last step, the remaining independent variables ranked from small to large values.

d. Percentage of contribution. In order to compare the contribution of each independent variable to dependent variable in a concise way, we developed a formula to calculate a percentage contribution of individual independent variable, which was deduced from the equation of multiple regression:

$$y = b_0 + b_1X_1 + b_2X_2 + \dots + b_kX_k$$

When value of the constant factor  $b_0$  is assumed as 0, namely,  $b_0 = 0$ , the multiple regression equation becomes  $y = b_1X_1 + b_2X_2 + \dots + b_kX_k$ , and percentage contribution of individual independent variable to dependent variable  $y$  can be calculated by the following formula:

$$\frac{\text{Mean value of individual variable} \times \text{its coefficient}}{\text{Sum of such products for all independent variables}} \times 100\%$$

The larger the percentage value, the more important the corresponding variables in joint contribution.

**RESULTS**

All patients underwent GI endoscopy, and the endoscopic results in two groups of patients are listed in Table 2. There was no statistical difference between the two groups of patients as to the endoscopic findings.

**Table 2 Endoscopic findings in two groups of patients**

	Esophagitis		HH(%)	Ulcer (%)
	I-II(%)	III-IV(%)		
E+DU(n=31)	74.6	25.4	49.0	100
E(n=72)	79.1	20.9	55.6	0
P value	>0.05	>0.05	>0.05	

E+DU = patient with esophagitis and duodenal ulcer  
E = patient with esophagitis only

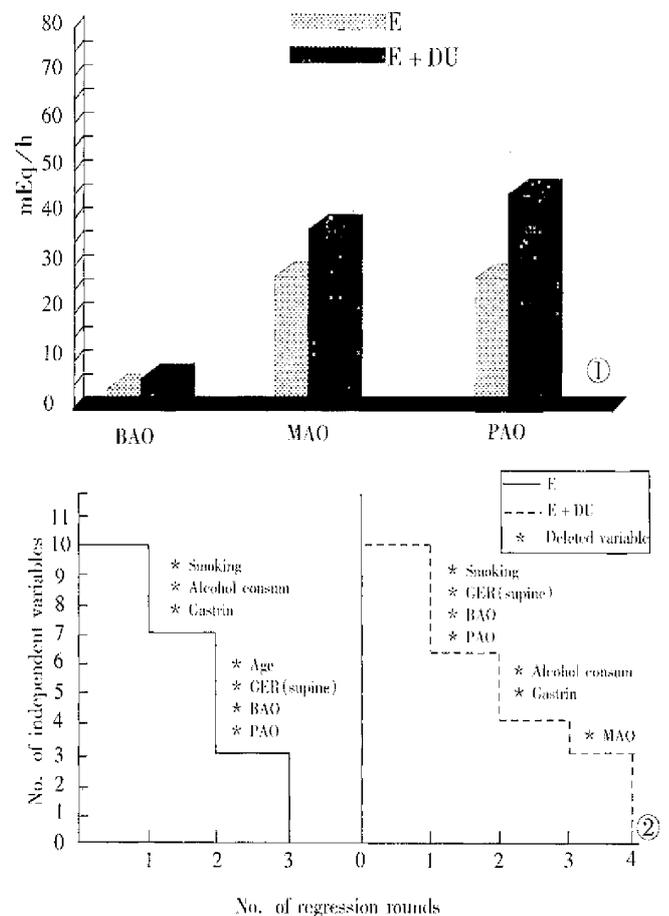
Figure 1 gives the parameters of gastric acid secretion in both groups of patients. There was no significant difference between the two groups of patients in the values of serum gastrin ( $P > 0.05$ ). The parameters of 24 h intraesophageal pH monitoring in patients with E+DU and those with esophagitis are shown in Table 3. There was no statistical difference between the two groups as to the parameters of GER ( $P > 0.05$ ).

**Table 3 Parameters of 24h intraesophageal pH-monitoring**

	GER-up* (% time)	GER-sup (% time)	Episode >5 min(No.)
E+DU	15.94±5.91	13.98±14.20	7.85±3.67
E	15.96±14.58	14.33±21.20	8.28±7.10
P value	>0.05	>0.05	>0.05

\*GER-up = GER in upright position  
GER-sup = GER in supine position

Figure 2 shows the results of multiple regression of dependent variable  $y$  (esophagitis) on 10 independent variables. In patients with E+DU, four rounds of regression with stepwise deletion analysis were performed. The first round of regression ended with deletion of independent variables X3 (smoking cigarette), X6 (GER in supine position), X7 (BAO) and X9 (PAO) because they showed very weak joint contribution or negative values. The remaining variables underwent a second round of regression with stepwise deletion, and 2 variables, i.e. X4 (alcohol consumption) and X10 (gastrin) were deleted. In the third round of regression, X8 (MAO) was deleted. The fourth round of regression on the remaining variables was completed, showing the coefficients of variables in order of importance: X2 (HH), 0.5696282; X5 (GER in upright position), 1.027288E-02; and X1 (age), 8.406402E-04. Similarly, in patients with E, the independent variables deleted in the first two rounds of regression were: smoking cigarette, alcohol consumption and gastrin; and age, GER in supine position, BAO and PAO. The third round ended with the following results: X5 (GER in upright position), 1.156702E-02; X8 (MAO), 9.72133E-04; and X2 (HH), 4.629944E-02 (Table 4).



**Figure 1** Gastric acid output in two groups of patients.  
**Figure 2** Flow chart of multiple regression analysis with stepwise deletion.

**Table 4 Contribution of independent variable to dependent variable**

Variables	Coefficients (b)		X value (mean)	Contribution (%)
	1st RR	Last RR		
E+DU				
X2(HH)	0.3943586	0.5696282	0.36363	52.39
X5(GER-up)	4.044132E-02	1.027288E-02	14.65455	38.08
X1(age)	2.869943E-02	8.406402E-04	44.81818	9.53
E				
X5(GER-up)	2.735608E-02	1.156702E-02	19.21875	80.56
X8(MAO)	3.405805E-02	9.72133E-04	28.34812	9.99
X2(HH)	0.1626756	4.629944E-02	0.562600	9.45

RR = round of regression

GER up = GER in upright position

In patients with E+DU, the percentage contributions of independent variables X2, X5 and X1 to y were 52.39%, 38.08% and 9.53%, respectively. Similarly, the percentage contributions of variables X5, X8 and X2 in patients with E to y were 80.56%, 9.99% and 9.54%, respectively.

## DISCUSSION

Gastric acid secretion is considered to be an important pathogenetic factor in the development of GER disease. In several studies, a basal acid output higher than normal has been found in patients with reflux esophagitis<sup>[17-19]</sup>. Collen *et al* have demonstrated that GER patients who did not respond to standard ulcer healing doses of H<sub>2</sub>-blocker showed gastric acid hypersecretion<sup>[20]</sup>. These results stress the point that gastric acid hypersecretion is a crucial factor for GER disease and for the resistance of GER patients to H<sub>2</sub>-blockers. However, our results showed that although the gastric acid output in patients with E+DU was significantly higher than in patients with E, there was no significant difference between two groups of patients as to severity of esophageal lesions and patterns of GER. These results suggest that no parallel relationship between GAO and severity of GER or esophageal lesions exists in patients with E+DU.

One approach to investigate the reason why the increased gastric acid output is not accompanied by aggravation of both GER patterns and severity of esophageal lesions is to assess quantitatively the role of the various potential pathogenetic factor involved, which may not only influence esophagitis but also interact. Therefore, multiple regression analysis with stepwise deletion is needed. According to this method, the value of any regression coefficient depends on all the other variables included in the regression. With stepwise deletion, the standard partial regression coefficient can be used as a measure of relative importance, the X being ranked in order of the size of their coefficients.

In our study, ten independent variables in each patient group were evaluated by multiple regression

analysis with stepwise deletion. In patients with E+DU, smoking, GER in supine position, BAO, PAO, alcohol consumption, gastrin, and MAO were deleted in a stepwise fashion because they failed to significantly affect esophagitis. Similarly, in patients with esophagitis without DU, seven variables such as smoking, alcohol consumption, gastrin, etc. were deleted by two rounds of regression. These results indicate that GER in upright position and HH are important determinants of esophagitis. Our results also demonstrate, on the contrary, that the gastric acid output is not an important pathogenetic factor responsible for GER disease.

The relationship between the development of esophagitis and the pattern of GER is also a debatable issue. Some authors have found that the development of esophagitis is related to an increased GER in supine position<sup>[21-24]</sup>. Others have argued that GER in upright position is the most important pathogenetic factor<sup>[25-28]</sup>. In our study, the result of multiple regression indicates that the GER in supine position appears to be a weak factor affecting esophagitis, whereas GER in upright position plays an important role in the pathogenesis of esophagitis in both groups of patients.

In conclusion, an increased gastric acid output in patients with E+DU does not aggravate both the pattern of GER and esophageal lesions because the gastric acid output fail to appear as a significant pathogenetic factor responsible for GER disease, whereas GER in upright position and presence of HH are significantly related to GER disease in both groups of patients.

## REFERENCES

- Zaninotto G, DeMeester TR, Schwizer W. The lower esophageal sphincter in health and disease. *Am J Surg*.1988;155(2):104-111
- Ahtaridis G, Snape WJ, Cohen S. Lower esophageal sphincter pressure as an index of gastroesophageal acid reflux. *Dig Dis Sic*. 1981;26(5):993-998
- Little AG, DeMeester TR, Kirchner PT. Pathogenesis of esophagitis in patients with gastroesophageal reflux. *Surgery*. 1980;88(1):101-107
- Berstad A, Weberg R, Fryshov Larsen I. Relationship of hiatus hernia to reflux oesophagitis: a prospective study of coincidence, using endoscopy. *Scand J Gastroenterol*. 1986;21(1):55-58
- Kaul B, Petersen H, Myrvold HE. Hiatus hernia in gastroesophageal reflux disease. *Scand J Gastroenterol*.1986;21(1):31-34
- Johnson LF, DeMeester TR, Haggitt RC. Esophageal epithelial response to gastroesophageal reflux, a quantitative study. *Dig Dis*. 1978;23(3):498-509
- Mittal RK, Lange RC, McCallum RW. Identification and mechanism of delayed esophageal acid clearance in subjects with hiatus hernia. *Gastroenterology*. 1987; 92(1):130-135
- Sloan S, Kahrilas PJ. Impairment of esophageal emptying with hiatal hernia. *Gastroenterology*.1991;100(5):596-605
- Howard PJ, Maher L, Prvde A. Symptomatic gastroesophageal reflux, abnormal oesophageal acid exposure, and mucosal acid sensitivity are three separate, though related, aspects of gastro-oesophageal reflux disease. *Gut*. 1991;32(1):128-132
- Janssens J, Vantrappen G, Ghillebert G. 24-hour recording of esophageal pressure and pH in patients with noncardiac chest pain. *Gastroenterology*. 1986;90(8): 1987-1984
- Savary M, Miller G. The esophagus. Handbook and atlas of endoscopy. First ed. Switzerland: *Gassmann*. 1987.
- Bianchi Porro G, Pace F. Comparison of three methods of intraesophageal pH recording in the diagnosis of gastroesophageal reflux. *Scand J Gastroenterol*. 1988;23(5):743-750
- Pace F, Sangaletti O, Bianchi Porro G. Daytime reduction of gastroesophageal reflux after healing of esophagitis and its value as an indicator of favourable response to maintenance treatment. *Gut*.1990;31(9):1025-1029
- Weiser HF, Siewert JR. Investigations with the 24-hour solid-state pH metry: correlation between gastroesophageal reflux extent and reflux sequelae. *Surg Gastroenterol*.1982;1(3):327-334

- 15 Raufman JP, Collins SM, Pandolfi SI. Reliability of symptoms in assessing control of gastric acid secretion in patients with Zollinger-Ellison syndrome. *Gastroenterology*, 1983;84(1):108-113
- 16 Snedecor GW, Cochran WG. Statistical methods. Iowa: The Iowa State University Press, 1979
- 17 Collen MJ, Ciarleglio CA, Stanczak VJ. Basal acid output in patients with gastroesophageal reflux disease. *Gastroenterology*, 1987;92(11):1350 (Abstract)
- 18 Mulholland MW, Reid NJ, Levine DS. Elevated gastric acid secretion in patients with Barrett's metaplastic epithelium. *Dig Dis Sci*, 1989;34(12):1329-1335
- 19 Barlow AP, DeMeester TR, Ball CS. The significance of the gastric secretory state in gastroesophageal reflux disease. *Arch Surg*, 1989;124(8):937-940
- 20 Collen MJ, Lewis JH, Benjamin SB. Gastric acid hypersecretion in refractory gastroesophageal reflux disease. *Gastroenterology*, 1990;98(5):654-661
- 21 Demeester TR, Johnson LF, Joseph CJ. Patterns of gastroesophageal reflux in health and disease. *Ann Surg*, 1976;184(4):459-470
- 22 Robertson D, Aldersley M, Shepherd H. Patterns of acid reflux in complicated oesophagitis. *Gut*, 1987;28(12):1484-1488
- 23 Lichter I. Measurement of gastro-oesophageal acid reflux: its significance in hiatus hernia. *Br J Surg*, 1974;61(3):253-258
- 24 Pujol A, Grande L, Ros E. Utility of inpatient 24-hour intraesophageal pH monitoring in diagnosis of gastroesophageal reflux. *Dig Dis Sci*, 1988;33(11):1134-1140
- 25 De Caestecker JS, Blackwell JN, Pryde A. Daytime gastro-oesophageal reflux is important in oesophagitis. *Gut*, 1987;28(5):519-526
- 26 Branicki FJ, Evans DF, Jones JA. A frequency-duration index (FDI) for the evaluation of ambulatory recordings of gastro-oesophageal reflux. *Br J Surg*, 1984;71(4):425-430
- 27 Rokkas T, Anggiansah A, Uzoehina E. The role of shorter than 24-h pH monitoring periods in the diagnosis of gastro-oesophageal reflux. *Scand J Gastroenterol*, 1986;21(6):614-620
- 28 Blackwell JN, Heading RC. When does gastro-oesophageal reflux occur in patients with peptic oesophagitis? (Abstract). *Gut*, 1980;21(5):A922

# Classification of gastric neuroendocrine tumors and its clinicopathologic significance

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**Subject headings** stomach neoplasms/pathology; neuroendocrine tumors/pathology; carcinoid tumor/pathology; gastritis, atrophic; G cell; immunohistochemistry; microscopy, electron

## Abstract

**AIMS** To study the pathologic classification of gastric neuroendocrine tumors and its clinicopathologic significance.

**METHODS** Paraffin-embedded sections of 52 gastric neuroendocrine tumors including 42 carcinoid tumors, and 10 cases of neuroendocrine carcinoma from 326 patients who underwent resection of stomach carcinomas were studied by immunohistochemical methods including 10 endocrine markers or hormones antibodies and endocrine cells in gastric neuroendocrine tumors and extratumoral mucosa were observed under electromicroscope.

**RESULTS** The 52 gastric neuroendocrine tumors were divided into three types: ① Gastrin dependent type of carcinoid (26 cases) accompanied by chronic atrophic gastritis (CAG) and tumor extension limited to the mucosa or submucosa complicated with hypergastrinemia and G cell hyperplasia. This type was consistently preceded by and associated with generalized proliferation of endocrine cells in the extratumoral fundic mucosa. ② Non-gastrin dependent type of carcinoids (16 cases) associated with neither CAG nor hypergastrinemia. This type was more aggressive; and ③ Neuroendocrine carcinomas (10 cases), which are highly aggressive tumors.

**CONCLUSIONS** A correct identification of different types of gastric endocrine tumors has major implications for the treatment and prognosis of the patients.

## INTRODUCTION

According to recent literature, gastric carcinoids incidence ranges from 11% to 41% of all gastrointestinal carcinoids<sup>[1,2]</sup>. Until a decade ago, however, it was regarded as pathological curiosity accounting for about 3%<sup>[3]</sup>. It was recognized that gastric neuroendocrine tumors cover a spectrum of neoplasms showing wide variations in their clinicopathological features, prognosis and pathogenetic mechanisms<sup>[4,5]</sup>. The classification of gastric neuroendocrine tumors (GNET) is of some value in its treatment and prognosis<sup>[6]</sup>. This paper is to study clinicopathologic classification of GNET for the correct management of patients.

## MATERIALS AND METHODS

A total of 42 cases of gastric carcinoid were collected in Parma University, Italy (29 cases) and China (13 cases). Ten cases of neuroendocrine carcinoma were identified from 326 resected gastric carcinomas by immunostaining and electromicroscopy.

Formalin-fixed and paraffin-embedded tissues were available and the blocks of specimens included tumor and extratumoral mucosa. A series of 15 sections (5 $\mu$ m thick) were cut from each block. Hematoxylin and eosin, Acilan blue pH 2.5-periodic staining were carried out. The cellular localizations of neuroendocrine substances were detected by the avidinbiotin<sup>a2</sup>peroxidase complex method (ABC kit, K355; Daco, Carpinteria, CA, USA). The primary antibodies and positive controls are listed in Table 1. All antibodies were polyclonal except serotonin and bombesin. Peroxidase was revealed using diaminobenzidine tetrahydrochloride (DAB) and H202. Control of specificity of the immunoreaction was performed by incubating consecutive sections with nonimmune serum instead of the primary antiserum or with the specific antisera preabsorbed with the excessive amounts of the respective antigens.

Eight cases of the 10 neuroendocrine carcinomas were examined under transmission electromicroscopy (Musashino, Tokyo, Japan).

Fasting serum gastrin was determined in 33 of 42 carcinoid patients and 8 of 10 neuroendocrine carcinomas.

## RESULTS

A total of 52 GNET cases were divided into three types according to Bordi<sup>[4]</sup> and Rindi<sup>[5]</sup>: Type 1, gastrin dependent carcinoid; type 2, non-gastrin de-

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\*Supported in part by grants from Medical Science Council of Chinese PLA, No.96MB01.

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Received 1997-04-17

pendent carcinoid; and type 3, neuroendocrine carcinoma. The immunostaining results for neuroendocrine markers and hormone antibodies are summarized in Table 2. Diffuse features of neuroendocrine differentiation were shown in all the 52 cases and ultrastructural evidence of endocrine type of cytoplasmic granules was observed in 8 cases of neuroendocrine carcinomas examined with transmission electronmicroscopy. Those tumors were classified as neuroendocrine tumors in stomach.

**Table 1 Antibodies used in the present study**

Code	Antigens	Working dilution	Positive control
M869	CgA	1:400	Pancreas
	SP	1:400	Pancreas
A568	GAST	1:300	Gastric antrum
A566	SS	1:400	Pancreas
A756	5-HT	1:20	Small bowel
A576	CT	1:800	Medullary carcinoma of thyroid gland
A619	PP	1:4000	Pancreas
A231	β-HCG	1:800	Human placenta
A571	ACTH	1:800	Pituitary gland
CA-08-219	BOM	1:20000	Duodenum

CgA: chromogranin A; SP: synaptophysin; GAST: gastrin; SS: somatostatin; 5-HT: serotonin; CT: calcitonin; PP: pancreatic polypeptide; β-HCG: human chorionic gonadotroph; ACTH: adrenocorticotrophin; GLU: glucagon; BOM: bombesin

**Table 2 Immunohistochemical results in 52 cases of gastric tumors**

	Type 1 (26)				Type 2 (16)				Type 3 (10)			
	+	±	-	ND	+	±	-	ND	+	±	-	ND
CgA	24	1	0	1	14	1	0	1	9	1	0	0
SP	16	3	4	3	4	6	4	1	2	5	0	3
GAST	4	2	17	3	2	1	11	2	1	1	8	0
SS	3	0	22	2	1	0	14	3	1	1	8	0
5-HT	10	1	15	0	9	0	5	2	2	0	7	1
CT	1	0	11	14	0	1	4	11	1	1	8	0
β-HCG	5	1	19	1	2	1	12	1	4	2	4	0
ACTH	1	1	12	12	0	1	3	12	2	1	5	2
PP	4	0	21	1	2	1	5	4	1	1	5	3
GLU	0	0	25	1	0	0	15	1	1	0	9	1
BOM	1	1	8	16	0	1	6	9	1	1	7	1

+: positive; -:negative; ±: doubtful; ND: study not done

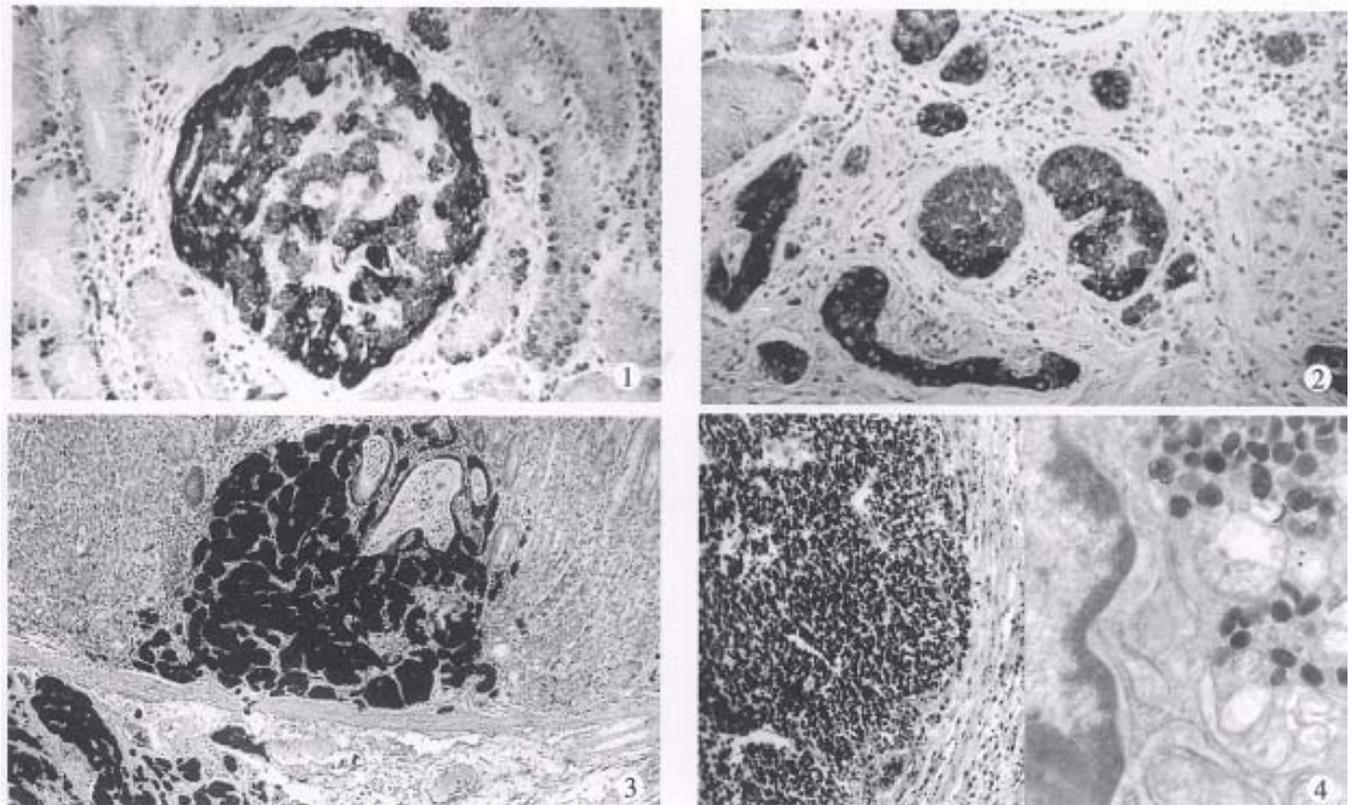
**Type 1.** Gastrin dependent carcinoid (26 cases), with a mean age of 58.2 years (range 27-88 years), a female to male ratio of 1.36:1. Eleven cases are single tumors appearing as polypoid lesions. Eight of them were <1cm in maximum diameter, 2 were 1 to 1.5cm, and only one was 2.5 cm. Fifteen multiple tumors were 1.0cm - 1.5cm in each one. In histological structure the carcinoids appeared either trabecular or in solid arrangement (Figure 1). Tumor cells were monomorphic and medium sized in regular shape and with round nuclei. Mitoses were either absent or seen occasionally. Sixteen cases confined to the gastric mucosa and 4 cases to the submucosa. One case was

found to have lymph node metastases, and no distal metastases was found.

This type of carcinoids is associated with CAG and proliferation of endocrine cells in extratumoral fundic mucosa. It includes hyperplasia as simple, linear, micronodular and adenomatoid hyperplasia and dysplasia as enlarging, fusing micronodules, invasive lesion and nodule with newly formed stroma. When at a diameter of 0.5 mm, it was defined as carcinoid tumor. The hyperplasia and dysplasia (Figure 2) of endocrine cells in extratumoral mucosa were found in all 26 cases. The fasting serum gastrin of 21 of 26 cases were examined before operation and all had hypergastrinemia, ranging from 700ng/L-1000ng/L (normal is 100ng/L). G cell hyperplasia in antrum was observed in 24 cases and antrum mucosa was investigated. Nine of 11 cases of single tumors underwent polypectomy, and 2 cases partial gastrectomy, and 15 multiple tumors received antrectomy. Eighteen cases were followed up, there were 3 deaths with a mean survival of 40 months, which was unrelated to the gastric carcinoid. After a mean of 64 months, 15 were alive.

**Type 2.** Non-gastrin dependent carcinoid (16 cases). The mean age was 53.8 years (range 42-61 years), a female to male ratio of 0.6:1. All tumors were single with a median diameter of 2.5cm. Tumors showing moderate enlargement of lobules and trabeculae and moderate cellular atypia included nuclear polymorphism, hyperchromasia, prominent nucleoli and slight increase in mitotic count (<1 per 2 HPF) (Figure 3). Small necrosis may also be found. The muscularis propria or serosa were invaded in 11 of 16 cases (68.8%). Local lymph node metastases were found in 9 (56%) cases and distant metastases were seen in 5 (31%) patients. The circulating gastrin of 12 of 16 patients was examined and had normal levels. No antral G cell hyperplasia was found in 13 of 16 cases. Two of 16 patients displayed hyperplasia of endocrine cells in extratumoral mucosa and none had dysplasia. Among 14 cases followed up died with a mean survival of 32 months and 10 were alive after a mean follow-up of 72 months.

**Type 3.** Neuroendocrine carcinomas (10 cases). The mean age was 62.8 years (range 57-78 years), 8 men and 2 women. All tumors were single, with an average size of 5.1cm (range 3.0cm - 8cm). Histologically, they were characterized by large, poorly defined, solid aggregates or diffuse sheets of round or spindle cells (Figure 4). Mitoses were usually abundant (>1 per HPF). Focal necrosis was common. The extratumoral mucosa was associated with chronic atrophic gastritis in six cases. Ultrastructural evidence of endocrine type cytoplasmic granules was found in 8 cases examined. The hypergastrinemia was found in only one of 5 cases.



**Figure 1** Type 1 gastric neuroendocrine tumorsgastrin dependent carcinoid. Histology shows typical carcinoid with trabecular arrangement. Tumor confined to the gastric mucosa, many tumor cells had positive reaction for chromogranin A. ABC method  $\times 450$

**Figure 2** The hyperplasia and dysplasia of endocrine cells in extratumoral mucosa were found in type 1 gastrin dependent carcinoid. Endocrine cells show positive reaction for chromogranin A. ABC method  $\times 450$

**Figure 3** Type 2 non-gastrin dependent carcinoid. Tomor invaded the muscularis propria. The hyperplasia of endocrine cells are not distinct. Diffuse synaptophysin in immunostaining of tumors. ABC method  $\times 250$

**Figure 4** Type 3 neuroendocrine carcinoma. Tumor cells are small and round, mitoses are abundant. Focal necrosis was found. HE $\times 250$  (left). Ultrastructural appearance of the neuroendocrine carcinoma with characteristic secretory granules.  $\times 28000$  (right)

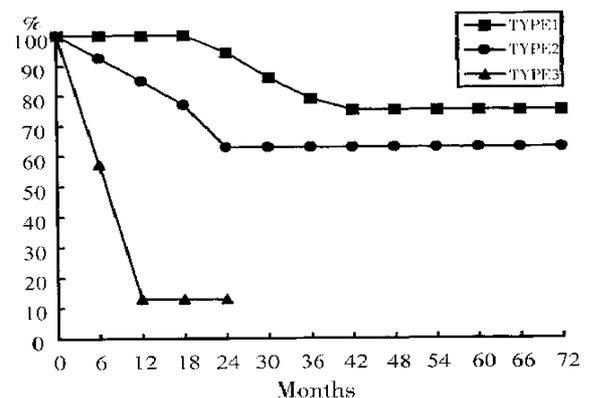
**Table 3** Clinicopathological characters of gastric neuroendocrine tumors

Type	Sex (F:M)	Mean age (a)	Tumor number	Infiltration	Metastases	Death	Treatment
1	1:3.6	58.2	Single	m,sm		no	Polypectomy
			Multiple	m,sm	3.8%	no	Antrectomy
1	0.6:1	53.8	Single	mp,s	56%	28%	Gastrectomy
3	1:4	62.8	Single	mp,s	80%	70%	Gastrectomy Chemotherapy

m: mucosa; sm: submucosa; mp: muscularis propria; s: sierosa

Carcinomas invaded the gastric wall, reaching the muscularis propria in three cases (30%), and serosa in seven (70%). Eight cases showed metastases to local lymph nodes and two to livers. Deaths were observed in seven cases with a mean survival of 8 months (range 2-10 months) after a mean follow-up of 54 months.

Table 3 and Figure 5 summarize the clinicopathological characters and survival of variations of gastric neuroendocrine tumors.



**Figure 5** The survival curve of three types of gastric endocrine tumors.

## DISCUSSION

The classification of gastric neuroendocrine tumors is subdivided into carcinoid and neuroendocrine carcinoma. Two subtypes of carcinoid tumors including gastrin dependent carcinoid and non-gastrin dependent carcinoid has further recognized in the study. Rindi *et al*<sup>[1]</sup> reported that carcinoids were subdivided into three subtypes which included: carcinoid tumors associated with atrophic gastritis<sup>[2]</sup>; carcinoid tumors associated with Zollinger Ellison syndrome<sup>[3,4]</sup>; carcinoid tumors of sporadic type. The carcinoid with ZES appears absent in ours.

The result of features of 26 cases of gastrin dependent carcinoid type is in agreement with some previous reports in which it was named as carcinoids associated with atrophic gastritis. This type is either single (polypoid lesions) or multiple tumor. The histological features show typical carcinoids. Tumor extension limited to the mucosa or submucosa. Gastrin dependent carcinoids are consistently associated with generalized proliferation of endocrine cells in the extratumoral fundic mucosa. A histopathological classification has been formulated for the spectrum of proliferative lesions presented by fundic endocrine cells of hypergastrinemic patients. The classification arranged in a sequence presumed to reflect the temporal evolution of the process and the increasing oncologic risk for the patients<sup>[5,6]</sup>. Our data also provide a rationale for the treatment of single carcinoids with tumor polypectomy followed by regular endoscopic follow-up. In the patients with multiple tumors, antrectomy is recommended. Postantrectomy regression of proliferating gastric endocrine cells was documented in several cases.

The 16 cases of non-gastrin dependent type which correspond to Solcia's carcinoids of sporadic type<sup>[7,8]</sup> had single tumors. They usually presented with typical carcinoid features by slight increase in mitotic count (<1 per HPF). Serum levels of gastrin were in the normal range. These tumors appear free from hypergastrinemia and should not benefit from antrectomy. Their aggressive behavior dictated a complete surgical ablation.

The type of neuroendocrine carcinomas composed mainly of cells was strongly positive for endocrine markers (Cg A, SP or hormonal products). Those positive cells were major parts of the tumor (>50%). Although neuroendocrine differentiation

is not a rare phenomenon in common gastric carcinoma (3.1%-53.5%)<sup>[9]</sup> but neuroendocrine carcinoma of the stomach was found infrequently. In this series, 10 (3.1%) were identified from 326 gastric carcinomas. The endocrine granules were observed by transmission electron microscopy. This type of carcinomas is high-grade one. Tumors were considered aggressive cancers with poor prognosis showing large tumors (mean 5.1cm) with deep invasion and metastasis. The surgical resection is the most appropriate form of treatment for this type and the usefulness of multiagent chemotherapy remains to be evaluated in larger series of patients.

Immunohistochemically, no significant specific differences were found in the three types of neuroendocrine tumors of stomach<sup>[4,10]</sup>. Types 1 and 2 of carcinoid were mostly composed of ECL cells which produce histamine<sup>[5,6]</sup>. The eight hormonal antibodies used in the study showed a various amount of positive expression in neuroendocrine tumors of stomach. Further investigations are needed to elucidate the significance of hormones produced by those tumors.

In conclusion, neuroendocrine tumors of the stomach cover a spectrum showing wide variations in their clinicopathological features, prognosis, and pathogenetic mechanisms. It has implications for the treatment.

## REFERENCES

- 1 Rindi G, Luinetti O, Cornaggia M, Capella C, Solcia E. Three subtypes of gastric argyrophil carcinoid and the gastric neuroendocrine carcinoma: clinicopathologic study. *Gastroenterology*, 1993;104(4):994-1006
- 2 Solcia E, Fiocca R, Sessa F, Rindi G, Gianatti A, Cornaggia M *et al.* Morphology and natural history of gastric endocrine tumors. In: Hakanson R, Sundler F, eds. *The stomach as an endocrine organ. Amsterdam: Elsevier*, 1991:473-498
- 3 Godwin JD. Carcinoid tumors: an analysis of 2837 cases. *Cancer*, 1975;49(2):560-569
- 4 Bordi C, Yu JY, Baggi MT, Davoli C, Pilato FP, Baruzzi G. Gastric carcinoids and their precursor lesions. A histologic and immunohistochemical study of 23 cases. *Cancer*, 1991;67(3):663-672
- 5 Rindi G, Bordi C, Rappel S, Rosa SL, Stolte M, Solcia E. Gastric carcinoids and neuroendocrine carcinomas: pathogenesis, pathology, and behavior. *World J Surg*, 1996;20(2):169-172
- 6 Bordi C. Endocrine tumours of the stomach. *Pathol Res Pract*, 1995;191(2):373-380
- 7 Creutzfeldt W. The achlorhydria carcinoid sequence: role of gastrin. *Digestion*, 1988;39(1):61-79
- 8 Solcia E, Rindi G, Fiocca R, Villani L, Buffa R, Ambrosiani L *et al.* Distinct patterns of chronic gastritis associated with carcinoid, neuroendocrine carcinoma or ordinary cancer and their role in tumorigenesis. *Yale J Biol Med*, 1992;112(3):793-1047
- 9 Luping W, Jiyao Y, Jingquan S, Yanie L. Neuroendocrine cells in gastric carcinoma and in extratumoral gastric mucosa. *Cell Visson*, 1996;3(1):47-53
- 10 Staren ED, Lott S, Saavedra VM, Jansson DS, Deziel DJ, Saclarides TJ *et al.* Neuroendocrine carcinomas of the stomach: a clinicopathologic evaluation. *Surgery*, 1992;112(6):1039-1047

# TRH microinjection into DVC enhances motility of rabbits gallbladder via vagus nerve \*

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**Subject headings** gallbladder; vagus nerve; dorsal vagal complex; thyrotropin releasing hormone; cholinergic M receptors; rabbits

## Abstract

**AIM** To investigate the effects of TRH in DVC on motility of the gallbladder in rabbits.

**METHODS** After fasted for 15h-18h, rabbits were anesthetized with urethane (1.0g/kg). Gallbladder pressure (GP) was measured by a frog bladder perfused with normal saline.

**RESULTS** After microinjection of TRH (8.8nmol, 1 $\mu$ l) into DVC, GP was raised and the frequency of phasic contraction of gallbladder (FPCGB) increased. All the doses of TRH (0.13, 0.25, 0.50, 0.80, 1.30nmol, 1 $\mu$ l) injected into DVC could excite the motility of gallbladder. As the dose of TRH was enlarged, the amplitude and duration of the reaction increased. Effects of TRH in DVC on motility of the gallbladder could be completely abolished by atropine (0.2mg/g, i.v.) or vagotomy, but could not be inhibited by phentolamine iv (1.5mg/g) or propranolol iv (1.5mg/g) or by transecting the spinal cord.

**CONCLUSION** Thyrotropin-releasing hormone in DVC can excite motility of gallbladder. This effect was mediated by vagus nerves and peripheral M receptor. Its physiological significance may be related to maintaining the phasic contraction of gallbladder in interdigestive period.

## INTRODUCTION

Our previous studies indicated that the dorsal vagal complex (DVC) might play an important role in regulating motility of extrahepatic biliary tree<sup>[1]</sup>, but we do not know which neurotransmitter mediates this effect.

Thyrotropin-releasing hormone (TRH) is one of important brain gut peptides<sup>[2]</sup>. There is a high density of TRH-immunoreactive nerve terminals in DVC<sup>[3]</sup>. Injecting TRH into DVC could excite the motility and secretion of stomach<sup>[2]</sup>. However, there is no report about TRH in DVC on the motility of extrahepatic biliary tree. In this study, TRH was injected into DVC of anesthetized rabbits, and then the effect on mean gallbladder pressure (GP) and frequency of phasic contraction of gallbladder (FPCGB) were observed, and the peripheral route of the effect of TRH was also investigated.

## MATERIALS AND METHODS

### Animal preparation

The experiments were conducted in 38 rabbits weighing 2.0 kg - 2.5 kg. Fasted for 15 h - 18 h, but allowed to drink water, the rabbits were anesthetized with urethane (1.0g/kg, iv.). A cannula was inserted into the trachea. Unilateral femoral artery was catheterized for measurement of blood pressure. The rectal temperature was maintained at 37°C-38.5°C.

### Measurement of GP

A frog bladder filled with saline was placed in gallbladder and connected to a transducer (TP-200T) by a catheter. The blood pressure and GP were recorded simultaneously by a polygraph (RM-6000, NIHON KOHDEN).

### Microinjection of TRH

The animal's head was fixed in a stereotaxis (I-C model, JIANG-WANG, China). According to the Messen's methods, a stainless steel guide tube (0.5mm in outer diameter) containing a needle was inserted into DVC (rostral from obex 0.5mm-1.0mm, left or right from midline 0.7mm-1.2mm, depth from the surface 0.5mm-1.2mm). Normal saline or TRH solution(1 $\mu$ l) was injected into DVC by a microsyringe connected to a cannula (0.3mm in outer diameter).

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Received 1997-12-05 Revised 1997-12-30

### Statistical analysis

Before the following tests were made, the basal level of GP of each animal was taken as control (i.e., basal GP as 0 kPa in place of real level). GP increase during the test, was considered as positive (+) value, while GP decrease was taken as negative (-). Student's *t* test was used for the statistical analysis. All values were  $\bar{x} \pm s_{\bar{x}}$ .  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### Normal GP

In interdigestive period, two kinds of motion of gallbladder were found, the tonic and phasic contractions. Tonic contraction, which means continuous and weak contraction, could maintain GP at a stable level ( $2.67 \times 10^3 \pm 190 \text{ Pa}$ ,  $n = 8$ ). Phasic contraction, with a duration of 5-20s and frequency of 1-3 times per minute, raised GP by 60kPa-200kPa rhythmically (Figure 1a).

### Microinjection of TRH into DVC

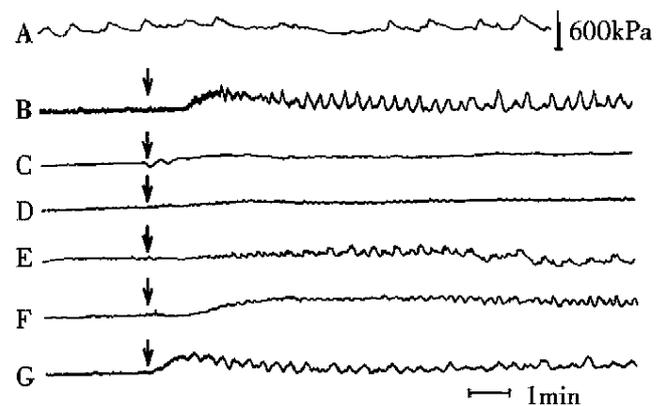
After TRH (0.8nmol, 1 $\mu$ l) was microinjected into DVC, the motility of gallbladder was enhanced. The effect occurred in 1min after the injection, reached its peak in 10 minutes and returned to normal within 20min-30min. At 5min after injection, GP increased by  $167 \text{ kPa} \pm 29 \text{ kPa}$  ( $n = 5$ ,  $P < 0.02$ ); FPCGB increased from  $1.14 \pm 0.26$  to  $2.28 \pm 0.26$  per min ( $n = 5$ ,  $P < 0.034$ ) (Figures 1b, 2-3). However, after normal saline (1 $\mu$ l) injected into DVC or TRH (0.8nmol, 1 $\mu$ l) into the control position in medulla (lateral 0.5mm-1.0mm to the DVC), the GP and FPCGB did not change significantly.

### Dose-response of TRH microinjected into DVC on the GP and FPCGB

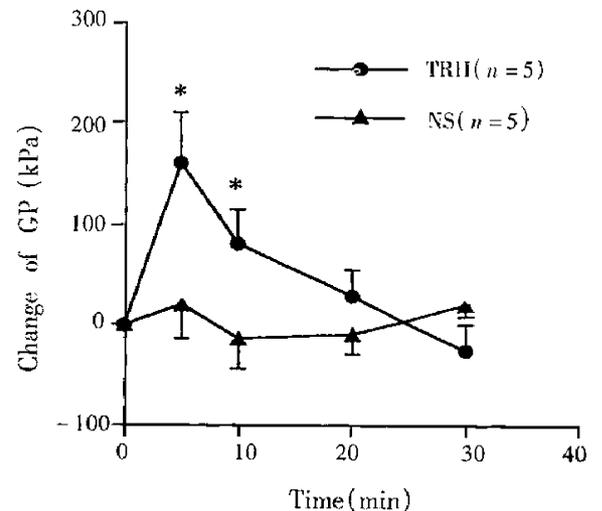
All the doses of TRH (0.13, 0.25, 0.50, 0.80, 1.30nmol, 1 $\mu$ l) microinjected into DVC could excite the motility of gallbladder. As the dose increased, the strength and duration of the effect of TRH on GP and PCGB went up, with a significant dose-response (Figures 4 and 5).

### Peripheral route of the TRH effect on the motility of gallbladder.

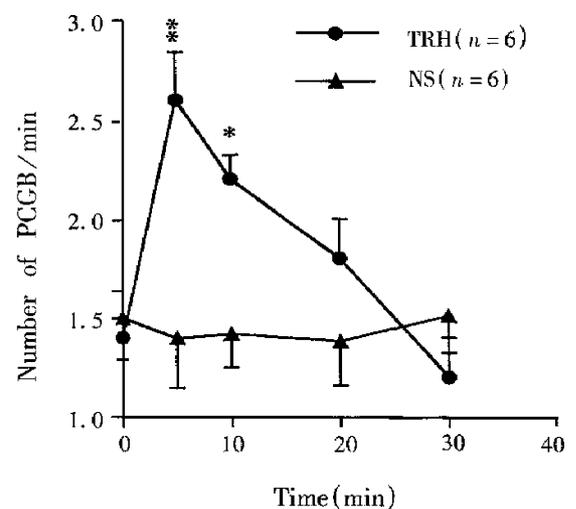
Ten minutes after the cervical vagotomy ( $n = 5$ ) or i.v. injection of atropine (0.2g/kg,  $n = 5$ ), microinjection of TRH (0.8nmol, 1 $\mu$ l) could no longer excite the motility of gallbladder. Within 30 minutes after the injection of TRH, the GP and FPCGB had no significant changes (Figure 1b, c). However, ten minutes after transection of the spinal cord from T1 or i.v. injection of phentolamine (1.5 mg/kg) or propranolol (1.5 mg/kg), injection of TRH into DVC could also enhance the motility of gallbladder (Figure 1d, e, f).



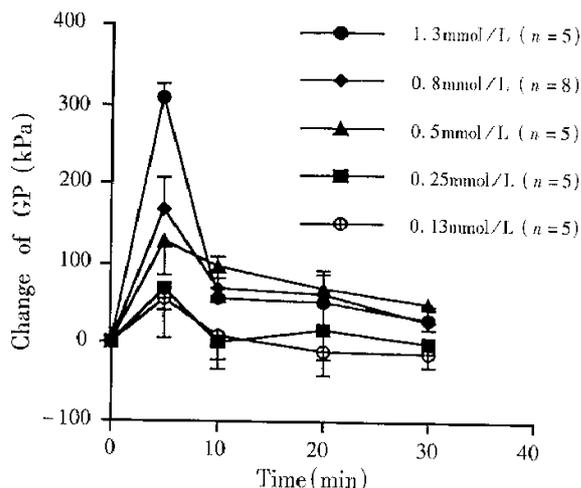
**Figure 1** The effects of TRH microinjected into DVC on the GP. A. control; B. TRH (0.8 nmol/1  $\mu$ l); C. i.v. atropine (0.2 mg/kg); D. vagotomy; E. i.v. phentolamine (1.5 mg/kg); F. i.v. propranolol (1.5 mg/kg); G. after transection of spinal cord through T1; G.  $\downarrow$  (0.8 nmol/1  $\mu$ l).



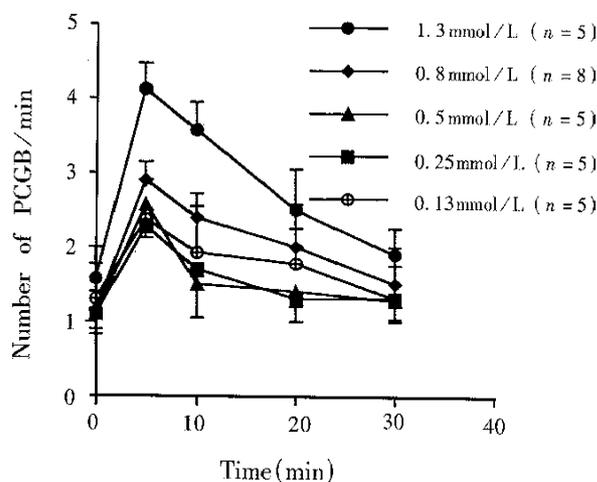
**Figure 2** The effects of microinjection of TRH (0.8 nmol/1  $\mu$ l) or NS on GP. \* $P < 0.05$  as compared with NS



**Figure 3** The effect of microinjection of TRH (0.8 nmol/1  $\mu$ l) or NS on the frequency of phasic contraction of gallbladder (FPCGB). \*\* $P < 0.01$ , \* $P < 0.05$  as compared with NS



**Figure 4** Dose-response curve for TRH stimulation on GP after microinjection into DVC.



**Figure 5** Dose-response curve for TRH stimulation on the number of phasic contraction of GB (PCGB) after microinjection into DVC.

**DISCUSSION**

In this study, we found that TRH microinjection into DVC raised GP and FPCGB. As an important brain-gut peptide, TRH in DVC plays an important role in regulating the function of gastrointestinal tract<sup>[2]</sup>. We found for the first time that TRH microinjected into DVC excited the motility of gallbladder in rabbits.

The effects of TRH microinjected into DVC on the motility and secretion of gut may be physiological. In DVC, there is a high density of TRH-immunoreactive nerve terminals which come from the nucleus raphe pallidus and raphe obscurus. Some of these terminals make synaptic contacts on the dendrites of gastric vagal motoneurons<sup>[5]</sup>. Multicolon TRH antibody injected into DVC can inhibit the gastric acid secretion<sup>[6]</sup>.

Through vagus nerve and peripheral cholinergic M receptors, TRH could excite the gallbladder

motility, but its physiological significance is unknown. Bile evacuation occurs during sham feeding, and is abolished by vagotomy<sup>[7]</sup>. After vagotomy or gastrectomy, the incidence of gallstones increases<sup>[8,9]</sup>. In this experiment, TRH in DVC raised GP and then facilitated the bile evacuation. Thus TRH may be a possible candidate for the transmitter in DVC mediating the cephalic bile output.

The present experiment showed that there was rhythmic phasic contraction of gallbladder in rabbits in the interdigestive period, which was similar to that found in dogs<sup>[10]</sup>. It was believed that this phasic contraction stirred the bile in gallbladder and prevented gallstones forming in the interdigestive period<sup>[11]</sup>. Our experimental results also showed that TRH microinjected into DVC enhanced the phasic contraction via vagus nerve. Thus TRH in DVC may be regarded as an important neurotransmitter in mediating the phasic contraction of gallbladder. Similar to Ura's data in dogs<sup>[11]</sup>, vagotomy almost inhibited the phasic contraction completely in our study. It was suggested that the tonic excitation of vagus nerve center may be important in maintaining this type of motion in the interdigestive period. TRH may participate in this effect.

In conclusion, we believed that TRH in DVC may play an important role in regulating the motility of gallbladder via vagal nerves and peripheral cholinergic M receptors, and may participate in cephalic gallbladder emptying during ingestion and maintain the phasic contraction of gallbladder in the interdigestive period.

**ACKNOWLEDGEMENT** We are grateful to Professor HE Zhu-Li, Director of the Department of English, Shandong Medical University, for polishing the manuscript.

**REFERENCES**

- 1 Liu CY, Liu JZ, Zhou JH, Wang HR, Li ZY. The effects of electric and chemical stimulation of DVC on the motility of biliary system. *Acta Acad Med Shandong*, 1997;35(3):207-210
- 2 Tache Y, Robertm L, Stephens TR, Ishikawa T. Central nervous system action of TRH to influence gastrointestinal function and ulceration. *Ann NY Scand Sci*, 1989;553(2):269-295
- 3 Richard BL, Margaret SK, Richard RM. Thyrotropin-releasing hormone-immunoreactive projections to the dorsal vagal motor nucleus and the nucleus of the solitary tract of the rat. *J Comp Neuro*, 1991;311(2):271-288
- 4 Palkovits M, Mezey E, Eskay R, Brwnstein MJ. Innervation of the nucleus of the solitary tract and the dorsal vagal nucleus by thyrotropin-releasing hormone-containing raphe neurons. *Brain Res*, 1996;373(2):246-251
- 5 Rinaman L, Richard RM. TRH immunoreactive nerve terminals synaps on the dendrites of the gastric vagal motoneurons in the rat. *J Comp Neuro*, 1990;294(2):235-251
- 6 Hernasez DE, Jenjers L, Emerick SG. Inhibition of gastric acid secretion by immunoneutralization of endogenous brain TRH. *Brain Res*, 1987;401(13):381-384
- 7 Fisher RS, Rock E, Malmud LS. Gallbladder emptying response to sham feeding in humans. *Gastroenterology*, 1986;90(5):1854-1857<sup>as</sup>
- 8 Hauters P, Roden A, Pourbaix A, Aupaix F, Couman P. Therasse G cholelithiasis: a serious complication after total gastrectomy. *Br J Surg*, 1988; 107(3):899-900
- 9 Ihasz M, Griffith CA. Gallstones after vagotomy. *Am J Surg*, 1981;141(1): 48-50
- 10 Matsumato T, Sarna SK, Condon RE, Dodds WJ, Mochinaga N. Caine gallbladder cyclic motor activity. *Am J Physiol*, 1985;255(2):G409-416
- 11 Ura K, Sarna K, Condon RE. Antral control of gallbladder cyclic motor activity on the fasting state. *Gastroenterology*, 1992;102(1):295-302

# Cloning and expression of NS3 cDNA fragment of HCV genome of Hebei isolate in *E. coli*

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**Subject headings** hepatitis C virus; NS3 gene; gene expression; DNA, viral; viral proteins; sequence analysis; polymerase chain reaction; enzyme-linked immunosorbent assay; *Escherichia coli*

## Abstract

**AIM** To obtain greater antigenicity of HCV NS3 protein.

**METHODS** The HCV NS3 cDNA fragment was amplified by reverse transcription polymerase chain reaction from the sera of the HCV infected patients. The DNA sequence was determined by dideoxy-mediated chain termination method using T7 polymerase. HCV NS3 protein was expressed in *E. coli*.

**RESULTS** Sequence analysis indicated that the HCV isolate of this study belongs to HCV-II; SDS-PAGE demonstrated an Mr 23 800 and an Mr 22 000 recombinant protein band which amount to 14% and 11% of the total bacterial proteins separately. Western blotting and ELISA showed NS3 protein possessed greater antigenicity.

**CONCLUSION** Recombinant HCV NS3 protein was expressed successfully, which provided the basis for developing HCV diagnostic reagents.

## INTRODUCTION

Since HCV (hepatitis C virus) was identified as the major cause of post-trans fusional non-A, non-B hepatitis by Choo *et al* in 1989<sup>[1]</sup>, the harm of this infectious disease to the human health has been gradually realized. Up to 50%-60% of the HCV hepatitis cases turn chronic, developed into cirrhosis and hepatocellular carcinoma. Due to lack of effective therapeutic methods, it is exceptionally important to prevent the transmission of HCV. As HCV is mainly transmitted through blood sources, the key to preventing the transmission of HCV is to screen the blood donors using specific and sensitive HCV diagnostic reagents. HCV NS3 antigen is necessary in anti-HCV diagnostic reagents because of the lower divergence in NS3 region of HCV genome and the strong antigenicity of NS3 protein. In addition, because of the early appearance and high incidence of the antibody against NS3 antigen. In this report, the HCV NS3 gene fragment was cloned and expressed in *E. coli*. The obtained NS3 antigen showed strong antigenicity, which provided the basis for developing HCV ELISA reagents.

## MATERIALS AND METHODS

### *Sera and extraction of HCV RNA*

Serum for cDNA synthesis was derived from a patient who comes from Hebei Province, which is positive for anti-HCV and HCV RNA. HCV RNA was extracted by single step of guanidinium-phenol-chloroform method<sup>[2]</sup>. Ninety-four serum specimens for ELISA were screened from 460 sera derived from patients with different types of hepatitis, which are anti-HCV positive tested by Abbott second generation HCV ELISA, provided by the Institute of Hepatitis, Youan Hospital.

### *Expression vectors*

Expression vectors are pRX vectors with trpE promoter<sup>[3]</sup> and pMY vector with p<sub>RP</sub>L promoter. pRX vector expresses fusion protein with 18 amino acid at N-termini of the protein induced by IPTG. pMY vector expresses non-fusion protein induced by heat at 42°C.

### *Primers designed for reverse transcription polymerase chain reaction*

HCV NS3 protein antigenicity analysis was conducted with computer according to HCV-BK<sup>[4]</sup> sequence.

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**Received** 1997-09-21 **Revised** 1998-02-22

Referred to the analysis results, the conservative region was selected and the primers were designed as follows:

Outer primer:F1 5'GTTGCGAAGGCGGTGGACTT 3'

R1 5'GTCGTCTCAATGGTGAAGGT 3'

Inner primer:F2 5'GGAATTCTCCGGCTGCATATGCA 3'

R2 5'CCATCGATAGGTATAGCCCGTCAT 3'

To facilitate subcloning, EcoR I and Cla I restriction endonuclease sites were added at the 5'-termini of the inner primer. The outer reverse primer was used in both cDNA synthesis and cDNA amplification.

### Reverse transcription polymerase chain reaction

According to the methods by Widell and Cristiano<sup>[5,6]</sup>, before reverse transcription, RNA template was denatured at 94°C for 5 min, then on ice promptly. Reverse transcription and first round of PCR were run in the same buffer at the same tube. Reaction volume was 100µl, containing AMV 1.6U and Taq DNA polymerase 2.5U. The reaction order is reverse transcription at 42°C for 30min, inactivation of reverse transcriptase 94°C for 3min, and PCR consisting of 94°C 55s, 42°C 1min, 72°C 1.5min for 5 cycles, and 94°C 55s, 55°C 1min, 72°C 1.5min for another 30 cycles. One tenth of the first round of PCR products were used at template to conduct the second round of PCR, the conditions were the same as the last 30 cycles of the first round of PCR. Finally, PCR products were analyzed on 15g/L agarose gel electrophoresis.

### Sequencing of cDNA fragment

The cDNA fragment was subcloned into M13 mp 18/19 vectors and single stranded DNA template was prepared. Sequencing reaction was performed using T7 sequencing kit. Nucleotide and amino acid homology analysis were conducted using Goldkey program.

### Construction of recombinant plasmids and expression of proteins

cDNA fragments and vectors were cleaved by restrictive endonuclease, ligated with T4 DNA ligase, the *E. coli* HB101 was transformed. The recombinant plasmids were identified through restrictive endonuclease digestion<sup>[7]</sup>. *E. coli* HB101 carrying recombinant plasmid were inoculated in M9 media. After incubation at 30°C for about two hours, the 3β-indolyl acrylic acid was added to the final concentration of 10mg/L, and the bacteria were cultured for 5 hours to express the protein. *E. coli* HB101 carrying recombinant plasmid pMYNS3 were cultured at 30°C for about two hours, then transferred to 42°C for another 5 hours to express the recombinant protein.

### Identification of the expressed proteins

Twelve percent SDS-PAGE and Western blotting were used to identify the expressed proteins. The sample was prepared as follows: removing 1ml of induced culture, centrifuging to collect the pellet, resuspending the pellet and boiling for 5min to lyse the bacteria with 2×SDS loading buffer. After the electrophoresis, the polyacrylamide gel was split into two pieces. One piece of gel was stained with Coomassie Brilliant Blue R-250, and scanned to analyze the expression level of the recombinant protein. The other piece was blotted to nitrocellulose membrane. Blotting condition was 0.8m·cm<sup>2</sup>·1.5 hours. The blotted membrane was incubated with blocking solution containing 10ml/L fetal bovine serum for one hour, then incubated with anti-HCV positive serum (diluted 1:100) at 4°C overnight. The following day, the membrane was incubated with sheep anti-human IgG-HRP (diluted 1:500) at room temperature for 30min, finally stained with substrate 3, 3'-diaminobenzidine.

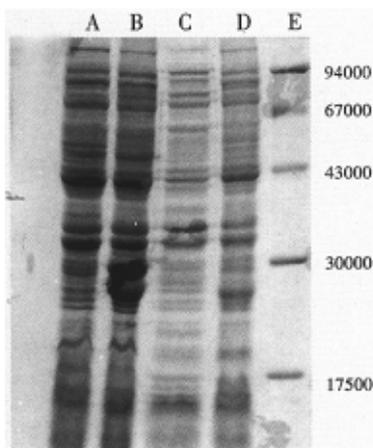
### ELISA

The recombinant protein expressed by pMYNS3 was purified by ion-exchange chromatography, and the purified protein was used as antigen to test 94 control positive sera by ELISA.

### RESULTS

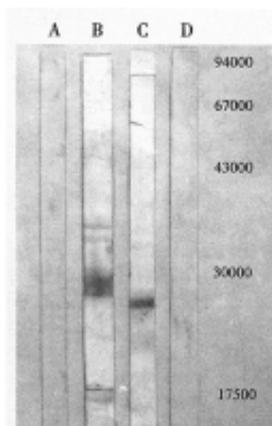
The PCR products were analyzed on 15g/L agarose gel electrophoresis, and the expected 634bp cDNA fragment was clearly seen. Sequencing results revealed that the cDNA fragment is 634bp, and proved to be HCV NS3 fragment in comparison of the sequence of cDNA fragment and the corresponding region of HCV-BK. Its nucleotide and amino acid homology with genotype II isolate HCV-BK, genotype I isolate HCV1, genotype III isolate HCV-J6 and genotype IV isolate HCV-J8 were 90.8% and 95%, 80% and 94.9%, 73.2% and 88.2%, 73.5% and 86.7%, respectively<sup>[8-10]</sup>. These results indicated that the isolate in this study belongs to HCV-II. In addition, we also compared its homology with the sequence of corresponding region of HCV isolates derived from the patients who came from Hebei and Taiwan separately, the nucleotide and amino acid sequence homology were 90.4% and 95.4%, 90.8% and 93.8%, respectively<sup>[11,12]</sup>. SDS-PAGE analysis demonstrated that pRXNS3 plasmid expressed a protein with a molecular weight of about Mr 23 800 which amounts to 14% of the total bacterial proteins (Figure 1). pMYNS3 plasmid expressed a molecular weight of about Mr 22 000 protein, which covers 11% of the total bacterial proteins (Figure 1). Two

forms proteins strongly reacted with anti-HCV antibodies in the sera in western blotting test (Figure 2). Using the purified pMYNS3 expressed recombinant protein as an antigen to test 94 control anti-HCV positive sera, 85 (90.4%) of 94 were detected. Among the 94 sera samples, 28 (96.6%) of 29 derived from patients with known transfusion history were detectable. Other sera derived from patients diagnosed clinically as having acute, chronic hepatitis and cirrhosis, the detection rates were 83.3% (10/12) for acute hepatitis, 86.8% (33/38) for chronic hepatitis and 93.3% (14/15) for cirrhosis, respectively.



**Figure 1** SDS-PAGE analysis of HCV NS3 fusion and non-fusion proteins expressed in *E. coli*

A: Control HB101/pRX      B: Induced HB101/pRXNS3  
C: Control HB101/pMY      D: Induced HB101/pMYNS3  
E: Molecular weight standards



**Figure 2** Western blot analysis of HCV NS3 recombinant proteins

A: Control HB101/pRX      B: Induced HB101/pRXNS3  
C: Induced HB101/pMYNS3      D: Control HB101/pMY

## DISCUSSION

In this report, the NS3 fragment of HCV genome was reversed transcribed and amplified successfully from the serum of a patient who comes from Hebei Province, and the cDNA fragment was sequenced

and expressed with high level in *E. coli*. The results of western blotting and ELISA showed greater antigenicity of the recombinant protein, which demonstrated good prospects of using the protein as an antigen to detect anti-HCV antibodies. The nucleotide and amino acid homology analysis indicated that the HCV isolate in this study should belong to HCV-II. Another HCV isolate derived from the same area is also HCV-II<sup>[11]</sup>, which suggested in some extent that the endemic HCV isolates in this region might be HCV-II. The sequence comparison of NS3 region of HCV between different isolates showed that NS3 region is the relative conservative region in HCV genome, in addition, the amino acid homology is higher than nucleotide homology, so using NS3 protein as antigen to detect anti-HCV antibodies will be characterized by a wide coverage of HCV infection. The prokaryotic expression vector pRX and pMY were used to express HCV NS3 protein. pRX vector with *trpE* promoter is induced by 3-indolyl acrylic acid and expresses a fusion-protein with 18 amino acid at N-termini of the protein. pMY vector with *p<sub>R</sub>P<sub>L</sub>* promoter is induced by heating at 42 °C and expresses non-fusion protein. We found that the expression level of fusion protein is higher than that of non-fusion protein. The possible explanation is that the proteinase of host bacteria will not recognize the recombinant fusion protein as non-self protein to lyse it. The fusion of bacterial protein to recombinant protein increases the stability of protein and raises the expression level. The comparison between the fusion and non-fusion protein in antigenicity showed no obvious difference. Because pMYNS3 is induced easily by heat, we used the protein expressed by pMYNS3 as coating antigen in ELISA. This study estimated the diagnostic value of NS3 recombinant antigen using 94 control anti-HCV positive sera which are detectable with Abbott second generation ELISA. The results showed that NS3 antigen can detect 85 (90.4%) of 94 of control sera, among these, 28 (96.6%) of 29 sera derived from hepatitis patients with known transfusion history were detected. These results consistent with the results of Abbott's ELISA test. The successful expression of HCV NS3 protein has provided an ideal basis for developing anti-HCV diagnostic reagents.

## REFERENCES

- 1 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*, 1989;244(4902):359-362
- 2 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 1987;162(1):156-159
- 3 Rimm DL, Pollard TD. New plasmid vectors for high level synthesis of eukaryotic fusion proteins in *Escherichia coli*. *Gene*, 1989;75(2):323-327
- 4 Takamizawa A, Mori C, Fuke I, Manabe S, Murahami S, Fujika J *et al.* Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol*, 1991;65(3):1105-1113<sup>a</sup>
- 5 Widell A, Mansson AS, Sundstr-n G, Hansson BG, Nordenfelt E. Hepatitis C virus RNA in blood donor sera detected by the polymerase chain reaction: comparison with supplementary hepatitis C virus antibody assays. *J Med Virol*, 1991;35

- (4):253-258
- 6 Cristiano K, Di Bisceglie AM, Hoofnagle JH, Feinstone M. Hepatitis C viral RNA in serum of with chronic non-A, non-B hepatitis: detection by the polymerase chain reaction using multiple primer sets. *Hepatology*, 1991;14(1): 51-55
- 7 Sambrook KJ, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed, New York: *Cold Spring Harbor Harbor Laboratory*, 1989:3-101
- 8 Choo Q-L, Richman KH, Han JH, Berger K, Lee C, Dong C et al. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA*, 1990;88(6):2451-2455
- 9 Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A et al. Nucleotide sequence of the hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J Gen Virol*, 1991;72(11):2697-2704
- 10 Okamoto H, Kurai K, Okada SI, Yamamoto K, Lizuka H, Tanaka T et al. Full length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. *Virology*, 1992;188(1): 331-341
- 11 Bi SL, Bai XH, Cong ME, Tian HW, Sun DG, Margolis HS et al. Primary structure and variation of Chinese hepatitis C virus genome. *Acta Virol*, 1993;9 (2):114-127
- 12 Chen PJ, Lin MH, Tai KF, Liu PC, Lin CJ, Chen DS. The Taiwanese hepatitis C virus genome: sequence determination and mapping the 5' termini of viral genomic and antigenomic RNA. *Virology*, 1992;188(1):102-113

# Short-term effect of *Salvia miltiorrhiza* in treating rat acetic acid chronic gastric ulcer and long-term effect in preventing recurrence

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**Subject headings** Danshen; disease models, animal; *Salvia miltiorrhiza*; stomach ulcer; acetic acid; cimetidine; gastric mucosa; recurrence; rats, Wistar; chronic diseases

## Abstract

**AIM** To study the short-term effect of Danshen (*Salvia miltiorrhiza*) on acetic acid induced chronic gastric ulcer in rats and its long-term effect in preventing recurrence.

**METHODS** Rats with acetic acid-induced gastric ulcer were treated with Danshen and cimetidine for 30 days. Traditional gastric mucosal auto-radiography and <sup>3</sup>H-TdR incorporation into gastric mucosa in vitro were employed to study the effects of Danshen in rat acetic acid-induced chronic gastric ulcer, including ulcer index (UI), ulcer inhibitory rate (IR) and label rate (LR).

**RESULTS** On the day 5, 30 and 126 of ulcer-making, the UI in the Danshen group was obviously lower than that in the cimetidine group and the control group (42.3±3.9, 3.6±1.2, 4.4±2.3; 49.1±3.6, 5.9±1.4, 9.2±1.3; 61.0±3.8, 8.9±2.5, 12.4±2.4, respectively,  $P<0.01$ ), the IR (%) in the Danshen group was obviously higher than that in the cimetidine group (31, 59, 64.8; 19, 33, 26, respectively), and the LR in the Danshen group was obviously higher than that in the cimetidine group and the control group (10.0±0.5, 16.2±0.8, 15.0±0.6; 9.0±0.5, 13.9±0.6, 10.8±0.7; 6.5±0.7, 10.1±0.5, 8.0±0.7, respectively,  $P<0.01$ ). There was no obvious difference in UI in the

Danshen group on day 30 as compared with that on day 126.

**CONCLUSION** Danshen is effective in promoting ulcer healing and preventing recurrence. The mechanism of action is to strengthen the gastric mucosal barrier and to promote the gastric mucosal cell proliferation along the edge of the ulcer.

## INTRODUCTION

Human peptic ulcer is characterized by repeated relapses and difficulty in prevention<sup>[1]</sup>. So, the emphasis of treatment of peptic ulcer should be put on preventing relapse. A rat model of acetic acid-induced chronic gastric ulcer has the features of repeated relapses and is similar to that of the human case<sup>[2,3]</sup>. This study is to observe the effect of the traditional Chinese medicine Danshen (*Salvia miltiorrhiza*) on the rat chronic gastric ulcer induced by acetic acid and its effect in preventing recurrence, and the relationship between the therapeutic effect and regeneration of gastric mucosal cells on the edge of the ulcer.

## MATERIALS AND METHODS

### Materials

Danshen water solution: prepared according to the reported method (240 g/L and 120 g/L)<sup>[4]</sup>. Cimetidine water solution: with cimetidine for injection (product of the 3rd Pharmaceutical Plant, Harbin), diluted to 2.4g/L and 1.2g/L water solution. <sup>3</sup>H-TdR supplied by China Atomic Scientific Institute, with radiation concentration of 18.5GBq/L.

### Methods

Rat model-making and grouping. Wistar male rats ( $n = 126$ ), weighing 210g ± 30g, were given 200ml/L acetic acid 0.05ml to create the model of chronic gastric ulcer, and then were divided into 3 groups randomly: Control group: allowed to drink water (pH 6.5) freely, continuing to day 126. Cimetidine group: During day 1-5 after ulcer was made, allowed to drink 2.4g/L cimetidine water solution (pH 6.5) freely; allowed to drink 1.2g/L

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\*Project supported by the Natural Science Foundation of China, No. 39070961.

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Received 1997-09-25 Revised 1997-12-18

cimetidine water solution freely on day 6-30; only water freely on day 31-126. Danshen Group: On day 1-5 after the ulcer is made, allowed to drink 240g/L Danshen water solution (pH 6.5) freely; 120g/L Danshen water solution freely on day 6-30; only water freely on day 31-126. Preliminary tests proved that each rat drank liquid on an average of about 35ml/d, without statistical difference.

Gastric mucosal auto-radiography. A 0.5 cm × 1cm tissue piece taken for the ulcer edge was put into 2ml 1640 culture fluid (pH 7.2-7.2) containing <sup>3</sup>H-TdR and 100ml/L beef serum, hatched in 37°C ±0.5°C CO<sub>2</sub> incubator for 1 hour, fixed for 3-6 hours in Carnoy liquid, and then embedded in paraffin, 5 μm slices were made for HE staining, coating H-4 emulsion in emulsion-coating box, and to expose for 14 days in 4°C refrigerator, and to develop, fix and take the nucleus over 5 silver granules as label cells.

Index and calculation. Ulcer index (UI)-ulcer maximum length × maximum width; ulcer inhibitory rate (IR) (%) = (1 - experimental group UI mean/control group UI mean) × 100%; label rate (LR) (%) = (label cells number/300 multiplication area cells) × 100%.

### Statistical analysis

All values were given as  $\bar{x} \pm s$ . Paired Student's *t* test was used. *P* values <0.05 were considered significant.

## RESULTS

Effect of Danshen in rat chronic gastric ulcers of different stages, UI and ulcer IR is shown in Table 1.

**Table 1 Rat chronic gastric ulcers of different stages, UI and IR ( $\bar{x} \pm s$ ) (%)**

Groups	Day 5		Day 30		Day 126			
	<i>n</i>	UI(mm) <sup>2</sup>	IR(%)	<i>n</i>	UI(mm) <sup>2</sup>	IR(%)		
Control	8	61.0±3.8		8	8.9±2.5 <sup>f</sup>	11	12.4±2.4	
Cimetidine	10	49.1±3.6 <sup>b</sup>	19	10	5.9±1.4 <sup>bf</sup>	33	11	9.2±1.3 <sup>b</sup>
Danshen	11	42.3±3.9 <sup>d</sup>	31	10	3.6±1.2 <sup>d</sup>	59	12	4.4±2.3 <sup>d</sup>

<sup>b</sup>*P*<0.01, vs control; <sup>d</sup>*P*<0.01, vs Cimetidine; <sup>f</sup>*P*<0.01, vs the same group on day 126.

**Table 2 Rat chronic gastric ulcers of different stages, IR of gastric mucosal cells (*n* = 6,  $\bar{x} \pm s$ ) (%)**

Groups	Day 5	Day 30	Day 126
Control	6.5±0.6	10.1±0.5	8.0±0.7
Cimetidine	9.0±0.5 <sup>b</sup>	13.9±0.6 <sup>b</sup>	10.8±0.7 <sup>b</sup>
Danshen	10.0±0.5 <sup>d</sup>	16.2±0.8 <sup>d</sup>	15.0±0.6 <sup>d</sup>

<sup>b</sup>*P*<0.01, vs control group, <sup>d</sup>*P*<0.01, vs cimetidine group.

Effect of Danshen in rat chronic gastric ulcers of different stages and gastric mucosal cell multiplication is shown in Table 2

## DISCUSSION

It is reported in the literature<sup>[5,6]</sup> that the natural healing process of acetic acid-induced gastric ulcer in rats is as follows: On day 1-35 UI decreased rapidly which is considered as acute stage; UI decreased steadily and slowly on day 36-80, which is a slow healing stage; UI slightly increased as the recurrence stage; UI increased obviously on day 120-140, the recurrence rate being the highest. In this experiment, we treated the ulcer with Danshen and cimetidine from day 1-30 to observe their therapeutic effects in the acute stage. During the period from day 31 to day 126 without the two drugs, we observed whether Danshen has a long-term effect in preventing ulcer recurrence. From Table 1, we can see the UI in the cimetidine group was obviously lower than in the control group (*P*<0.01), and that in the Danshen group was even lower than that in the cimetidine group (*P*<0.01). As for the ulcer IR Danshen group was definitely superior to the cimetidine group. This indicated that Danshen might promote ulcer healing. In comparison of the UI of each group on day 30 and day 126, significant difference was found in both the control and cimetidine groups (*P*<0.01), but in the Danshen group there was no significant difference on day 30 and day 126. This indicated that Danshen has significant effect in preventing recurrence. From Table 2, one could see a higher LR in the cimetidine group than in the control group (*P*<0.01), and the Danshen group had a higher rate than the cimetidine group (*P*<0.01). So, the mechanism of Danshen in promoting ulcer healing and preventing ulcer recurrence was an enhancement of gastric mucosal cell multiplication along the edge of ulcer and also a fortification of the mucosal barrier.

## REFERENCES

- 1 Tao J. On some problems of peptic ulcer. *Jpn Med Introd*, 1992;8(13): 386-387
- 2 Zhang SH, Chen L, Chen DM, Shi AR. A method of making rat acetic acid ulcer model. *Dissectol J*, 1987;10(2):158-159
- 3 Houteng YM, Sangyun KZ. Experiment gastric ulcer model. *Clin Science*, 1990;26(1):87-94
- 4 Li HQ, Chang LG, Ju BQ, Liu RD. The study of the experiment that Danshen resists ulcer action. *J China Med Uni*, 1985;4(2):89-92
- 5 Umeda Y, Nakamura K. Effects of Trimprostol on healing and recurrence of acetic acid-induced gastric ulcer in rats. *Jpn J Pharmacol*, 1986;42(8):457-463
- 6 Baimu ZY, Shanqi SY, Shisan GX, Shenbian MS, Shunei YY, Qiantian XE et al. Recurrence and relapse of acetic acid ulcer as well as effect of strengthening defence factor medicine Proamipide. *Folia Pharmacol Jpn*, 1988;92(6):389-395

# Sishengtang decoction in alleviation of toxic and side effects of transarterial embolization \*

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**Subject headings** Sishengtang decoction; liver neoplasms/therapy; embolization, therapeutic; immunity, cellular; killer cell, natural; killer cell, lymphokine-activated; leucocytes

## Abstract

**AIM** To observe the therapeutic effects of Sishengtang decoction in alleviating the toxic and side effects of transarterial embolization (TAE).

**METHODS** Fifty-four patients with liver cancer were divided randomly into Sishengtang decoction group (34 cases) and control group (20 cases). The changes of clinical symptoms and peripheral hemogram and some cellular immune functions were observed before and two weeks after TAE.

**RESULTS** Sishengtang decoction was superior to the control group in improving the digestive tract reaction. The leucocytes of peripheral blood and cellular immune functions (activities of NK cells and LAK cells) of control group decreased obviously after TAE, while that of Sishengtang decoction group decreased slightly, without obvious difference as compared with that of preoperation.

**CONCLUSIONS** Sishengtang decoction might improve the clinical symptoms and increase the leucocytes of peripheral blood and the cellular immune functions of TAE patients.

## INTRODUCTION

After clinical and experimental studies of antiradioactive reaction produced by Sishengtang decoction<sup>[1]</sup>, we studied systematically the therapeutic effects of this decoction in alleviating the toxic and side effects of chemotherapy, and observe the changes of peripheral hemogram and the cellular immune functions.

## MATERIALS AND METHODS

### General data

Of 54 patients with liver cancer, submitted for TAE from May 1995 to October 1996, 48 were males and 6 females, aged from 31 to 74 years, averaging 48.8 years. Twenty-one cases were in stage III and 33 were in stage III clinically. These patients were divided into Sishengtang decoction group (34 cases) and control group (20 cases). There were 29 males and 5 females in the Sishengtang decoction group, with an average of 49.2 years of age, and 14 cases in stage II and 20 in stage III. In the control group, there were 19 males and 1 female, averaging 48.1 years in age, 7 in stage II and 13 in stage III.

### Component of the decoction

Sishengtang decoction contains mainly: *Radix Astragali sen Hedysari* 30g, *Radix Rehmanniae* 10g, *Rhizoma Atractylodis Macrocephalac* 9g and *Somen Colcis* 15g. Components were regulated according to symptoms (RCAS): *Radix Asparagi* 15g and *Radix Dhipogonis* 15g were added for mouth dryness; *Radix Codonopsis Pilosnlac* 30g and *Poria* 15g were added for lassitude; *Rhizoma Dioscoreae* 9g, *Endothelinm Cornenm Gigeriaw Galli* 12g, *Fructus Crataegi* 12g, *Fructus Hordei Germinatus* 12g and *Massa Fermentata Medicinalis* 12g were added for anorexia.

### Administration and dosage

Sishengtang decoction and RCAS were used concomitantly in the Sishengtang decoction group. They were given a week before TAE, at 1 dose daily divided in the morning and evening. Only RCAS was used in the control group, administration and dosage were the same as the Sishengtang decoction group.

### Observation

The changes in clinical symptoms of all the 54 patients were recorded one day and 5 days respectively after TAE and the peripheral hemogram and some

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\*Project supported by the National Natural Science Foundation of China, No.39390850.

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Received 1997-12-02 Resived 1998-01-04

cellular immune functions were measured 1 week before and 2 weeks after TAE<sup>[2]</sup>. The activities of NK and LAK cells were detected by radioimmunoassay, with K<sub>502</sub> and Raji, as target cells. Detailed processes were in accordance with Reference 2 and 3.

### Statistical treatment

The data were expressed as  $\bar{x} \pm s$ , and the comparison between the two groups was made by  $\chi^2$  test and *t* test.

## RESULTS

### Changes in clinical symptoms

In 3 main clinical symptoms observed, the effect of Sishengtang decoction was superior to that of the control group (Table 1).

### Changes in laboratory indexes

There was no obvious difference in peripheral hemogram and some cellular immune functions between Sishengtang decoction group and control group before therapy, but with no obvious decline of these two indexes in the control group after therapy (Table 2).

## DISCUSSION

TAE is one of the most effective therapies for the patients who can not be operated on and for some of the patients with recurrent liver cancer after operation. But the toxic and side effects of TAE, such as decreased leucocytes in peripheral blood, reaction of digestive tract and decline of body immune

functions, not only result in great pain for patients, but also become a main factor that influences the therapeutic effects of TAE. So it has been an important topic to seek effective traditional Chinese medicine or complex prescriptions which can reduce the toxic and side effects of chemotherapy.

Sishengtang decoction is a basic prescription, made according to the theory of traditional Chinese medicine and clinical experience from the specialists to relieve the patients from the toxic and side effects of radiotherapy and chemotherapy. In this prescription, *Radix Astragali sen Hedysari* invigorates the vital energy and the spleen; *Radix Rehmanniae* nourishes *yin* and invigorates the Kidney; *Rhizoma Atractylodis Macrocephalae* and *Semen Coicis* invigorate the spleen and regulate the middle warmer, and prevent the greasy of *Radix Astragali sen Hedysari* and *Radix Rehmanniae*. This prescription acts to invigorate both vital energy and *yin*, take care of Spleen and Kidney, not only prevent the exhaustion of congenital *yin* and *yang*, but also protect the acquired essential substance from food. Four elements of this prescription are all used unpreparedly in order to prevent too much dryness or greasiness of the herbal characters, which is of great significance in maintaining the process of digestion of the middle warmer and relieving the digestive tract reaction. According to the modern pharmacological analysis, *Radix Astragali sen Hedysari* and *Rhizoma Atractylodis Macrocephalae* can improve body immune function and increase leucocytes in peripheral blood; and *Semen Coicis* has the anti-cancer effects if used unprepared. When used together, these four elements will have the functions to reduce toxicin and promote their effects.

**Table 1** Effects of Sishengtang decoction and RCAS on clinical symptoms of TAE patients ( $\bar{x} \pm s$ )

Groups	<i>n</i>	Significant improvement	Improvement	No improvement	Improvement rate(%)
Sishengtang decoction(34)					
Lassitude	28	14	12	2	93.9
Poor appetite	23	12	9	2	91.3
Nausea	23	11	9	3	86.9
Control (20)					
Lassitude	17	6	6	5	70.1 <sup>a</sup>
Poor appetite	12	5	3	4	66.7 <sup>a</sup>
Nausea	14	4	7	3	78.9 <sup>a</sup>

<sup>a</sup>*P*<0.05 vs Sishengtang decoction

**Table 2** Laboratory indexes of TAE patients in Sishengtang decoction group and control group ( $\bar{x} \pm s$ )

Groups	<i>n</i>	WBC( $\times 10^9/L$ )	PLT( $\times 10^9/L$ )	NK activities(%)	LAK activities(%)
Sishengtang decoction	34				
Before treatment		4.62 $\pm$ 0.62	123.00 $\pm$ 37.45	32.03 $\pm$ 7.25	34.07 $\pm$ 6.69
After treatment		4.44 $\pm$ 0.63	116.23 $\pm$ 29.42	31.04 $\pm$ 6.92	32.03 $\pm$ 6.76
Control	20				
Before treatment		5.31 $\pm$ 0.82	31.92 $\pm$ 47.93	141.20 $\pm$ 5.22	32.87 $\pm$ 4.85
After treatment		3.87 $\pm$ 0.38	126.80 $\pm$ 37.58	26.16 $\pm$ 7.77 <sup>a</sup>	27.16 $\pm$ 5.74 <sup>a</sup>

<sup>a</sup>*P*<0.05 vs before treatment in control

In the improvement of clinical symptoms and results of laboratory examinations, RCAS, used in the control group, though can partially improve the clinical symptoms, its effects are not as good as Sishengtang decoction. As for the increase of leucocytes in peripheral blood, the effects of Sishengtang decoction group is also superior to that of the control group. The results indicated that Sishengtang decoction can alleviate the toxic and side effects of chemotherapy.

In term of the cellular immune functions of TAE patients, the activities of NK and LAK cells of the control group were decreased markedly, but without significant difference in the Sishengtang group compared with that of preoperation. The results showed that Sishengtang decoction might in-

crease the leucocytes in peripheral blood and cellular immune functions, which is important in promoting the recovery of patients and enhancing the effects of TAE itself. The mechanism may be related to its enhancement and protection of blood production by marrow<sup>[4]</sup>.

## REFERENCES

- 1 Ling CQ, Chen LQ, Chen Z, Huang XQ. Clinical and experimental study of the resistance effects of Sishengtang decoction on toxic and side effects in radiotherapy and chemotherapy. *J Tradit Chin Med*, 1993;34(12):733
- 2 Chao XT. Basic and clinical study of Interleukin-2. *Beijing: Beijing Science and Technology Publisher*, 1990:48-53
- 3 Zhu ZY. Methodology of practical medical tests. *Beijing: People's Military Medical Publisher*, 1992:828-829
- 4 Ling CQ, Shi JH, sdsdsd Z, Zhang HW. The experimental study of Sishengtang decoction in promoting and protecting marrow blood making. *China J Basic Med Tradit Chin Med*, 1997;5(2):68

# DNA methylation and carcinogenesis in digestive neoplasms

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**Subject headings** DNA methylation; mutation; DNA methyltransferase; genes, suppressor tumor; digestive system neoplasms; p53 gene; gene expression

The DNA of vertebrates contains tissue specific patterns of methylated cytosine residues. These methylation patterns are transmitted by clonal inheritance<sup>[1]</sup> through the strong preference of mammalian DNA (cytosine-5) methyltransferase (DNA-MTase) for hemimethylated DNA<sup>[2]</sup>, and are established during early embryogenesis and gametogenesis<sup>[3]</sup>. Newly replicated DNA lacks this methylation in the nascent strand. Shortly after the passage of the replication fork, a maintenance DNA-MTase methylates CpG dinucleotides on the newly synthesized strand, thereby recreating the spectrum of methyl groups that existed prior to replication. DNA methylation (5-methylcytosine<sup>[5-mCyt]</sup> content of DNA) plays a considerable role in both normal development and carcinogenesis<sup>[4]</sup>, but there seems to be conflicting reports concerning the role of DNA methylation in carcinogenesis. It has been hypothesized that hypomethylation of DNA facilitates aberrant gene expression in tumorigenesis<sup>[5]</sup>. Some believe that hypermethylation of DNA leads to the causative alteration in tumorigenesis which involves inactivating tumor suppressor genes and marking chromosome regions for deletion<sup>[6]</sup>. Some overlooked the importance of alterations in gene expression and thought that mutation played the key role<sup>[4]</sup>. All these help to achieve a better understanding of the mechanisms underlying carcinogenesis. We believe that carcinogenesis is a multistep/multistage process that occurs in animals<sup>[7]</sup> with more carcinogenesis than mutagenesis, and that DNA methylation played multiple roles in the transformation from a normal cell into a frank malignancy.

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**Received** 1997-09-13 **Revised** 1997-10-20

## DNA METHYLATION AND CANCER

Mutations which occur at CpG dinucleotides in vertebrate DNA can be attributed to the hydrolytic deamination of 5-mCyt and are easily recognized because of the nature of base substitution. Deamination of 5-mCyt dinucleotides results in the formation of thyminephosphoguanosine (TpG). This still does not account for the high frequency of mutagenesis observed at CpG sites. Differences in the repairing efficiencies of promutagenic lesions may be partly responsible for this discrepancy. The guanosine-thymine (G-T) misrepairs resulting from deamination of 5-mCyt are believed to be more difficult for the cell to repair, as thymine is a normal component of DNA.

Mutations in the evolutionarily conserved codons of the p53 tumor suppressor gene are common in diverse types of human cancer. The p53 mutational spectrum differs among cancers of the colon, esophagus and liver. Transitions predominate in colon, whereas G:C to T:A transversions are the most frequent substitutions observed in cancers of the liver. Mutations at adenine-thymine (A:T) base pairs are seen more frequently in esophageal carcinomas than in other solid tumors. Most transitions in colorectal carcinomas are at CpG dinucleotide mutational hot spots. G-T transversions in esophageal carcinomas are dispersed among numerous codons<sup>[8]</sup>. One copy of the short arm of chromosome 17, which harbors the p53 gene, is lost in many human tumors including those of the colon and rectum. In the colon carcinomas 75% - 80% show a loss of both p53 alleles, one through deletion, the other through a point mutation. The point mutations are usually missense, giving rise to an altered protein<sup>[9]</sup>. Cytosine-thymine (C-T) transitions at CpG sites are the most prevalent mutations found in the p53 tumor suppressor gene in human colon tumors and in the germline (Li-Fraumeni syndrome). All of the mutational hot spots are methylated to 5-mCyt, and it has been hypothesized that the majority of these mutations are caused by spontaneous hydrolytic deamination of this base to thymine<sup>[10]</sup>. It is estimated that 75%-90% of hepatocellular carcinoma (HCC) cases are attributable to hepatitis B virus. HBV DNA integrates into HCC cells at random sites in the genome. It contains the X gene, which codes for a protein HBX that modu-

lates the transactivation of many cellular genes and is a candidate viral oncoprotein. HBX protein binds with p53 *in vitro* and *in vivo*<sup>[11]</sup>, inhibits p53 sequence-specific DNA binding and transactivation activities, partially disrupts p53 oligomerization and prevents p53 binding to transcription-repair coupling factor ERCC3<sup>[12]</sup>. HBV infection alone does not influence the rate of p53 mutation, and aflatoxin B1 (AFB1) exposure is the most important factor influencing mutation prevalence. AFB1 plays an etiological role in HCC carcinogenesis and indicates a synergy between HBV and AFB1<sup>[13]</sup>. In patients with liver tumors from geographical areas where both AFB1 and HBV are cancer risk factors, most mutations are at one nucleotide pair of codon 249<sup>[8]</sup>. Multiple genetic alterations occur in gastric carcinomas, including point mutation of the *ras* oncogene and the p53 tumor suppressor gene, amplification of the *c-met*, *k-sam*, and *c-erbB-2/neu* genes, and loss of heterozygosity (LOH) of the *bcl-2*, *APC*, and *DCC* genes. Genetic changes in the transforming growth factor beta (TGF- $\beta$ ) type II receptor gene or altered expression of its messenger RNA<sup>[14]</sup> occur commonly in gastric cancer cells resistant to growth inhibitory action of TGF- $\beta$ . Inactivation of the p53 gene through mutations and the allelic deletion may play an important role in gastric tumorigenesis. These mutations may cause a conformational change in the p53 protein, resulting in the loss of p53 suppression of the gastric cell growth partly through disruption of the association of p53 protein with a cellular component<sup>[15]</sup>. Allelic loss and mutation of the p53 gene are detected in over 60% of gastric cancers regardless of the histological type. Several gastric cancer cell lines have shown p53 gene abnormalities<sup>[16]</sup>. The *c-met* gene encoding the receptor for hepatocyte growth factor (HGF) is often amplified in advanced gastric cancer, particularly in 39% of scirrhous carcinomas, whereas *c-met* amplification is extremely rare in esophageal and colorectal cancers<sup>[17]</sup>. The interaction of *c-met* overexpressed tumor cells and HGF from activated stromal cells is involved in the morphogenesis and progression of gastric cancer. The *K-sam* gene encoding receptor for keratinocyte growth factor is amplified preferentially in the poorly differentiated scirrhous cancer<sup>[18]</sup>. However, no *K-sam* amplification is seen in esophageal and colorectal cancers. In general, *K-sam* amplification is independent of *c-met* amplification in scirrhous carcinoma, and *c-erbB-2* gene amplification is detected only in well differentiated gastric cancer. Moreover, overexpression of *c-erbB-2* is closely correlated with liver metastases. *c-Ki-ras* point mutation is observed in 9%-18% of well differentiated gastric cancers but not in poorly differentiated ones<sup>[19]</sup>.

## MECHANISMS OF DNA METHYLATION IN CARCINOGENESIS

Both general hypomethylation and regional hypermethylation coexist in the genome of a wide variety of human and animal cancers<sup>[5,6]</sup>. It has been suggested that changes in methylation may not play a causal role in carcinogenesis and could be consequences of the transformed state of tumor cells and that C-T transitions brought about by increased expression of the DNA methyltransferase play the key role<sup>[4]</sup>. Beside DNA MTase induced mutations additional factors appear to be involved in the cancer process. Cancers originate from a single cell that is changed dramatically by a series of alterations to the genome, e.g., mutation and changes in methylation altered gene expression. Mutagenesis plays a role in carcinogenesis. Mutated gene must be expressed to exert an effect.

It has been suggested that reduction of DNA MTase activity would lead to marked hypomethylation which can inhibit tumorigenesis<sup>[4,20]</sup>. Since DNA methylation is critical in development and differentiation<sup>[21]</sup> of tumors, it is reasonable to propose that hypomethylation at an intermediate level plays a key role in carcinogenesis while excessive hypomethylation may not be compatible with the life of the affected cells (e.g., owing to massive deregulation of gene expression). By this we mean that initiated cells may die under the severe conditions of hypomethylation and would not be available to form tumors. Thus the fact that inhibition of methylation may decrease tumor formation does not prove that excessive DNA MTase activity is the sole mechanism underlying carcinogenesis.

There are mechanistically and theoretically plausible genotoxic mechanisms that support the roles of both hypomethylation and hypermethylation of DNA (i.e. epigenetic changes) in carcinogenesis. These involve mainly alterations in normal gene expression (including tumor suppressor genes). Altered DNA methylation not only affects gene expression but also facilitates mutations, as 5-mCyt can deaminate spontaneously to Thymine (T). This indicates that elevated expression of the DNA MTase may lead to increased 5-mCyt, and this can increase the probability of C to T transitions<sup>[4,22,23]</sup>. Moderate DNA-MTase increases are not merely bystander effects, but could rather constitute one of the earliest and fundamental changes of neoplastic development<sup>[23]</sup>. Hypomethylation of DNA is associated with increased gene expression<sup>[24]</sup>. Additionally, a decreased capacity or fidelity of maintaining the normal methylation status of DNA may underlie the sensitivity of some mouse strains to liver tumorigenesis, in which increased expression of oncogenes appears to be involved by facilitating tumor promotion<sup>[5,25,27]</sup>. The

principal characteristic of the promotion stage of the carcinogenesis that distinguishes it from the stages of initiation and progression is its operational reversibility, i.e., clones of initiated cells regress when the promoting agent is withdrawn. The promotion stage ends when a lesion attains the capacity for growth in the absence of a promotion stimulus, i.e., when it is no longer reversible and can progress to a frank malignancy<sup>[7]</sup>. Hypermethylation of specific regions of DNA has been identified in cancer cells. For example, the Von Hippel Landau (VHL) tumor suppressor gene is hypermethylated and inactivated in a fraction of renal cell lines and tumors that did not have mutations in the coding regions sequenced<sup>[6]</sup>. Regional chromosomal hypermethylation is related to areas believed to contain tumor suppressor genes at various target sites. This regional hypermethylation in portions of the genome normally unmethylated may inactivate tumor suppressor genes. The functional significance would be the same as an inactivating mutation or as the loss of an allele. Regional hypermethylation of the retinoblastoma gene appears to inhibit transcription of this tumor suppressor genes<sup>[26]</sup>. Hypermethylation of the 5' CpG island of CDKN2/p16/MTS (tumor suppressor gene located on chromosome 9p21) is frequent in cell lines and primary lung tumors, gliomas and head and neck squamous cell carcinomas. Furthermore, inactivation through DNA methylation can occur not only in neoplasms where deletion is frequent in cell lines (breast, renal cells) but also in those which are not commonly associated with loss of p16 through homozygous deletion (colon and prostate). Hypermethylation of the p16 gene promoter region is a common abnormality of p16 gene in human cancer<sup>[27]</sup>. Nearly all acute leukemias and 50% of chronic leukemias and lymphomas show extensive methylation of the estrogen receptor (ER) gene CpG island<sup>[28]</sup>. Mutation in the p53 tumor suppressor gene is believed to occur in more than half of all solid tumors<sup>[29]</sup>. Approximately 24% of point mutations of p53 in human cancers are C-T transitions at CpG dinucleotides<sup>[29]</sup>. All of the mutational hot spots in the p53 gene occurring at CpG are methylated<sup>[30-32]</sup>, suggesting an involvement of 5-mCyt as an endogenous mutagen. It is important to not only determine which tumor suppressor genes lie in specific regions and their normal functions but also demonstrate a causative role, perhaps by reversing their inactivation. Altered DNA methylation leads to 5-mCyt which has intrinsic hypermutability as compared with C via deamination. This causes C-T transitions<sup>[22]</sup>. In the presence of low levels of S-adenosyl methionine, DNA-MTase may be able to form uracil leading to C-T transitions<sup>[22]</sup>. Therefore, the high rate of mutation at CpG dinucleotides may be due, in part, to DNA-

MTase mediated deamination<sup>[4,23]</sup>, and inhibition of DNA mismatch repair<sup>[23]</sup>. Additionally, 5-mCyt may influence carcinogenesis via inhibition of DNA repair, leading to fixation of promutagenic lesions<sup>[5,25]</sup>. However, the high percentage of mutations at CpG sites can not be explained solely by the presence of 5-mCyt, as the methylation patterns of the p53 gene are tissue independent, suggesting that tissue specific methylation does not contribute to the different mutation patterns at CpG sites seen in tumors<sup>[32]</sup>. This supports the contention that the effect of DNA methylation alterations may vary in different tissues and in the genesis of different tumor types.

The complex nature of the methylation role in region of gene expression is illustrated by the insulin growth factor type II receptor (Igf2r) gene. Both hypomethylation of the 5' flanking region and methylation of a specific CpG site in an intron are required for the occurrence of expression. The latter appears to be an imprinting signal, and hypomethylation at this site may silence the gene even if the 5' flanking region remains hypomethylated<sup>[33]</sup>.

The epigenetic properties of DNA methylation are heritable and unlike the mutagenic effects of 5-mCyt which do not involve alterations of the primary DNA sequence. Methylation of cytosine residues contained in CpG islands of certain genes has been inversely correlated with gene activity, but it is still unclear whether this methylation is actually responsible for different activity states of a gene or is merely the result of such changes. Methylation at a CpG islands may lead to decreased gene expression by a variety of mechanisms including disruption of local chromatin structure, inhibition of transcription factor DNA, or by recruitment of proteins which interact specifically with methylated sequences indirectly preventing transcription factor binding<sup>[5,34]</sup>. Increased methylation and heterochromatization of CpG islands have been proposed as a mechanism for silencing the expression of non-essential genes during the establishment of immortal cell lines.

## CONCLUSION

The current literature provides a compelling basis for suggesting that mutations arising secondarily to deamination of 5-mCyt, C or both are an important source of critical point mutations. Mutation, altered gene expression, hypomethylation and hypermethylation may be all related to carcinogenesis which are not mutually exclusive. Hypomethylation, hypermethylation and mutations may be important based on different situations. The examination of DNA methylation status provides the potential to discover alterations in gene expression, cell proliferation, mutation, chromatin aberrations and inactivation/

deletion of tumor suppressor genes in multifaceted approach that fits the multistep process of carcinogenesis. This notion can be supported by the depiction of human colon carcinogenesis in which roles for hypomethylation of DNA, mutation and tumor suppressor gene inactivation are considered to be relevant to the ultimate tumor formation<sup>[5,25]</sup>. There is a need to address the functional significance of specific changes in methylation (e.g., how the binding of transacting factors to specific genes is affected by methylation), and changes in methylation that occur in target tissues prior to the appearance of frank malignancies. The overall goal should be an understanding of changes in methylation and how they facilitate movement of cells through the different stages of carcinogenesis. This can be accomplished by keeping in perspective the fact that cancer is a disease of the whole entity, and thus there is a need to focus, though not exclusively, on *in vivo* studies.

## REFERENCES

- Wigler M, Levy D, Perucho M. The somatic replication of DNA methylation. *Cell*, 1981;24(1):33-40
- Graunbaum Y, Cedar H, Razin A. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature*, 1982;295(5850):620-622
- Chaillet JR, Vogt TF, Beier DR, Leder P. Parental specific methylation of an imprinted transgene is established during gametogenesis and progressive changes during embryogenesis. *Cell*, 1991;66(1):77-83
- Laird PW, Grusby JL, Fazeli A, Dickson S, Edward JW, Li E *et al.* Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, 1995;81(2):197-205
- Count JL, Goodman JL. Hypomethylation of DNA: an epigenetic mechanism involved in tumor promotion. *Mol Carcinogen*, 1994;11(1):185-188
- Herman JG, Latif F, Weng YK, Lerman ML, Zbar B, Liu S *et al.* Silencing of the VHL tumor suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci USA*, 1994;91(21):9700-9704
- Pitot HC, Dragon YP. Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J*, 1991;5(9):2280-2286
- Hollestein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. *Science*, 1991;253(5015):49-53
- Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature*, 1991;351(6326):453-456
- Schumutte C, Yang AS, Nguyen TDT, Beart RW, Jones PA. Mechanisms for the involvement of DNA methylation in colon carcinogenesis. *Cancer Res*, 1996;56(10):2375-2381
- Feitelson MA, Zhu M, Duan LX, London WT. Hepatitis BX antigen and p53 are associated *in vitro* and in liver tissues from patients with primary hepatocellular carcinoma. *Oncogene*, 1993;8(5):1109-1117
- Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR, Harris CC. Hepatitis B virus X protein inhibits p53 sequence specific DNA binding transcriptional activity, and association with transcription factor ERCC3. *Proc Natl Acad Sci USA*, 1994;91(6):2230-2234
- Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT *et al.* Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*, 1992;339(8799):943-946
- Park K, Kim SJ, Bang YJ, Park JG, Kim NK, Robert AB *et al.* Genetic changes in the transforming growth factor beta type II receptor (TGF- $\beta$ ) gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF- $\beta$ . *Proc Natl Acad Sci USA*, 1994;91(19):8772-8776
- Matozoki T, Sakamoto C, Suzuki T, Matsuda K, Uchida T, Nakano O *et al.* p53 gene mutations in the human gastric cancer: wild type p53 but not mutant p53 suppresses growth of human gastric cancer cells. *Cancer Res*, 1992;52(16):4335-4341
- Tahara E, Sembo S, Tahara H. Molecular biological observations in gastric cancer. *Seminars in Oncology*, 1996;23(3):307-315
- Kuniyasu H, Yasu W, Kitada Y, Yokozaki H, Ito H, Tahara E. Frequent amplification of the met gene in scirrhous type stomach cancer. *Biochem Biophys Res Commun*, 1992;189(1):227-232
- Katoh M, Terada M. Oncogenes and tumor suppressor genes. In: Nishi M, Ichikawa H, Nakajima T *et al.*, eds. Gastric cancer. Tokyo: Springer-Verlag, 1993:196-208
- Tahara E. Molecular mechanisms of stomach carcinogenesis. *J Cancer Res Clin Oncol*, 1993;119(5):265-272
- Macleod AR, Szyf M. Expression of antisense to DNA methyltransferase mRNA induces DNA demethylation and inhibits tumorigenesis. *J Biol Chem*, 1995;270(14):8037-8043
- Li E, Beard C, Jaenisch R. Role for demethylation in genomic imprinting. *Nature*, 1993;366(6453):362-365
- Yang AS, Shen JC, Zingg JM, Mi S, Jones PS. Hha I and Hha II DNA methyltransferases bind DNA mismatches methylate uracil and block DNA repair. *Nucleic Acids Res*, 1995;23(8):1380-1387
- Wu J, Herman JG, Wilson G, Lee YR, Chiu Yen RW, Mabry M *et al.* Expression of prokaryotic Hha I DNA methyltransferase is transforming and lethal to NIH 3T3 cells. *Cancer Res*, 1996;56(3):616-622
- Ferguson AT, Lapidus RG, Baylin SB, Davidson NE. Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer can reactivate estrogen receptor gene expression. *Cancer Res*, 1995;55(11):2279-2283
- Counts JL, Goodman JL. Molecular and cellular mechanisms. In: Liver regeneration and carcinogen. *New York: Academic Press*, 1995:227-255
- Greger V, Debus N, Lohmann D, Hopping W, Passage E, Horsthemke B. Frequency and parental origin of hypermethylated Rb1 alleles in retinoblastoma. *Hum Genet*, 1994;94(5):491-496
- Herman JG, Merlo A, Mao L, Lapidus RG, Issa JJP, Davidson NE. Inactivation of the CDKN2/p16/MTS gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res*, 1995;55(20):4525-4530
- Issa JJP, Zehnbauser BA, Civin CI, Collector MI, Sharkis SJ, Davidson NE *et al.* The estrogen receptor CpG island is methylated in most haemopoietic neoplasms. *Cancer Res*, 1996;56(5):973-977
- Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer aetiology and molecular pathogenesis. *Cancer Res*, 1994;54(18):4855-4878
- Rideout WM, Coetzee GA, Olumi AF, Jones PA. 5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science*, 1990;249(4974):1288-1290
- Mague AN, Jones PA. Ubiquitous and tenacious methylation of the CpG site in codon 248 of the p53 gene may explain its frequent appearance as a mutation hot spot in human cancer. *Mol Cell Biol*, 1994;14(6):4225-4232
- Tornaletti S, Pfeifer GP. Complete and tissue independent methylation of CpG sites in the p53 gene: implications for mutations in human cancers. *Oncogene*, 1995;10(8):1493-1499
- Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H *et al.* Maternal specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell*, 1993;73(1):61-71
- Bird AP. The essentials of DNA methylation. *Cell*, 1992;70(1):5-8

# Progress in endoscopic management of pancreas diseases

LI Zhao-Shen

**Subject headings** pancreatic diseases/therapy; cholangiopancreatography, endoscopic retrograde; pancreatitis/therapy; pancreatic neoplasms/therapy

Endoscopy has been widely used in the treatment of many biliary tract disorders and as an alternative to many surgical procedures. The remarkable advances achieved in the performance of therapeutic endoscopic retrograde cholangio-pancreatography (ERCP) have placed endoscopy in the forefront for treating pancreato-biliary diseases<sup>[1]</sup>. It has established itself as a valuable tool. However, it is only recently that these techniques have been applied and adopted to disorders of the pancreas for their frequent and potentially serious complications and technical problems, etc. Generally, therapeutic ERCP of the pancreas is still in its infancy.

Recently, endoscopic treatment, especially therapeutic ERCP for pancreas diseases has developed rapidly, such as acute pancreatitis, chronic pancreatitis, pancreatic pseudocysts or pancreas divisum. These will be reviewed as follows.

## ACUTE PANCREATITIS

### *Acute gallstone pancreatitis*

Gallstones are one of the major etiological factors of acute pancreatitis. 60% of the patients with acute pancreatitis are found to have gallstones, and 68% of the severe cases have<sup>[2,3]</sup> gallstones in common bile duct. Conservative medical management alone for gallstone pancreatitis fails in 20% - 30% of patients, with more complications and higher mortality. In 1974, Cotton and Beales showed that acute pancreatitis could not be considered contraindicated for ERCP<sup>[4]</sup>. Recently, urgent (within 24-72 hours) ERCP and endoscopic sphincterotomy ES have been performed in those patients with suspected acute gallstone pancreatitis. Urgent ERCP usually shows normal pancreatic duct, and being less helpful to the diagnosis of mild pancreatitis and surgical indications. However, it does not prolong hospital stays or increase morbidity. Controlled study showed that patients benefited from early ERCP and ES as demonstrated by lower morbidity (24%

vs 64%), shorter hospitalization (9.5% days vs 17 days), and lower mortality (2% vs 8%). No serious procedure-induced complications were observed in the studies<sup>[2]</sup>. It suggested that urgent ERCP and ES are safe and effective for patients with acute biliary pancreatitis.

Fan *et al*<sup>[5]</sup> reported their results with early ERCP and ES in 195 patients with suspected acute gallstone pancreatitis, among whom 127 (65%) had gallstones in common bile duct or gall bladder. Those who underwent ERCP had nobiliary sepsis, and the mortality was also low. Patients with a mild attack of pancreatitis should be closely observed and an immediate intervention is not necessary. Urgent ERCP and ES should be reserved for patients with an attack of pancreatitis predicted to be severe, those whose pancreatitis does not subside with conservative measures over 72 hours.

### *Acute recurrent pancreatitis*

Acute recurrent pancreatitis is defined as two or more attacks of pancreatitis associated with at least twice normal serum amylase levels<sup>[6]</sup>. Those patients are always without a positive history of alcohol abuse or gallstone. ERCP can disclose an etiological factor such as bile duct stones, choledochocoles, papillary tumors, duodenal duplication cysts, intraductal strictures, pancreas divisum, or papillary stenosis in 40%-50% of cases. In about a half of those, ERCP can reveal a motor abnormality of Oddi sphincter (normal  $\frac{1}{4}$ 30mmHg) or dysfunction of Oddi sphincter. The management of acute recurrent pancreatitis includes balloon dilatation of the stricture and endoprosthesis. If the recurrent attacks of pancreatitis relate closely to Oddi sphincter dysfunction, endoscopic sphincterotomy of pancreatic duct sphincter should be performed as a routine. This procedure can decrease the pressure of the pancreatic duct, and relieve symptoms of pancreatitis. At present, pancreatic sphincterotomy is not recommended for patients without sphincteric dysfunction and a normal pancreatic duct<sup>[2]</sup>.

## CHRONIC PANCREATITIS

Chronic pancreatitis is a disease that is difficult to manage. It has three types of clinical presentations, including abdominal pain (70% of cases may be related to the increased pancreatic duct pressure), diarrhea (20%-30%), endocrine failure in the form of diabetes (3%-37%). ERCP can reveal pancreatic duct stricture, stones in pancreatic duct or pan-

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Received 1997-10-06

creatic pseudocysts. So the endoscopic management includes pancreatic sphincterotomy, balloon dilatation of strictures, pancreatic stent and pseudocyst drainage.

To those patients with pancreatic duct stricture, a stiff nylon catheter or a Soehendra dilating catheter is introduced over a guidewire to dilate the stricture up to 7-Fr. The catheter is then removed, and over the guidewire an endoprosthesis (5-or 7-Fr) is inserted. Most strictures will recur soon after dilation alone. Improvement of pain was seen in 54% - 90% of cases after pancreatic stenting. Pancreatic stenting might occlude side branches and subsequently cause formation of cystic lesion in the side branches. The stent can also induce inflammatory changes of the duct which mimics chronic pancreatitis<sup>[7]</sup>. There is a tendency to leave the endoprosthesis in place for as short a period as possible (about 6 w). Recurrence of abdominal pain often indicates stent blockage and the necessity to replace or remove the stent. Additional side flaps outside the papilla may prevent the stent from migrating into the pancreatic duct<sup>[8]</sup>. The migrated stent can be extracted by endoscope immediately or surgical intervention if necessary. Endoscopic stenting is a useful alternative to surgical treatment and has lower morbidity and mortality. It does not impair the remaining pancreatic function, and surgery is still possible, if necessary.

It is not clear whether pancreatic stones are the cause or the results of recurrent attacks of pancreatitis. They can be extracted by balloon catheter or baskets. If stones can not be removed completely during the first endoscopic session, a stent can be inserted beyond the stones for adequate drainage until the second attempt. Larger stones above a stricture can be fragmented within the pancreatic duct by using extracorporeal shock wave lithotripsy (ESWL). In series of 123 patients, stones were fragmented successfully in 99% cases with the main pancreatic duct being completely cleared in 59%. After a mean follow-up of 14 months, complete or partial pain-relief could be obtained for 85% of the patients<sup>[12]</sup>. Electromagnetic lithotripsy (EML) can also be applied. Smith reported 15 cases, and complete clearance of pancreatic duct was seen in 60% of patients.

#### **PANCREATIC PSEUDOCYSTS OR PANCREATIC ABSCESSSES<sup>[2,9-11]</sup>**

Patients with pancreatic pseudocysts have clinical features as chronic pancreatitis, they also have obstruction of biliary and upper gastrointestinal tract. Endoscopic treatment includes nasopancreatic drainage, cystogastrostomy or cystoduodenostomy and endoprosthesis with lower morbidity and mortality in comparing with surgical procedures. Chronic pseudocysts more than 4cm-6cm should be drained soon after the diagnosis was made. Na-

sopancreatic drainage is always used to perform cystogastrostomy or cystoduodenostomy if the pseudocyst is closely in contact with the gastric or duodenal wall, as evidenced by CT or endosonography. Endoscopic cystoduodenostomy (ECD) is the first choice for treatment of paraduodenal cysts, whereas endoscopic cystogastrostomy (ECG) is an alternative procedure for the drainage of retrogastric cysts. Binmoeller *et al* reported their results of 53 patients who had undergone transpapillary drainage of ECD and/or ECG, cysts disappeared in 95% of them. Cremer *et al* reported 33 cases, and no recurrence of pseudocysts or complications was seen. Symptoms were relieved completely in 90% of the patients.

Endoscopic drainage is also safe and effective in pancreatic abscesses. Binmoeller *et al* reported 10 cases. Abscesses completely resolved in 8 patients. During a mean follow-up of 10 months, none of the patients had recurrence.

#### **PANCREAS DIVISUM**

Pancreas divisum is a congenital abnormality found in 5%-7% of patients<sup>[6]</sup>. Most investigators believe that the relatively small size of the minor papilla, which drains the largest part of the pancreas, could cause pancreatic congestion, leading to pain or pancreatitis. Endoscopic sphincterotomy of the minor papilla and endoprosthesis are always performed, and symptoms improve significantly in 83%-90% of cases.

Endoscopic sphincterotomy of the minor papilla can enlarge outflow of the pancreatic duct, but stricture recurs quickly and causes attacks of pancreatitis, so a stent (5- or 7-Fr) is always inserted after the sphincterotomy. Lehman *et al* reported that patients with recurrent pancreatitis benefited from endoscopic therapy more often than those with chronic pancreatitis or chronic pain during the mean follow-up of 1.7 years<sup>[12]</sup>. In patients with pancreas divisum and pancreatitis, stenting of the minor papilla was associated with a 49% complication rate, and was always moderate or severe. Endoscopic stent was not effective but associated with 80% of complications in those cases with pain only and a normal ERCP<sup>[13]</sup>.

The long-term success of stent treatment is unknown. Stenting may cause dilation of the minor papilla sphincter. The results of this study suggest that impairment of pancreatic drainage by the minor papilla is probably an etiological factor for recurrent pancreatitis in patients with pancreas divisum.

#### **PERIPHERIC TUMORS OF VATER'S AMPULLA**

ERCP is mainly used for the diagnosis of peripheral tumors of Vater's ampulla. Endoscopic appearance, radiography, forceps biopsy, collection of pancreatic cells and pancreatic secretion can afford evidences of pathology and pathology. To those benign tumors such as adenomas and malignant ones that

cannot be removed, complete removal and conservative medical treatment should be performed, respectively. Binmoeller *et al*<sup>[14]</sup> reported 25 cases of removal of benign adenomas with standard polypectomy snare, all were successful. Its criteria were: size less than 4 cm; and benign histologic findings on forceps biopsy (minimum of six biopsies). It is dangerous with more complications, so its indications are those who refused to undergo surgical treatment and those at high surgical risk.

## COMPLICATIONS

The complications of endoscopic management of pancreatic disorders are much higher than those of biliary tract diseases (about 25%).

The common complications of endoscopic treatment are pancreatitis, hemorrhage, perforation and infection. Abdominal discomfort and sepsis are frequent in the management of endoscopic sphincterotomy and stone extraction. Complications of cystogastrostomy and cystoduodenostomy are mainly hemorrhage and perforation, and technique-related infection, for example, the infection of cysts. Stent dysfunction, inducing clogging migration of the stent and erosion of the opposite wall of the duodenum, appear to be frequent and major long-term problem. Stent clogging is reduced through self-expandable metal stents, but experience in the pancreatic duct is limited and these stents are not easy to be removed. Most complications of endoscopic therapy can be successfully treated conserva-

tively or endoscopically, and surgical procedure is also needed if necessary.

In summary, selection of patients for the various treatment options should be strict and is probably important for optimum results of therapy because of their technique and complications, especially in children<sup>[15]</sup>.

## REFERENCES

- 1 Siegel JH, Cohen SA. Therapeutic pancreaticobiliary endoscopy. *Gastroenterology*, 1995;3(1):28-40
- 2 Huibregtse K, Smits ME. Endoscopic management of disease of the pancreas. *Am J Gastroenterol*, 1994;89(8):66-77
- 3 Carr-Locke DL. Role of endoscopy in gallstone pancreatitis. *Am J Surg*, 1993; 165(4):519-520
- 4 Cotton PJ, Beales JSM. Endoscopic pancreatography in the management of acute pancreatitis. *Br Med J*, 1974;1(5):608-611
- 5 Fan ST, Lai ECS, Mok EPT, Lo CM, Zheng SS, Wong J *et al*. Early treatment of acute biliary pancreatitis by endoscopic papillotomy. *N Engl J Med*, 1993;328(2):228-232
- 6 Bedford RA, Howerton OH, Geenen JE. The current role of ERCP in the management of benign pancreatic disease. *Endoscopy*, 1994;26(2):113-119
- 7 Sherman S, Alvarez C, Roberts M, Ashlev S, Reber H, Lehman G *et al*. Polyethylene pancreatic duct (PD) stent induced changes in the normal dog pancreas. *Gastrointest Endosc*, 1993;39(2):A332
- 8 Johanson J, Schmalz M, Geenen JE. Simple modification of a pancreatic duct stenting to prevent proximal migration. *Gastrointest Endosc*, 1993;39(1): 62-64
- 9 Binmoeller KF, Walter A, Seifert H, Soehendra N. Endoscopic stenting for pancreatic pseudocysts in 53 patients. *Gastrointest Endosc*, 1993;39(2):A240
- 10 Binmoeller KF, Walter A, Seifert H, Soehendra N. Endoscopic therapy for pancreatic abscesses. *Gastrointest Endosc*, 1993;39(2):A239
- 11 Howell DA, Mnggia RA, Bosco JJ, Biber BP. Transpapillary pancreatic duct endoprosthesis in the management of pancreatic pseudocysts. *Gastrointest Endosc*, 1993;39(2):A317
- 12 Lehman GA, Sherman S, Nisi R, Hawes RH. Pancreas divisum: results of minor papilla sphincterotomy. *Gastrointest Endosc*, 1993;39(1):1-8
- 13 Rosch J, Allescher HD. Update in gastroenterologic endoscopy: a review of endoscopy (abstracts presented at 1993 DDW in Boston). *Endoscopy*, 1993;25(4): 401-422
- 14 Binmoeller KF, Boaveture S, Ramsperger K, Soehendra N. Endoscopic snare excision of benign adenoma of the papilla of Vater. *Gastrointest Endosc*, 1993;39(2):127-131
- 15 Brown KO, Goldschmiedt M. Endoscopic therapy of biliary and pancreatic disorders in children. *Endoscopy*, 1994;26(9):719-723

# Combined measurement of serum tumor markers in patients with hepatocellular carcinoma \*

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**Subject headings** liver neoplasms/diagnosis; carcinoma, hepatocellular/diagnosis; tumor markers, biological/blood; alpha-fetoproteins/blood; sialic acid/blood; fucosidase/blood; enzyme linked immunosorbent assay; spectrophotometry

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms, and its prognosis is very poor if diagnosed late, therefore, early detection is important. As we know not all HCC can secrete AFP, and AFP levels may be normal in as many as 40% of patients with early HCC and 15%-20% of patients with advanced HCC<sup>[1]</sup>. Therefore, we selected AFP, alpha-L-fucosidase (AFU) and sialic acid (SA) in combination for detecting HCC.

## MATERIALS AND METHODS

Subjects were divided into three groups: ① HCC group consisted of 30 patients (26 males and 4 females) with a mean age of  $50.4 \pm 12.7$  years; ② liver cirrhosis group consisted of 30 patients (24 males and 6 females) with a mean age of  $45.3 \pm 8.4$  years; and ③ control group consisted of 30 healthy subjects (28 males and 2 females) with a mean age of  $33.2 \pm 4.8$  years whose liver function tests were normal. Diagnosis of HCC was in accordance with the criteria of the National HCC Association of China in 1977<sup>[2]</sup>.

The fasting sera from all subjects were stored at  $-18^{\circ}\text{C}$ . AFP was measured by ELISA. The kits were provided by Xiamen Advanced Scientific Institute<sup>[3]</sup>. AFU was measured according to Troost's method and expressed as nkat/L<sup>[3]</sup>. The kits were provided by Sanming Lanbo Biological Technique Institute. SA was measured by spectrophotometry. The kits were purchased from Dongou Biological Technical Institute. The data were expressed as  $\bar{x} \pm s$  and analyzed statistically by the Student's *t* test.

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\*Supported by the Educational Commission of Fujian Province, No. JQ93026.

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Received 1997-10-06

## RESULTS

The serum levels of AFP, AFU and SA in the three groups are shown in Table 1.

The serum levels of AFP, AFU and SA in patients with HCC were significantly higher than those in patients with cirrhosis ( $P < 0.01$ ) and in the control subjects ( $P < 0.01$ ). No significant differences were found between the latter two groups.

The cutoff value was defined as  $\bar{x} + 2s$  (AFP  $\leq 30 \mu\text{g/L}$ , AFU  $\leq 180 \text{ nkat/L}$  and SA  $\leq 630 \text{ mg/L}$ ).

The positive rates and significance of AFP, AFU and SA in the three groups are shown in Tables 2 and 3.

Results of combined measurement with two positives among the three tumor markers are shown in Table 4.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate were 86.7%, 98.3%, 93.7%, 96.3% and 94.4%, respectively.

**Table 1** The serum levels of AFP, AFU and SA among patients in the three groups ( $\bar{x} \pm s$ )

Groups	AFP( $\mu\text{g/L}$ )	AFU(nkat/L)	SA(mg/L)
Control	10.2 $\pm$ 9.8	106.0 $\pm$ 36.5	513.7 $\pm$ 57.8
Cirrhosis	14.4 $\pm$ 9.0	126.8 $\pm$ 52.1	522.7 $\pm$ 70.5
HCC	71.7 $\pm$ 38.8 <sup>bd</sup>	284.5 $\pm$ 102.6 <sup>bd</sup>	636.7 $\pm$ 76.6 <sup>bd</sup>

<sup>b</sup> $P < 0.01$ , compared with controls, <sup>d</sup> $P < 0.01$ , compared with cirrhosis.

**Table 2** Positive rates of AFP, AFU and SA among the three groups

Groups	AFP		AFU		SA	
	<i>n</i>	(%)	<i>n</i>	(%)	<i>n</i>	(%)
Controls	1	(3.3)	1	(3.3)	2	(6.7)
Cirrhosis	2	(6.7)	4	(13.3)	5	(16.7)
HCC	21	(70.0)	23	(76.7)	21	(70.0)

**Table 3** Significance of AFP, AFU and SA (%)

	AFP	AFU	SA
Sensitivity	70.0(21/30)	76.7(23/30)	70.0(21/30)
Specificity	95.0(57/60)	91.7(55/60)	88.3(53/60)
Positive predictive value	87.5(21/24)	82.1(23/28)	75.0(21/28)
Negative predictive value	86.4(57/66)	88.7(55/62)	85.5(53/62)
Accuracy	86.7(78/90)	86.7(78/90)	82.2(74/90)

**Table 4 Results of combined measurement of serum AFP, AFU and SA in the three groups**

Groups	Postivity	
	n	%
Controls	0	0.0
Cirrhosis	1	3.3
HCC	26	86.7

Patients with HCC showed both positive AFU and AFP in 16/30 patients and negative results in 2/30 patients. Of the remaining patients, 5 were positive in AFP and negative in AFU, and 7 were negative in AFP and positive in AFU (Table 5). No correlation was found between AFP and AFU ( $P < 0.05$ ).

**Table 5 Comparison between AFP and AFU in patients with HCC**

AFP	AFU		Total
	Positive	Negative	
Positive	16	5	21
Negative	7	2	9
Total	23	7	30

## DISCUSSION

So far, AFP still remained the most sensitive and specific marker of HCC. Our results showed that the sensitivity of AFP in patients with HCC was 70%, concordant with the reports by many other authors<sup>[4,5]</sup>.

AFU is a lysosomal enzyme involved in the catabolism of the fucose-containing glycoconjugates. In accordance with the study of Deugnier, et al, the serum level of AFU activity in patients with HCC was increased<sup>[6]</sup>. Its positive rate in our study was 76.7% (23/30), similar to other reports (75%-76.7%)<sup>[4-7]</sup>. This may be related to increased enzyme release by tumor cells<sup>[6,7]</sup>.

Our study showed that serum AFP and AFU activity was independent with no correlations between

them as shown in Table 5. Therefore, AFU may be considered as an additional useful marker for detection of HCC.

The SA was considered to be associated with behaviour of malignant tumors. In our studies, its positive rate in patients with HCC was 70% as reported by many other authors<sup>[8,9]</sup>. The serum level of SA in HCC was also high and was positive in 71.4% when AFP was negative.

The main purpose of using combined measurement of tumor markers is to eliminate the false negative and false positive results as reported by many authors<sup>[1,4,5,10]</sup>. We used combined measurement of serum AFP, AFU and SA of patients with HCC. With this measurement, any two positives among the three tumor markers as the diagnostic criteria, the sensitivity, specificity, positive predictive value, negative predictive value and rate of accuracy in HCC patients were 86.7%, 98.3%, 93.7%, 96.3% and 94.4%, respectively. Six of 9 patients with HCC who had negative AFP had positive results in both AFU and SA. None of 3 patients with liver cirrhosis and controls who had positive AFP had positive results in AFU and/or SA. Therefore, combined measurement of serum AFP, AFU and SA is of practical significance in diagnosis of HCC.

## REFERENCES

- Giardna MG, Matarazzo M, Varriale A, Morante R, Napoli A, Martino R. Serum alpha L fucosidase: a useful marker in the diagnosis of hepatocellular carcinoma. *Cancer*, 1992;70(5):1044-1048
- Editorial Board of Practical Oncology. *Practical oncology*. Beijing: People's Health Publisher, 1979:112-142
- Troost J, Van der Heijden M, Staal G. Characterization of alpha-L-fucosidase from two different families with fucosidosis. *Clin Chim Acta*, 1976;73(2):329-346
- He M, Shi YR, Zou W. Combined assay of serum AFU and AFP in patients with primary hepatic carcinoma. *Chin J Clin Oncol*, 1996;23(1):21-23
- Shen W, Shen DM. Preliminary study of serum  $\alpha$ -L-fucosidase assay in diagnosis of hepatocellular carcinoma. *Chin J Intern Med*, 1989;28(7):397-399
- Deugnier Y, David V, Brissot P, Mabo P, Delamaire D, Messner M et al. Serum  $\alpha$ -L-fucosidase: a new marker for diagnosis of primary hepatic carcinoma. *Hepatology*, 1984;4(5):889-892
- Zhang SM, Wu MC, Chen H, Tu ZX, Cui ZF, Yu ZX et al. Value of serum glycosidase spectrum in the diagnosis of hepatocellular carcinoma. *Chin J Oncol*, 1992;14(2):143-145
- Liu JL, Cai ZD, Liu RT, Zhang QS, Zhang YK, Che LH et al. The significance of serum sialic acid in the diagnosis of primary hepatic carcinoma. *J Pract Internal Med*, 1991;11(11):576-577
- Pan J, Geng JM, Zou JX, Wen GY. Improved colorimetry assay of serum sialic acid in the patients with primary hepatic carcinoma. *J Clin Lab Sci*, 1991;9(3):124-125
- Tu ZX, Yin ZF, Cui ZF, Wu MC. Prediction of primary liver cancer with alpha-fetoprotein. *Acad J Sec Mil Med Univ*, 1991;12(5):417-419

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ISSN 1007-9327 CN 14-1219/R World J Gastroenterol 1998; 4(2):183-184

## Effect of various drinking water on human micronucleus frequency in high risk population of PHC

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\*Supported by the National "8th Five-Year Plan" on the project "the Comprehensive Prevention and Treatment of Liver Cancer", No.85-914-01-04.

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**Received:** 1997-09-02

**Subject headings:** liver neoplasms/etiology; carcinoma, hepatocellular/etiology; water; micronucleus tests; lymphocytes; risk factors

Liu e, Zhang QN, Li WG.Effect of various drinking water on human micronucleus frequency in high risk population of PHC. World J Gastroentero, 1998;4(2):183-184

### INTRODUCTION

The relationship between PHC and contamination of drinking water has been reported in some papers [ 1,2 ]. In Qidong, a PHC high-incidence area, the epidemiological investigation showed that there may be one or various carcinogens in the drinking water from various sources [ 3 ], but these data were epidemiological results. Using a simple and effective micronucleus testing technique to evaluate environmental contamination, we observed the micronucleus effect of human peripheral lymphocytes induced by the drinking water from various sources. We reviewed and analyzed previous research data, together with the results of epidemiological investigation, in order to provide more convincing experimental evidence for the pathogenesis of PHC induced by drinking water.

### MATERIAL AND METHODS

#### *Experimental materials*

Water samples. Water samples were taken from house pool water (HPW), field canal water (FCW) and tap water (TW or deep well water). Water samples of these 3 types were collected in the same area, filtered, boiled and concentrated 100 times for use. Double distilled water (DDW) was used as the control. Blood samples. Obtained from 38 year old male volunteers with no diseases in physical check up.

### **Experimental methods**

**Preparation of micronucleus.** Put 0.3ml venous blood from volunteers into each culture flask containing heparin 50mg/L, and then add PHA 0.2ml, RPMI-1640 culture solution (contains 20% bovine serum) 4.5ml, and water samples 0.05ml-0.15ml. Keep the flask at 37 °C in an incubator for 72 hours, and shake them every 12 hours. Micronucleus was prepared according to the method reported by us [ 4 ] .

**Indices observed.** Cell growth of each experimental group each day, and micronucleus. Using a double blind test, we checked each sample under oil immersion objective to choose transformed lymphocytes which are intact and clear. Micronucleus number was counted with ‰ in 2000 lymphocytes of each blood sample. The standard to evaluate micronucleus has been reported [ 5 ] .

### **Experimental grouping**

Each of the 3 experimental groups (HPW, FCW and TW) was divided into 3 subgroups according to different doses of water samples (0.05ml, 0.10ml and 0.15ml) together with DDW, there were totally 10 experimental groups.

## **RESULTS**

### **Morphologic observation of cultural cells in each experimental group**

The cytotoxicity of 3 subgroups of HPW was very strong, with only a few transformed lymphocytes, and damaged membrane structure of many cells, so that the micronuclei could not be observed and the micronucleus frequency (MNF) could not be counted. The cell growth was in relatively good condition in FCW. The majority of lymphocytes were transformed with a full appearance and had intact membrane, but the cytotoxicity was enhanced with increasing doses of experimental water, and numbers of micronuclei induced were also increased. The situation of cell growth in TW groups was better than that of FCW groups. There was a weak cytotoxicity, MNF in each subgroup was lower than that of FCW groups. The cell growth condition of DDW groups was the best. There were lots of clear, transformed lymphocytes with intact membrane, abundant cytoplasm and few micronuclei.

### **Dose-response relationship of MNF induced by water in each experimental group**

Compared with DDW group, the MNF induced by FCW groups was the highest, and that of TW water groups was relatively lower, which rose with increasing doses of water in each experimental group (Figure 1).

**Figure 1** (PDF) Dose-response relationship of MNF induced by various water samples.

## **DISCUSSION**

Primary hepatocellular carcinoma (PHC) is one of ten common malignant tumors in the world. Qidong is a high-risk area of PHC in China, and its annual mortality remains at above 50/100000 . Genetic epidemiological and experimental research data showed that PHC was caused by a complex of etiologic factors, including HBV infection, liver disease history, heredity, sex, etc [ 6,7 ] . Contamination of drinking water may be one of the causes of PHC. The results of this study showed that the degree of cytotoxicity of drinking water from various sources was in the order of HPW > FCW > TW > DDW and the effect of various drinking water samples on inducing micronuclei of human peripheral lymphocytes was FCW > TW > DDW, and MNF was elevated with the increasing dose of each experimental group. Previous research data showed that the incidence rates of PHC varied in Qidong residents who drank water from various sources. The incidence was HPW (141.40/100000 ), FCW (72.32/100000 ), river water (43.45/100000), shallow well water (22.26/100000) and deep well water (11.70/100000) [ 3 ] ,

with significant difference ( $P < 0.01$ ). This suggests that MNF induced by drinking water from different sources correlates with the incidence of PHC in the population who drink the corresponding water. In other words, if the residents drink badly polluted water, the effect on micronucleus will be strong, and incidence rate of PHC will be high. Our experimental results are consistent with the results of epidemiological investigation. These data have confirmed the close relationship between drinking water and PHC incidence in the high-risk population of PHC, and the polluted drinking water may be one of the causal factors in PHC.

Recent reports showed that microcystin contents (MC), a kind of PHC carcinogen from a certain poisonous algae, was detected in the drinking water of Haimen area, a neighbour of Qidong City. The positive rates ( $> 50\text{ng/L}$ ) of MC in drinking water from various sources were 17.3% (house pool water), 31.9% (river water), 4.3% (shallow well water) and 0.0% (deep well water). The average contents of each of these water samples were 101ng/L, 160ng/L, 68ng/L and 0ng/L, respectively.

The difference was significant ( $P < 0.01$ ) [8]. The more serious the contamination of drinking water, the higher is its MC content. The relationship between MC and PHC incidence rate in residents drinking water from various sources should be further studied. It can be suggested that drinking deep well water, or improving the quality of drinking water may be of great significance in the prevention of PHC.

## REFERENCES

- 1 Su DL. Drinking water and liver cancer. Chin J Prev Med, 1980;14(2):65-68
- 2 Zhang LS, Yang KZ, Hou JL, Meng GR, Huang ZY, Wu JL. The effect of micronucleus on contaminative water in the high morbid area of liver cancer and study on carcinogenic potentiality. Cancer, 1988;7(1):1-4
- 3 Shen ZC, Li WG, Chen JG, Xie JR. The epidemiological research on relationship between various drinking water types and incidence of PHC. Jiangsu Med J, 1985;11(1):28-31
- 4 Liu E, Zhang QN. The simple slide-making method of micronucleus of lymphocytes cultured in vitro. J Clin Lab Sci, 1992;10(2):105
- 5 Zhang QN, Li WG, Liu E, Huang QS, Yu SY, Zhu YJ. The effect of Se-intaking on micronucleus frequency of peripheral lymphocytes in HBsAg positive patients who were induced by AFB-1. Cancer Res Prev Treat, 1993;20(1):3
- 6 Shen FM, Le MK, Cong HM, Wang JM. Complex segregation analysis of primary hepatocellular carcinoma in Chinese families: interaction of inherited susceptibility and hepatitis B viral infection. Am J Hum Genet, 1991;49(2):88-92
- 7 Liu E, Wu Y, Zhang BC. Epidemiologic and clinical features of primary hepatocellular carcinoma. Jiangsu Med J, 1993;19(5):247-248
- 8 Chen G, Yu SZ, Wei GR, Chen GC, Xu XY, Huang YH et al. Studies on microcystin contents in different drinking water in highly endemic area of liver cancer. Chin J Prev Med, 1996;30(1):6-9

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# Molecular biology and the diagnosis and treatment of liver diseases

Howard J. Worman, Feng Lin, Naoto Mamiya and Paul J. Mustacchia

**Subject headings** liver disease; molecular biology; viral hepatitis; metabolic diseases; recombinant DNA; rational drug design; gene therapy

Molecular biology has made a tremendous impact on the diagnosis and treatment of liver diseases<sup>[1]</sup>. This review will provide several recent examples. Emphasis will be placed on how molecular biology has influenced the diagnosis of viral hepatitis, autoimmune and metabolic liver diseases. The use of recombinant DNA technology for drug development and the possibility of gene therapy as a treatment modality will then be discussed.

## DIAGNOSIS OF VIRAL HEPATITIS

### *Hepatitis B*

Approximately 350 000 000 individuals are chronically infected with the hepatitis B virus (HBV) worldwide and the disease is endemic in eastern Asia including China<sup>[2]</sup>. As viral protein antigens can be readily detected in serum, chronic HBV infection can usually be diagnosed by serological assays for the presence of hepatitis B surface antigen (HBsAg). HBsAg is detectable in virtually all infected individuals and the hepatitis B e antigen (HBeAg) is detected in most individuals with high levels of viral replication. Serological assays, usually enzyme linked immunosorbant assays (ELISAs), can utilize either proteins purified from serum or recombinant proteins expressed in yeast or tissue culture cells. ELISAs for detection of antigens can utilize monoclonal antibodies.

Because HBV infection can usually be diagnosed by serological assays, molecular biological methods to measure or detect nucleic acids are usually not necessary in routine clinical diagnosis. In some instances, however, measurement of viral nucleic acids from serum may be helpful in the

assessment of patients with chronic hepatitis B. Viral nucleic acid concentrations in serum can be assessed by hybridization to complementary DNA sequences and by a branched chain DNA (bDNA) assay<sup>[3]</sup>. Such assays can be quantitated to provide estimates of the concentration of viral DNA in serum. As serum viral DNA concentrations may be predictive of prognosis or of response to treatment and prognosis, estimation of viral loads may be useful clinically.

Polymerase chain reaction (PCR) amplification<sup>[4,5]</sup> of HBV DNA is also relatively easy as HBV is a double stranded DNA virus whose genome is fairly stable in blood and tissue. Sequencing of amplified DNA can be performed to identify mutant viruses of clinical significance. Truncating mutations in the HBV precore gene have been identified that prevent secretion of the e antigen but allow the continued assembly of infectious virus<sup>[6,7]</sup>. Such mutant strains may be actively replicating even though HBeAg is not detectable using serological assays.

### *Hepatitis C*

In Western countries, molecular biology has arguably had its greatest clinical impact with regards to liver diseases in the diagnosis of viral hepatitis C. Although the virus that causes hepatitis C cannot be propagated in cell culture, molecular biological methods have enabled the identification of this virus and determination of the sequence of its entire genome. This has revolutionized the practice of hepatology.

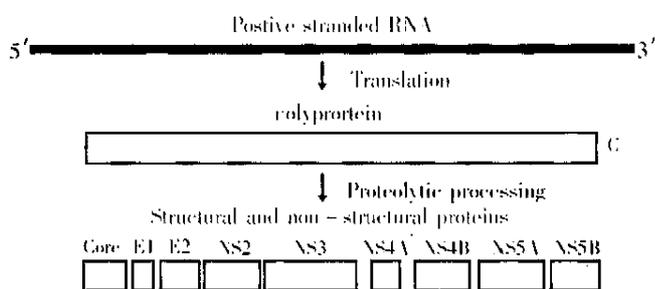
The hepatitis C virus (HCV) was identified in 1989 by investigators at Chiron Corporation<sup>[8]</sup>. HCV was identified by antibody screening of cDNA expression libraries made from DNA and RNA from the plasma of chimpanzees infected with serum from humans with what was then called non-A, non-B hepatitis. The expression library was screened with serum antibodies from patients with non-A, non-B hepatitis. This work led to the isolation of cDNA clones that were derived from portions of the HCV genome and encoded fragments of viral polypeptides. The authors also showed that HCV was a positive stranded RNA virus<sup>[8]</sup> and that the vast majority of individuals with chronic non-A, non-B hepatitis had antibodies against this virus<sup>[9]</sup>.

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Received: 1998-5-25

Following the identification of fragments of the HCV genome, the entire genome was cloned and sequenced in several laboratories<sup>[10-13]</sup>. This work showed the HCV genome to be a positive stranded RNA of approximately 10 000 nucleotides with a single open reading frame encoding a polyprotein of 3010 to 3033 depending upon the strain (Figure 1). The polyprotein is processed by host cell and virally encoded proteases into several structural and non-structural polypeptides (Figure 1). The structural proteins are the core and two envelope polypeptides. The several non structural proteins have various different enzymatic functions.



**Figure 1** Schematic diagram of the HCV genome and proteins. The positive stranded RNA of about 10000 nucleotides is translated into a polyprotein of approximately 3 000 amino acids. This polyprotein is proteolytically cleaved into several smaller proteins. Core, E1 and E2 are structural polypeptides. Core protein is the virus nucleocapsid and E1 and E2 are viral envelope proteins. A small polypeptide known as P7 (not shown) is also produced by additional cleavage between E2 and NS2. The major non-structural proteins are NS2, NS3, NS4 and NS5. NS4 is further processed into NS4A and NS4B and NS5 into NS5A and NS5B. NS2 and part of NS3 are proteases that process the viral polyprotein. NS3 also has RNA helicase activity. NS4A is a cofactor for the NS3 protease and NS5B is an RNA-dependent, RNA polymerase. The functions of NS4B and NS5A are less well understood but NS5A is thought to play a role in determining sensitivity to interferon.

ELISAs utilizing recombinant HCV polypeptides have been available since the discovery of the virus to detect antibodies against HCV. In individuals with risk factors, the presence of anti-HCV antibodies and an elevated serum alanine aminotransferase activity is highly sensitive for the diagnosis of infection. However, false positive antibody tests are not uncommon and some individuals with HCV infection develop antibodies at low titers that may not be detected.

In the past few years, assays have become routinely available for the detection of HCV RNA in serum. In the bDNA assay, viral RNA is captured by virus-specific nucleotide probes followed by hybridization to branched DNA

molecules which are detected by a chemiluminescent substrate system<sup>[3,14]</sup>. The bDNA assay can be quantitated and is relatively easy to perform in the routine clinical laboratory. However, bDNA is of lower sensitivity than PCR that is now available to detect HCV RNA in serum and tissue. Since the HCV genome is RNA, reverse transcription must be performed before HCV complementary DNA sequences can be amplified by PCR. The amplified cDNA products are separated by agarose gel electrophoresis and detected by a variety of methods such as ethidium bromide staining or Southern hybridization. Care must be taken in the clinical laboratory to avoid contamination when using reverse transcription-PCR to detect HCV RNA, however, the standardization of this method in excellent clinical laboratories has made it the gold-standard for the detection of HCV infection. PCR has even been made semi-quantitative by using competitive inhibitors or highly standardized assay procedures.

The ability to readily reverse transcribe, amplify and sequence HCV RNA has led to the identification of several HCV genotypes<sup>[15]</sup>. By using different PCR primers and probes to amplify and detect different sequences from various isolates, routine HCV genotype analysis is possible in the clinical laboratory<sup>[16,17]</sup>. Different genotypes may differ in the severity of disease they cause and the response to treatment<sup>[18]</sup>.

### Hepatitis G

Sensitive molecular biological techniques led to the identification of a new virus in 1995 and 1996 in individuals with hepatitis<sup>[19,20]</sup>. This virus, termed both hepatitis G virus (HGV) and GB-C virus, is a novel Flavivirus with approximately 25% sequence identity to HCV. The initial identification of this virus was originally met with considerable enthusiasm as it was proposed to be a relatively common cause of non-A, non-B, non-C acute and chronic hepatitis. However, subsequent studies<sup>[21,22]</sup> have questioned the etiological role of HGV/GB-C as a cause of serious liver disease. Most investigators now feel that HGV/GB-C infection is quite common but that it does not cause clinically significant liver disease.

### AUTOIMMUNE LIVER DISEASES

Autoimmune liver diseases such as primary biliary cirrhosis, autoimmune hepatitis and sclerosing cholangitis are associated with the presence of various autoantibodies. Although the pathophysiological significance of these autoantibodies are generally unclear, their detection is central to diagnosis. Basic molecular biological methods have made possible the identification and

cDNA cloning of some of the intracellular protein antigens and the predominant epitopes recognized by disease-specific autoantibodies. This work has led to the development of assays for their detection that can be used in the clinical laboratory.

Illustrative examples on the identification of intracellular antigens recognized by autoantibodies in a liver disease come from work on primary biliary cirrhosis (PBC). Almost all individuals with this disease have autoantibodies directed against the E2 subunits of mitochondrial oxo-acid dehydrogenases<sup>[23-25]</sup> and about 25% of patients have autoantibodies against gp210, an integral membrane protein of the nuclear pore complex<sup>[26,27]</sup>. Autoantibodies of these specificities are virtually 100% specific for primary biliary cirrhosis.

Screening of bacteriophage lambda cDNA expression libraries with autoantibodies from patients with PBC was used to identify the E2 subunits of the pyruvate dehydrogenase complex, the branched chain 2-oxo-acid dehydrogenase complex and the 2-oxo-glutarate dehydrogenase complex as the mitochondrial autoantigens in this disease<sup>[24,25,28-30]</sup>. ELISAs utilizing expressed recombinant proteins have been devised that are sensitive and specific for the detection of antibodies against these proteins<sup>[23,31,32]</sup>. Determination of the immunodominant epitopes the E2 subunits of all three oxo acid dehydrogenases has allowed for the construction a designer hybrid clone that contains the major epitope of each<sup>£Ü32£Ý</sup>. An immunoassay utilizing a polypeptide expressed from this hybrid clone is highly sensitive and specific for the diagnosis of PBC<sup>[32]</sup>.

In 1990, nuclear pore membrane glycoprotein gp210 was identified as an autoantigen recognized by approximately 25% of individuals with PBC<sup>[33]</sup>. Using overlapping cDNA for gp210, its immunodominant epitope which was mapped to a stretch of 15 amino acids in this protein of over 1880 amino acids<sup>[34]</sup>. Based on this information, two ELISAs have been developed for the detection of autoantibodies, one which utilizes a recombinant HJfusion protein expressed in bacteria<sup>[35]</sup> and the other which uses a synthetic polypeptide<sup>[36]</sup>.

As PBC is a relative uncommon diseases, assays to detect autoantibodies again oxo-acid dehydrogenase subunits and gp210 have not received much commercial attention. These examples nonetheless demonstrate how molecular biology can impact on the diagnosis of a relatively rare disease.

## GENE DISCOVERY AND METABOLIC LIVER DISEASES

Advances in positional cloning and genomics have led to the identification of the genes responsible for

some of the major metabolic diseases that affect the liver. In the past decade, genes mutated in Wilson disease, hereditary hemochromatosis, congenital hyperbilirubinemias and inherited cholestatic disorders have been discovered (Table 1). These discoveries will permit the use of molecular diagnostic methods to diagnose these disorders. An understanding of the defective genes will also lead to new treatment options. This section reviews some of the major discoveries of genes responsible for metabolic liver diseases in the 1990s. The entry number for On line Mendelian Inheritance in Man (OMIM; <http://www3.ncbi.nlm.nih.gov/Omim/>) is given for each diseased that is discussed.

**Table 1** Some inherited diseases that affect the liver and the genes that were indentified in the 1990s by molecular biological and genetic methods.

Disease	Gene
Wilson disease	Cu-transporting ATPase
Hereditary hemochromatosis	HFE
Crigler-Najjar syndrome	UDP-glucuronosyltransferase
Dubin-Johnson syndrome	cMOAT
Benign Recurrent Intrahepatic Cholestasis	P-type ATPase
Progressive familial Intrahepatic Cholestasis Type 1	P-type ATPase

### *Wilson disease (OMIM # 277900)*

Wilson disease is an autosomally inherited disorder that causes changes in the basal ganglia and liver that respectively lead to neuropsychiatric disease, hepatitis and cirrhosis. Abnormalities in serum ceruloplasmin, urinary copper excretion and copper accumulation in the liver have for many years suggested a primary defect in copper metabolism as the cause. In 1985, the Wilson disease gene was shown to be linked to the esterase D locus on chromosome 13<sup>[37]</sup>. Over the next eight years, the Wilson disease locus was more precisely localized by genetic analysis of various families and individuals and was placed at the junction of band q14.3 and q21.1 on chromosome 13 in 1993 by using fluorescence in situ hybridization studies of chromosomal aberrations<sup>[38]</sup>. Finally, in 1993, several yeast artificial chromosomes spanning this region were molecularly cloned and a gene encoding a P-type ATPase that was mutated in individuals with Wilson disease was identified<sup>[39-41]</sup>. This ATPase was highly similar to the ATPase previously shown to be responsible for Menke disease, another disorder of copper metabolism, and is thought to be a copper-translocating ATPase. At least 70 different mutations have since been described in this copper-ATPase gene in individuals with Wilson disease<sup>[42]</sup>.

### *Hereditary hemochromatosis (OMIM #235200)*

Hereditary hemochromatosis is the most common inherited disease in individuals of European

descent<sup>[43]</sup>. Homozygous individuals suffer from the complications of excess iron deposition in the liver, heart, joints and some endocrine organs. Excessive hepatocyte iron causes hepatitis and cirrhosis. The disease often goes undiagnosed which is especially unfortunate because phlebotomy is effective treatment.

In the 1970's, hereditary hemochromatosis was linked to the HLA-A locus on chromosome<sup>[44-46]</sup>. In 1996, investigators at Mercator Genetics<sup>[47]</sup> used linkage disequilibrium and full haplotype analysis to identify a candidate gene for hemochromatosis on chromosome 6. They termed this gene HLA-H because of its homology to other MHC class I family members; the current accepted designation of this gene is HFE. A guanine to adenine transition at coding nucleotide 845 of this gene resulted in a cysteine to tyrosine substitution at amino acid residue 282 in the protein in 85% of 178 patients they examined. Several other studies subsequently confirmed the mutations identified by the group at Mercator Genetics<sup>£Û48-50£Ý</sup> and the cysteine to tyrosine substitution in HFE is now felt to be present in between 70% and 100% of individuals with hereditary hemochromatosis<sup>[51]</sup>. Another mutation in HFE that converts histidine 63 to aspartate also increases the relative risk of for the development of hemochromatosis in individuals who are heterozygous for the cysteine to tyrosine substitution at amino acid 282<sup>[47,52]</sup>.

When the HFE gene was discovered, it was unclear how a protein with homology to MHC class I proteins could be involved in the regulation of iron metabolism. Recently, however, it has been shown that the protein encoded by HFE associates with the transferrin receptor and decreases the affinity of transferrin receptor for iron bound transferrin<sup>[53-55]</sup>. The cysteine to tyrosine mutant protein does not interact with transferrin receptor and hence does not decrease its affinity for iron-bound transferrin, while the histidine to aspartate mutant protein associates with transferrin receptor but does not decrease its affinity for iron bound transferrin as much as the wild type protein<sup>[54]</sup>. These findings can explain how mutations in the HFE gene product cause iron overload.

#### ***Crigler-Najjar syndrome type 1 (OMIM #218800)***

Individuals with Crigler-Najjar syndrome type 1 have a complete absence of activity of the UDP-glucuronosyltransferase isoform that catalyzes the conjugation of bilirubin to mono and diglucuronides<sup>[56,57]</sup>. Affected individuals present with severe childhood disease manifested by jaundice, kernicterus, resultant abnormal neurologic development and early death. Patients with Crigler-

Najjar syndrome type 2 have a partial deficiency of this enzyme activity and generally survive through adulthood without significant problems<sup>[57]</sup>. A single gene of chromosome 2 called UGT1 encodes bilirubin, phenol, and other UDP glucuronosyltransferase isozymes with identical carboxyl termini<sup>[58]</sup>. Several different mutations in UGT1 have since been described in individuals with Crigler Najjar syndrome type 1<sup>[58-61]</sup>.

#### ***Dubin-Johnson syndrome (OMIM #237500)***

Dubin-Johnson syndrome results from the inability of conjugated bilirubin to be secreted from hepatocytes. A cDNA for a rat multispecific organic anion transporter (cMOAT), a protein homologous to the multidrug resistance proteins that is located in the canalicular membrane of hepatocytes, was characterized in 1996<sup>£Û62£Ý</sup>. In 1997, the human orthologue of rat cMOAT was mapped to chromosome 10q24<sup>[63]</sup>. The human cMOAT cDNA was cloned and sequenced in 1997<sup>[64]</sup> and mutations in this gene have been identified in individuals with Dubin Johnson syndrome<sup>[64,65]</sup>.

#### ***Benign recurrent intrahepatic cholestasis (OMIM #243300) and progressive familial intrahepatic cholestasis type 1 (OMIM #211600)***

Benign recurrent cholestasis type 1 or Summerskill syndrome is characterized by intermittent episodes of cholestasis without extrahepatic bile duct obstruction. The symptoms usually spontaneously resolve after periods of weeks to months and patients generally have a good prognosis. In 1994, a candidate gene was localized by linkage disequilibrium to chromosome 18<sup>[66]</sup>, and in 1997, the localization was further refined to chromosome 18q21<sup>[67]</sup>. Progressive intrahepatic cholestasis or Byler disease was first described in the Old Order Amish. This condition is severe and usually leads to death within the first decade of life. Because both benign recurrent intrahepatic cholestasis type 1 and progressive familial intrahepatic cholestasis have similar symptoms, albeit different prognoses, it was suspected that they may be caused by different mutations in the same gene. In 1995, the gene for progressive familial intrahepatic cholestasis was mapped to chromosome 18q21-22, the benign recurrent intrahepatic cholestasis region, in an extended Amish kindred<sup>[68]</sup>. In 1998, a gene at this locus that encodes a P-type ATPase was identified<sup>[69]</sup>. Different mutations in the gene encoding this P-type ATPase were identified in individuals with benign recurrent intrahepatic cholestasis and progressive familial intrahepatic cholestasis type 1. The P-type ATPases responsible for these two diseases likely actively transports bile

salts across cell membranes. Depending upon the specific mutation in this P-type ATPase, a cholestatic disorder with a relatively benign or very severe phenotype can result.

## MOLECULAR BIOLOGY AND DRUG DEVELOPMENT

### *Recombinant vaccines to prevent viral hepatitis*

Molecular biology has had an impact on the development of safe vaccines to prevent viral hepatitis. The greatest impact has been in the development of vaccines against HBV. Highly effective HBV vaccines have been produced by recombinant DNA technology<sup>[70,71]</sup>. For these preparations, HBV surface antigen is generally produced as a recombinant protein in yeast. In a landmark study published in 1997, it was demonstrated that universal hepatitis B vaccination reduced the incidence of hepatocellular carcinoma in children in Taiwan<sup>[72]</sup>. Universal vaccination with relatively inexpensive preparations produced by recombinant DNA technology could lead to the eradication of this disease. The use of recombinant proteins for vaccination against hepatitis viruses other than HBV has lagged. Preliminary results in monkeys suggest that vaccination with a recombinant protein containing part of the viral capsid antigen may be protective against hepatitis E<sup>[73]</sup>.

### *Recombinant interferons for viral hepatitis*

Interferon alpha is used in the treatment of chronic hepatitis B and C<sup>[74]</sup>. Cloning of the cDNA for interferon alpha has made it possible to produce it using recombinant DNA technology. Several slightly different recombinant preparations with various amino acid substitutions are available. Although much leaves to be desired regarding long-term cure rates of treated individuals, recombinant interferons currently provide a partially effective first line treatment option for individuals with hepatitis B and C.

### *Rational drug design*

Determination of the three-dimensional structures of target molecules facilitates the design of compounds that inhibit or possibly enhance the functions of the target. For example, inhibitors of an enzyme can be rationally designed if the three-dimensional structure of the active site is known. Structure based design can be combined with high throughput combinatorial methods to rapidly identify compounds that are effective against a target.

The ability to express portions of proteins from recombinant cDNA clones has tremendously simplified the ability to purify starting materials for

structural studies. Improvements in methods for X-ray crystallography and nuclear magnetic resonance spectroscopy have also reduced the amount of time and starting material required for accurate structure determination. These advances have recently had an impact in the rational design of drugs to treat liver diseases as exemplified by the structure determinations of several non structural proteins of HCV (Table 2) . The NS3 polypeptides has two functional domains, one with protease activity that cleaves the viral polyprotein at several sites and the other with RNA helicase activity that unwinds viral RNA. Both of these activities are necessary for viral replication. Expression of recombinant polypeptides of the protease and helicase domains of NS3, as well as the protease domain complexed with its co-factor NS4A, has permitted their three dimensional structures to be determined<sup>[75-78]</sup>. Based on this knowledge, pharmaceutical and biotechnology companies are in the process of rationally designing inhibitors of these essential viral enzymatic functions.

**Table 2** HCV non-structural proteins with three-dimensional structures determined and published in the literature

Polypeptide	Function	Reference
NS3 protease domain	Protease that cleaves HCV polyprotein at several locations	75,76
NS4A (complexed to NS3)	Cofactor for NS3 protease	76
NS3 RNA helicase domain	Unwinds viral RNA	77, 78

Novel processes have also recently been used to determine the structures of hepatitis viral proteins. Despite many efforts, the core protein of HBV has eluded crystallization. However, high resolution cryoelectron microscopy has recently been used to establish the three-dimensional structure of complexes of this protein<sup>[79,80]</sup>. This knowledge can also provide a rational foundation to develop inhibitors of HBV particle assembly.

With regards to liver diseases, rational drug design is not limited to hepatitis virus. For example, the domain of the hemochromatosis protein HFE that binds to transferrin receptor has been expressed as a recombinant protein and its three-dimensional structure determined by X-ray crystallography<sup>[55]</sup>. This knowledge can lead to the potential design of drugs that bind to transferrin receptor in a similar fashion to inhibit cellular iron uptake that may be increased in hereditary hemochromatosis because of defective HFE binding.

### *Gene therapy*

Gene therapy for gastrointestinal and liver diseases have been reviewed in detail elsewhere<sup>[81,82]</sup>. A detailed discussion of hepatic gene therapy is beyond

the scope of this paper and only some of the major areas are touched upon here. Hepatic gene therapy will probably have the greatest initial impact in the treatment of inherited metabolic diseases but variations on classical techniques may also find utility in the treatment of viral diseases, cancer and in the production of vaccines.

In *ex vivo* hepatic gene therapy, hepatocytes are removed from the patient, cultured *in vitro* and the desired expression vector is introduced into the cultured hepatocytes. The transduced or transfected hepatocytes are then introduced into the portal vein and lodge in the patient's liver. Hepatocyte directed *ex vivo* gene therapy has already been performed in human subjects with familial hypercholesterolemia using the low density lipoprotein (LDL) receptor gene<sup>[83]</sup>. Several treated patients had persistent and significant reductions in serum cholesterol and LDL concentrations.

*In vivo* gene therapy is also an option for the treatment of liver diseases. In *in vivo* gene therapy, gene transfer vectors are introduced directly into the patient and taken up by the liver. Vectors for hepatic *in vivo* gene therapy include DNA complexed with proteins, DNA in liposomes, naked DNA and hepatotropic viral vectors such as those based on adenovirus.

Besides the transfer of human genes, other nucleic acid based therapies may be useful in the treatment of liver diseases. Antisense oligonucleotides<sup>[84]</sup> can be used to inhibit the expression of genes such as those essential for the replication of hepatitis viruses. Ribozymes are catalytic RNA molecules that can also be used for similar purposes<sup>[85]</sup>. Direct injection of DNA vectors into human cells may also be used for the production of vaccines against viral hepatitis<sup>[86]</sup>. All of these techniques are in their infancy, however, clinical trials will likely be common in the next decade.

## CONCLUSIONS

Molecular biology will continue to have a tremendous impact on the diagnosis and treatment of liver diseases. The basic research conducted in the past three decades will revolutionize the way hepatology, and all of medicine, is practiced. As a result, common conditions such as viral hepatitis B and C and inherited metabolic diseases affecting the liver such as hemochromatosis will hopefully vanish from the world.

## REFERENCES

- 1 Worman HJ. Molecular biological methods in the diagnosis and treatment of liver diseases. *Clin Chem*, 1997;43:1476-1486
- 2 Mamiya N, Worman HJ. Epidemiology, prevention, clinical features and therapy of hepatitis B, C, and G. *Curr Opin Infect Dis*, 1997;10:390-397
- 3 Urdea MS. Synthesis and characterization of branch DNA for the direct and quantitative detection of CMV, HBV, HCV and HIV. *Clin Chem* 1993; 39:725-726
- 4 Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA *et al*. Enzymatic amplification of beta globin genomic sequences and restriction site

- analysis for diagnosis of sickle cell anemia. *Science*, 1985;230:1350-1354
- 5 Mullis KB, Faloona FA. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol*, 1987;155:335-350
- 6 Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A *et al*. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet*, 1989;2:588-591
- 7 Ulrich PP, Bhat RA, Kelly I, Brunetto MR, Bonino F, Vyas GN. A precore-defective mutant of hepatitis B virus associated with eantigen-negative chronic liver disease. *J Med Virol*, 1990;32:109-118
- 8 Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*, 1989;244:359-362
- 9 Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH *et al*. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science*, 1989;244:362-364
- 10 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T *et al*. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA*, 1990; 87:9524-9528
- 11 Choo, QL, Richman KH, Han JH, Berger K, Lee C, Dong C *et al*. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA*, 1991; 88:2451-2455
- 12 Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A *et al*. Nucleotide sequence of the genomic RNA hepatitis C virus isolated from a human carrier: comparison with reported isolates from conserved and divergent regions. *J Gen Virol*, 1991;72:2697-2704
- 13 Takamizawa A, Mori C, Fuke I, Manabe S, Murakami S, Fujita J *et al*. Structure and organization of the hepatitis C genome isolated from human carriers. *J Virol*, 1991;65:1105-1113
- 14 Urdea MS. Branched DNA signal amplification. *Biotechnology*, 1994;12:926-928
- 15 Simmonds P, Smith DB, McOmish F, Yap PL, Kolberg J, Urdea MS *et al*. Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS5 regions. *J Gen Virol*, 1994;75:1053-1061
- 16 Lau JY, Mizokami M, Kolberg JA, Davis GL, Prescott LE, Ohno T *et al*. Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. *J Infect Dis*, 1994;171:281-289
- 17 Lau JY, Davis GL, Prescott LE, Maertens G, Lindsay KL, Qian K *et al*. Distribution of hepatitis C virus genotypes determined by line probe assay in patients with chronic hepatitis C seen at tertiary referral centers in the United States. *Ann Intern Med*, 1996;124:868-876
- 18 Zein NN, Rakela J, Krawitt EL, Reddy KR, Tominaga T, Persing DH *et al*. Hepatitis C virus genotype in the United States: epidemiology, pathogenicity, and response to interferon therapy. *Ann Intern Med*, 1996;125:634-639
- 19 Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG *et al*. Isolation of novel virus like sequences associated with human hepatitis. *Nature Med*, 1995;1:564-569
- 20 Linnen J, Wages J Jr, Zhang Keck ZY, Fry KE, Krawczynski KZ, Alter H *et al*. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science*, 1996;271:505-508
- 21 Alter MJ, Gallagher M, Morris T, Moyer LA, Meeks EL., Krawczynski K *et al*. Acute Non-A-E hepatitis in the United States and the role of hepatitis G virus infection. *N Engl J Med*, 1997;336:741-746
- 22 Alter HJ, Nakatsuji Y, Melpolder J, Wages J, Wesley R, Shih WK *et al*. The incidence of transfusion-associated hepatitis G virus infection and its relation to liver disease. *N Engl J Med*, 1997;336:747-754
- 23 Van de Water J, Cooper A, Surh CD, Coppel R, Danner D, Ansari A *et al*. Detection of autoantibodies to recombinant mitochondrial proteins in patients with primary biliary cirrhosis. *N Engl J Med*, 1989;320:1377-1380
- 24 Gershwin ME, Coppel RL, Mackay IR. Primary biliary cirrhosis and mitochondrial autoantigens insights from molecular biology. *Hepatology*, 1988; 8:147-151
- 25 Gershwin ME, Mackay IR. Primary biliary cirrhosis: paradigm or paradox for autoimmunity. *Gastroenterology*, 1991;100:822-833
- 26 Worman HJ, Courvalin JC. Autoantibodies against nuclear envelope proteins in liver disease. *Hepatology*, 1991;14:1269-1279
- 27 Courvalin JC, Worman HJ. Nuclear envelope protein autoantibodies in primary biliary cirrhosis. *Semin Liver Dis*, 1997;17:79-90
- 28 Coppel RL, McNeilage LJ, Surh CD, Van de Water J, Spithill TW, Whittingham S *et al*. Primary structure of the human M2 mitochondria autoantigen of primary biliary cirrhosis: dihydrolipoamide acetyltransferase. *Proc Natl Acad Sci USA*, 1988;85:7317-7321
- 29 Fussey SPM, Guest JR, James OFW, Bassendine MF, Yeaman SJ. Identification of the major M2 autoantigens in primary biliary cirrhosis. *Proc Natl Acad Sci USA*, 1988;85:8654-8658
- 30 Surh CD, Danner DJ, Ahmed A, Coppel RL, Mackay IR, Dickson ER *et al*. Reactivity of primary biliary cirrhosis sera with a human fetal liver cDNA line of branched chain alpha keto acid dehydrogenase dihydrolipoamide acetyltransferase, the 52 kD mitochondrial antigen. *Hepatology*, 1989;9:63-68
- 31 Leung PSC, Iwayama T, Prindiville T, Chuang DT, Ansari AA, Wynn RM *et al*. Use of designer recombinant mitochondrial antigens in the diagnosis of primary biliary cirrhosis. *Hepatology*, 1992;15:367-372
- 32 Moteki S, Leung PS, Coppel RL, Dickson ER, Kaplan MM, Munoz S *et al*. Use of a designer triple expression hybrid clone for three different lipoyl domain for the detection of antimitochondrial autoantibodies. *Hepatology*, 1996;24:97-103
- 33 Courvalin JC, Lassoued K, Bartnik E, Blobel G, Wozniak RW. The 210 kilodalton nuclear envelope polypeptide recognized by human autoantibodies in primary biliary cirrhosis is the major glycoprotein of the nuclear pore. *J Clin Invest*, 1990;86:279-285
- 34 Nickowitz RE, Worman HJ. Autoantibodies from patients with primary biliary cirrhosis recognize a restricted region within the cytoplasmic tail of nuclear pore membrane glycoprotein gp210. *J Exp Med*, 1993;178:2237-2242

- 35 Tartakovsky F, Worman HJ. Detection of gp210 autoantibodies in primary biliary cirrhosis using a recombinant fusion protein containing the predominant autoepitope. *Hepatology*, 1995;21:495-500
- 36 Bandin O, Courvalin JC, Poupon R, Dubel L, Homberg JC, Johanet C. Specificity and sensitivity of gp210 autoantibodies detected using an enzyme-linked immunosorbent assay and a synthetic polypeptide in the diagnosis of primary biliary cirrhosis. *Hepatology*, 1996;23:1020-1024
- 37 Frydman M, Bonne-Tamir B, Farrer LA, Conneally PM, Magazanik A, Ashbel S *et al.* Assignment of the gene for Wilson disease to chromosome 13: linkage to the esterase D locus. *Proc Natl Acad Sci USA*, 1985;82:1819-1821
- 38 Kooy RF, Van der Veen AY, Verlind E, Houwen RHJ, Scheffer H, Buys CHCM. Physical localisation of the chromosomal marker D13S31 places the Wilson disease locus at the junction of bands q14.3 and q21.1 of chromosome 13. *Hum Genet*, 1993;91:504-506
- 39 Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nature Genet*, 1993;5:327-337
- 40 Petrukhin K, Fischer SG, Pirastu M, Tanzi RE, Chernov I, Devoto M *et al.* Mapping, cloning and genetic characterization of the region containing the Wilson disease gene. *Nature Genet*, 1993;5:338-343
- 41 Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B *et al.* The Wilson disease gene is a copper transporting ATPase with homology to the menkes disease gene. *Nature Genet*, 1993;5:344-350
- 42 Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW. The Wilson disease gene: spectrum of mutations and their consequences. *Nature Genet*, 1995;9:210-217
- 43 Edwards CQ, Griffen LM, Goldgar D, Drummond C, Skolnick MH, Kushner JP. Prevalence of hemochromatosis among 11065 presumably healthy blood donors. *N Engl J Med*, 1988;318:1355-1362
- 44 Simon M, Bourel M, Fauchet R, Genetet B. Association of HLA-A3 and HLA-B14 antigens with idiopathic haemochromatosis. *Gut*, 1976;17:332-334
- 45 Stevens FM, Walters JM, Watt DW, McCarthy CF. Inheritance of idiopathic haemochromatosis. *Lancet*, 1977;1:1107
- 46 Cartwright GE, Skolnick M, Amos DB, Edwards CQ, Kravitz K, Johnson A. Inheritance of hemochromatosis: linkage to HLA. *Trans Assoc Am Phys*, 1978;91:273-281
- 47 Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A *et al.* A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genet*, 1996;13:399-408
- 48 Beutler E, Gelbart T, West C, Lee P, Adams M, Blackstone R *et al.* Mutation analysis in hereditary hemochromatosis. *Blood Cells Molec Dis*, 1996;22:187-194
- 49 Jazwinska EC, Cullen LM, Busfield F, Pyper WR, Webb SI, Powell LW *et al.* Haemochromatosis and HLA H. *Nature Genet*, 1996;14:249-251
- 50 Jouanolle AM, Gandon G, Jezequel P, Blayau M, Campion ML, Yaouanq J *et al.* Haemochromatosis and HLA H. *Nature Genet*, 1996;14:251-252
- 51 Cuthbert JA. Iron, HFE, and hemochromatosis update. *J Invest Med*, 1997;45:518-529
- 52 Beutler E. The significance of the 187G (H63D) mutation in hemochromatosis. *Am J Hum Genet*, 1997;61:762-764
- 53 Parkkila S, Waheed A, Britton RS, Bacon BR, Zhou XY, Tomatsu S *et al.* Association of the transferrin receptor in human placenta with HFE, the protein defective in hereditary hemochromatosis. *Proc Natl Acad Sci USA*, 1997;94:13198-13202
- 54 Feder JN, Penny DM, Irrinke A, Lee VK, Lebr<sup>n</sup> JA, Watson N *et al.* The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proc Natl Acad Sci USA*, 1998;95:1472-1477
- 55 Lebr<sup>n</sup> JA, Bennett MJ, Vaughn DE, Chirino AJ, Snow PM, Mintier GA *et al.* Crystal structure of the hemochromatosis protein HFE and characterization of its interaction with transferrin receptor. *Cell*, 1998;93:111-123
- 56 Crigler JF JR, Najjar VA. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics*, 1952;10:169-179
- 57 Arias IM, Gartner LM, Cohen M, Ben-Ezzer J, Levi AJ. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency: clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. *Am J Med*, 1969;47:395-409
- 58 Ritter JK, Chen F, Sheen YY, Tran HM, Kimura S, Yeatman MT *et al.* A novel complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. *J Biol Chem*, 1992;267:3257-3261
- 59 Bosma PJ, Roy Chowdhury J, Huang TJ, Lahiri P, Oude Elferink RPJ, Van Es, HHG *et al.* Mechanisms of inherited deficiencies of multiple UDP-glucuronosyltransferase isoforms in two patients with Crigler-Najjar syndrome, type I. *FASEB J*, 1992;6:2859-2863
- 60 Ritter JK, Yeatman MT, Kaiser C, Gridelli B, Owens IS. A phenylalanine codon deletion at the UGT1 gene complex locus of a Crigler-Najjar type I patient generates a pH-sensitive bilirubin UDP-glucuronosyltransferase. *J Biol Chem*, 1993;268:23573-23579
- 61 Aono S, Yamada Y, Keino H, Sasaoka Y, Nakagawa T, Onishi S *et al.* A new type of defect in the gene for bilirubin uridine 5-prime-diphosphate-glucuronosyltransferase in a patient with Crigler-Najjar syndrome type I. *Ped Res*, 1994;35:629-632
- 62 Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL *et al.* Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science*, 1996;271:1126-1128
- 63 van Kuijk MA, Kool M, Merck GFM, Geurts van Kessel A, Bindels RJM, Deen PMT *et al.* Assignment of the canalicular multispecific organic anion transporter gene (CMOAT) to human chromosome 10q24 and mouse chromosome 19D2 by fluorescent in situ hybridization. *Cytogenet Cell Genet*, 1997;77:285-287
- 64 Paulusma CC, Kool M, Bosma PJ, Scheffer GL, Borg FT, Scheper RJ *et al.* A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin Johnson syndrome. *Hepatology*, 1997;25:1539-1542
- 65 Wada M, Toh S, Taniguchi K, Nakamura T, Uchiyama T, Kohno K *et al.* Mutations in the canalicular multispecific organic anion transporter (CMOAT) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome. *Hum Molec Genet*, 1998;7:203-207
- 66 Houwen RHJ, Baharloo S, Blankenship K, Raeymaekers P, Juyn J, Sandkuijji *et al.* Genome screening by searching for shared segments: mapping a gene for benign recurrent intrahepatic cholestasis. *Nature Genet*, 1994;8:380-386
- 67 Sinke RJ, Carlton VEH, Juijn JA, Delhaas T, Bull L, van Berge Henegouwen GP *et al.* Benign recurrent intrahepatic cholestasis (BRIC): evidence of genetic heterogeneity and delimitation of the BRIC locus to a 7-cM interval between D18S69 and D18S64. *Hum Genet*, 1997;100:382-387
- 68 Carlton VEH, Knisely AS, Freimer NB. Mapping of a locus for progressive familial intrahepatic cholestasis (Byler disease) to 18q21-q22, the benign recurrent intrahepatic cholestasis region. *Hum Molec Genet*, 1995;4:1049-1053
- 69 Bull LN, van Eijk MJT, Pawlikowska L, DeYoung JA, Juijn JA, Liao M *et al.* A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. *Nature Genet*, 1998;18:219-224
- 70 McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Millr WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature*, 1984;307:178-180
- 71 Lemon SM, Thomas DL. Vaccines to prevent viral hepatitis. *N Engl J Med*, 1997;336:196-204
- 72 Chang MH, Chen CJ, Lai MS, Hsu HM, Wu TC, Kong MS *et al.* Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. *N Engl J Med*, 1997;336:1855-1859
- 73 Tsarev SA, Tsareva TS, Emerson SU, Govindarajan S, Shapiro M, Gerin JL *et al.* Successful passive and active immunization of cynomolgus monkeys against hepatitis E. *Proc Natl Acad Sci USA*, 1994;91:10198-10202
- 74 Hoofnagle JH, Di Bisceglie AM. The treatment of chronic viral hepatitis. *N Engl J Med*, 336:347-356
- 75 Love RA, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW *et al.* The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell*, 1996;87:331-342
- 76 Kim JL, Morgenstern KA, Lin C, Fox T, Dwyer MD, Landro JA *et al.* Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell*, 1996;87:343-355
- 77 Yao N, Hesson T, Cable M, Hong Z, Kwong AD, Le HV *et al.* Structure of the hepatitis C virus RNA helicase domain. *Nature Struct Biol*, 1997;4:463-467
- 78 Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA *et al.* Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure*, 1998;6:89-100
- 79 Bottcher B, Wynne SA, Crowther RA. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature*, 1997;386:89-91
- 80 Conway JF, Cheng N, Zlotnick A, Wingfield PT, Stahl SJ, Steven AC. Visualization of the 4 helix bundle in the hepatitis B virus capsid by cryoelectron microscopy. *Nature*, 1997;386:91-94
- 81 Wilson JM, Askari FK. Hepatic and gastrointestinal gene therapy. In: Yamada T, ed. Textbook of Gastroenterology (Yamada T, ed. Gastroenterology Updates, Vol. 1) Philadelphia: J.B. Lippincott Co. 1996:1-20
- 82 Xu CT, Pan BR. Current status of gene therapy in gastroenterology. *World J Gastroenterol*, 4:85-89
- 83 Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D *et al.* Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. *Nature Genet*, 1994;6:335-441
- 84 Askari FK, McDonnell WM. Antisense oligonucleotide therapy. *N Engl J Med*, 1996;334:316-318
- 85 Christoffersen RE, Marr JJ. Ribozymes as human therapeutic agents. *J Med Chem*, 1995;38:2023-2037
- 86 McDonnell WM, Askari FK. DNA vaccines. *N Engl J Med*, 1996;334:42-45

# ***Regulation of hepatic function by brain neuropeptides***

Masashi Yoneda

**Subject headings** liver/physiology; thyrotropin releasing hormone; corticotropin releasing factor; neuropeptide Y; central nervous system; bombesin;  $\beta$ -endorphin; D-Ala-Met enkephalinamide

Since the central nervous system was first demonstrated to be involved in regulation of gastric function by Pavlov<sup>[1]</sup>, and Selye<sup>[2]</sup> established the stress theory that psychopsychiatric stress altered physiological functions<sup>[3]</sup>, electrical stimulation or lesion of specific brain nuclei identified specific sites in the hypothalamus, limbic system, and medulla that influence gastrointestinal functions. A plethora of peptides have been characterized in the brain by immunohistochemical and molecular biological techniques<sup>[4]</sup>. The development of retrograde tracing techniques combined with immunohistochemistry reveals that these peptides are localized in the nerve fibers or cell bodies of the hypothalamus and medulla which are important sites for autonomic nervous outflow to the gastrointestinal tract<sup>[5,6]</sup>. Based on these studies, Taché first reported the effect of central neuropeptides in regulation of gastric functions<sup>[7,8]</sup>. Since then, more than 40 peptides have been examined and it is well established that many neuropeptides, such as thyrotropin releasing hormone (TRH), corticotropin releasing factor (CRF), neuropeptide Y (NPY), bombesin and somatostatin, mediate a central nervous system induced stimulation or inhibition of gastrointestinal function<sup>[9,10]</sup>. On the other hand, the liver is also richly innervated<sup>[11,12]</sup> and retrograde tracing

technique has revealed hepatic innervation through the vagus originating in the medulla<sup>[13]</sup>, where abundant neuropeptides exist. This review introduces the current knowledge of central nervous system regulation of hepatic functions by various neuropeptides.

## **THYROTROPIN RELEASING HORMONE (TRH)**

TRH exists in the central nervous system and abundant TRH immunoreactive nerve terminals and TRH receptors are localized in the dorsal vagal complex including the vagal motor nucleus and the nucleus of the solitary tract<sup>[14,15]</sup>. Neuropharmacological studies demonstrated that TRH displayed a vast array of central nervous system-mediated actions unrelated to its physiological role in the regulation of the pituitary thyroid axis<sup>[16]</sup>. TRH injected into the cerebrospinal fluid or into specific brain nuclei exerted a variety of behavioral effects. Accumulated evidence also demonstrated that TRH had potent central nervous system mediated stimulatory effects on gastrointestinal secretion, motility and transit, as well as on the development of gastric ulceration in rats and other animals<sup>[7,8,17,18]</sup>. Mapping studies using microinjection of TRH or TRH analogs into selective nuclei have identified brain sites important for stimulation of gastric secretion and motility. The gastric secretory response has been elicited by microinjection of TRH into the lateral and the ventromedial hypothalamus<sup>[19]</sup>. More sensitive sites have been identified in the brainstem including the dorsal vagal complex, the nucleus ambiguus and the raphe pallidus<sup>[20-22]</sup>.

Hepatic blood flow is composed of hepatic arterial and hepatic portal blood supplies. In rats portal blood flow constitutes 80% of hepatic circulation. Portal blood flow was altered by electrical stimulation of autonomic nerves and specific brain nuclei which are important sites for autonomic nervous regulation<sup>[23]</sup>. Stimulation of sympathetic nerves caused constriction of hepatic arterial and portal vessels, resulting in a decrease of liver blood volume and flow in hepatic artery, and an increase in portal pressure<sup>[24]</sup>. Electrical stimulation of the hypothalamus produced changes in intestinal blood flow and, consequently, in portal vein and in intrahepatic arterial and portal beds<sup>[25]</sup>. Moreover, stimulation of the medial and posterior hypothalamus has been reported to increase hepatic arterial resistance and decrease portal blood flow

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**Received** 1998-04-29

through sympathetic nerves<sup>[26]</sup>. On the other hand, using an in vivo microscopic technique in rats, dilatation of the liver sinusoid following electrical vagal stimulation and acetylcholine application has been observed<sup>[27,28]</sup>.

We studied the effect of central TRH analog on hepatic microcirculation in anesthetized rats<sup>[29]</sup>. We used the stable TRH analog, RX 77368, which has the same receptor affinity and about the times the potency of natural TRH. In rats under urethan anesthesia, hepatic microcirculation was assessed by the hydrogengas clearance method. Intracisternal injection (the injection into the cisternal magna of the brain) of TRH analog induced stimulation of hepatic blood flow by 17%-74% in a dose dependent manner ranging from 5ng to 100ng. This stimulatory response of hepatic blood flow to central TRH analog occurred during the first 15-minute observation period, reached a plateau at 30 minutes and returned to basal value at 60 minutes. On the other hand, intravenous injection of the TRH analog did not modify hepatic blood flow, confirming the central but not peripheral action of the TRH analog. Although this increased hepatic blood flow by central TRH analog was not modified by spinal cord transection, it was reversed by atropine, vagotomy, and N-G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide and indomethacin, indicating that TRH acted in the brain to stimulate hepatic blood flow through vagal, muscarinic, prostaglandin and nitric oxide pathways. Mapping studies by microinjection of TRH analog into the medullary nuclei revealed that the left but not right dorsal vagal complex was a responsive site for TRH on modulation of hepatic blood flow<sup>[30]</sup>. This finding agreed well with anatomical evidence that hepatic vagal nerve is originated in the left, but not right, dorsal vagal complex<sup>[13]</sup>.

Although in adult animals the liver is normally in a state of growth arrest, once the liver is damaged or impaired because of hepatic injury or liver resection, hepatic regeneration or proliferation is immediately started. Seventy percent partial hepatectomy<sup>[31]</sup> performed in rats initiated a very striking response in the liver remnant through hypertrophy and hyperplasia, and returned to its original size in 7-10 days<sup>[31]</sup>. Hepatocytes in the non stimulated liver were essentially arrested in the G<sub>0</sub> state. After partial hepatectomy, growth related events started almost instantly as the cells underwent the transition from G<sub>0</sub> to G<sub>1</sub> state (prereplicative phase), which lasted about 12 hours, at which point DNA synthesis (S state) began and peaked at about 24 hours. Mitosis a similar course 6-8 hours later<sup>[32]</sup>. Many factors, such as hormones, peptides and cytokines, were thought to be involved and may interact synergistically to initiate and maintain hepatic proliferation. The autonomic

nervous system was also suggested to play a role in the liver regeneration and regulation of hormones and growth factors related to hepatic proliferation. Partial hepatectomy suppressed the sympathetic nerve activity<sup>[33]</sup> and plasma adrenaline and noradrenaline increased immediately after partial hepatectomy and  $\alpha$ -blockade reversed DNA synthesis induced by hepatectomy<sup>[34,35]</sup>, indicating the involvement of an adrenergic effect on hepatic regeneration. The parasympathetic nervous system was also suggested to play an important role in hepatic proliferation. Subdiaphragmatic vagotomy more strongly suppressed DNA synthesis after partial hepatectomy as compared with the splanchnicectomy<sup>[36]</sup>, and selective hepatic branch vagotomy suppressed or delayed liver regeneration in partially hepatectomized rats<sup>[37]</sup>. Moreover, lesion of the ventromedial hypothalamus has recently induced hepatic DNA synthesis through the vagal nerve<sup>[38]</sup>.

The effect of central administration of TRH analog, RX 77368, on hepatic proliferation has recently been studied in conscious adult rats<sup>[39]</sup>, because central TRH was known to activate vagal efferent fibers<sup>[40,41]</sup>. Rats was injected with TRH analog intracisternally and hepatic DNA synthesis was assessed by thymidine incorporation into the hepatic DNA fraction and BrdU accumulation 6-72 hours later. Hepatic proliferation was stimulated by intracisternal TRH analog (10ng) with a peak response at 48 hours after peptide injection and returned to basal at 72 hours. This stimulatory effect by central TRH on hepatic proliferation was dose related, ranging from 5ng to 100ng assessed at 24 hours. Intravenous TRH analog did not influence hepatic proliferation, confirming a central but not peripheral action of TRH. Stimulation of hepatic proliferation by central TRH was abolished by hepatic branch vagotomy, atropine and indomethacin, suggesting that TRH acts in the brain to stimulate hepatic proliferation through vagal, muscarinic and prostaglandin pathways. However, N-G-nitro-L-arginine methyl ester did not reverse central TRH induced stimulation of hepatic proliferation as it did central TRH induced hepatic circulation, indicating that stimulation of hepatic proliferation is not secondary to the change in hepatic circulation. These data suggest that TRH in the central nervous system may be involved in the vagal regulation of hepatic proliferation.

These findings led us to speculate that central TRH might also protect against experimental liver damage, so effect of central injection of TRH on CCl<sub>4</sub> induced liver injury has been investigated<sup>[42,43]</sup>. Rats were coadministered CCl<sub>4</sub> (2ml/kg, ip) with TRH analog injected intracisternally and liver damage was assessed by serum alanine aminotransferase (ALT) levels. Intracisternal, but not intravenous, injection of TRH analog dose-

dependently protected against  $\text{CCl}_4$  induced liver damage and this protective effect of central TRH was also blocked by hepatic vagotomy.

#### CORTICOTROPIN RELEASING FACTOR (CRF)

CRF is one of the brain neuropeptides, and effect of central CRF on physiological, pharmacological, and pathophysiological regulations of the gastrointestinal tract have been reported. Injection of CRF into the cerebrospinal fluid or the brain nuclei, such as paraventricular nucleus or locus ceruleus inhibited gastric motility and secretion<sup>[43-46]</sup>, and enhanced colonic motility through the autonomic nervous system<sup>[47,48]</sup>. Since CRF is known to act in the hypothalamus and stimulate the sympathetic nervous outflow, the opposite effect to TRH on hepatic function was expected when injected in the central nervous system.

We studied the effect of central CRF on hepatic microcirculation in anesthetized rats<sup>[49]</sup>. In rats under urethan anesthesia, hepatic microcirculation was assessed by the hydrogen gas clearance method and laser Doppler. Intracisternal injection of CRF induced inhibition of hepatic blood flow by 18%-36% in a dose dependent manner, ranging from 1 $\mu\text{g}$  to 5 $\mu\text{g}$ . This inhibitory response of hepatic blood flow to central CRF was noted during the first 15 minute observation period, reached a plateau at 30-60 minutes and maintained for more than 120 minutes. On the other hand, intravenous injection of the CRF did not modify hepatic blood flow, confirming the central but not peripheral action of the TRH analog. Although this decreased hepatic blood flow by central CRF was not modified by atropine and vagotomy, it was reversed by hepatic sympathectomy and 6-hydroxydopamine, which depleted noradrenergic fibers, indicating that CRF acted in the brain to inhibit hepatic blood flow through sympathetic and noradrenergic pathways. The effect of central injection of CRF on  $\text{CCl}_4$  induced liver injury has also been investigated<sup>[50]</sup>. Rats were injected with  $\text{CCl}_4$  (2ml/kg, sc) and CRF was injected just before and 6 hours after  $\text{CCl}_4$  administration. Liver damage was assessed by serum ALT levels. Intracisternal injection of CRF dose dependently aggravated  $\text{CCl}_4$  induced liver damage and this aggravating effect of central CRF was blocked by 6-hydroxydopamine and chemical sympathectomy.

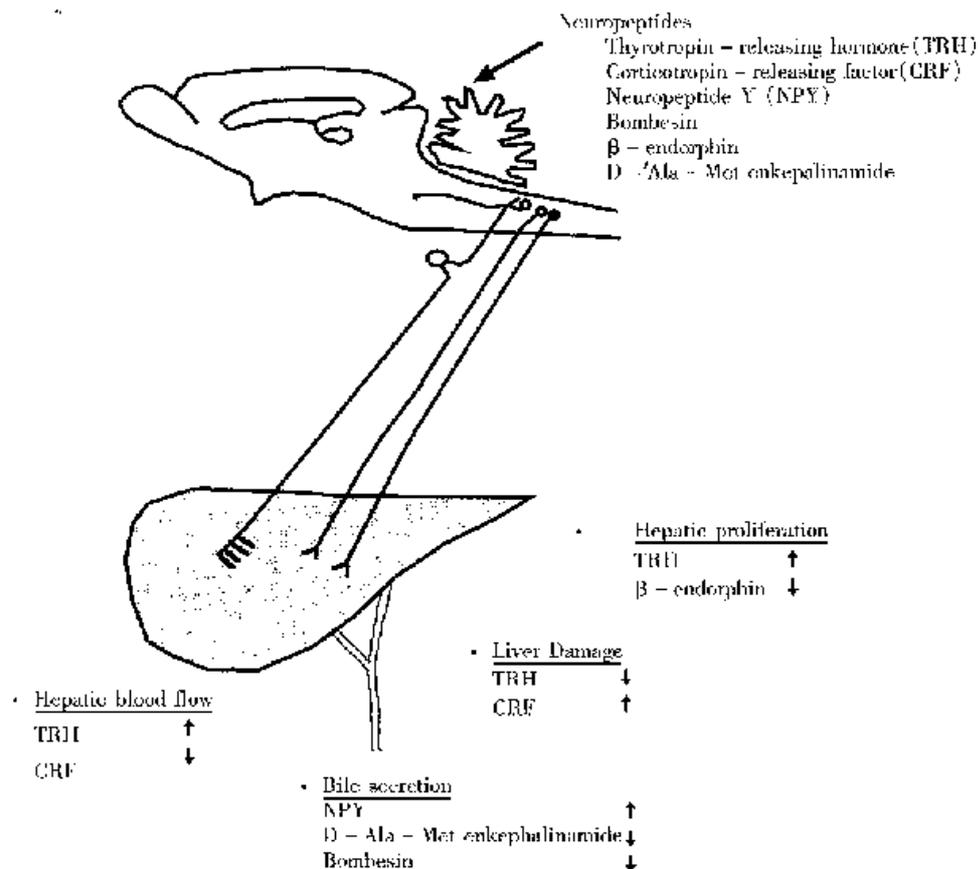
#### NEUROPEPTIDE Y (NPY)

NPY, a 36 amino acid peptide of the pancreatic polypeptide family, was first isolated from porcine brain<sup>[51,52]</sup>. NPY was localized mainly in the peripheral nervous system<sup>[53]</sup>, where it contributed to the innervation of the digestive organs, including the biliary tree<sup>[54]</sup>. In the brain, NPY nerve fibers and terminals, and NPY receptors were localized in the paraventricular nucleus of the hypothalamus and the dorsal vagal complex<sup>[55-57]</sup> which are important sites for the autonomic nervous system<sup>[58]</sup>. Central

administration of NPY affected feeding behavior and visceral function<sup>[57]</sup>. The bile duct was richly innervated by autonomic nerves<sup>[59]</sup> and electrical stimulation of sympathetic and parasympathetic nerves and stimulation or lesion of certain hypothalamic regions<sup>[60]</sup> altered bile secretion<sup>[61,62]</sup>. With respect to the gastrointestinal tract, injection of NPY into the cerebrospinal fluid stimulates gastric acid and pepsin secretion, and pancreatic exocrine and endocrine secretion in rats and dogs<sup>[63-65]</sup>. Farouk *et al*<sup>[66]</sup> and we<sup>[67]</sup> have found an effect of central NPY on bile secretion in dogs and rats. The effect of intracisternal injection of NPY on bile secretion in urethan anesthetized rats was investigated<sup>[67]</sup>. Rats were anesthetized with urethan (1.5g/kg, ip) and the common bile duct was cannulated to collect bile samples. Sodium taurocholic acid (30 $\mu\text{mol}\cdot\text{kg}\cdot\text{h}$ ) was infused intravenously to compensate for bile acid loss due to biliary drainage. Intracisternal NPY (0.02nmol-0.12nmol) dose-dependently stimulated bile secretion by 9%-20%. The secretory response occurred within the first 20-40 minutes after injection and lasted 120 minutes. On the other hand, intravenous injection of NPY (0.12nmol) did not modify bile secretion, confirming that NPY did not leak from the cerebrospinal fluid to the peripheral circulation. Thus, central NPY stimulated bile secretion in a bile acid independent and bicarbonate dependent bile flow because central NPY increased only biliary bicarbonate secretion but not biliary bile acid, phospholipid or cholesterol secretion. In other words, central NPY stimulated ductal bile secretion. Although cervical cord transection, bilateral adrenalectomy or pretreatment with N-G-nitro-L-arginine methyl ester did not alter intracisternal NPY induced stimulation of bile secretion, atropine and bilateral cervical vagotomy completely abolished the stimulatory effect of intracisternal NPY on bile secretion, indicating that NPY acted in the brain to stimulate bile acid independent bile secretion through vagal and muscarinic pathways. Mapping studies by microinjection of NPY into the medullary nuclei have shown that the left dorsal vagal complex is a responsive site, like TRH on hepatic circulation, for NPY on stimulation of bile secretion<sup>[68]</sup>.

#### OTHER NEUROPEPTIDES

Besides TRH, CRF and NPY, a few peptides were suggested to act in the central nervous system to modulate hepatobiliary function.  $\beta$ -endorphin has produced 70% inhibition in liver DNA synthesis in six day old rats<sup>[69]</sup>. Intracerebroventricular injection of bombesin with a dose of 10 $\mu\text{g}$  induces bicarbonate dependent inhibition of bile secretion in rats<sup>[70]</sup>. Moreover, intracisternal injection of opioid peptide, DAla-Met enkephalinamide decreased bile secretion in urethan anesthetized rats<sup>[71]</sup>.



**Figure 1** Schematic illustration of interaction between neuropeptides in the central nervous system and hepatobiliary function.

### SUMMARY AND CONCLUSIONS

Several peptides have been established to act in the brain to influence hepatic function (Figure 1). Bile secretion is modified by central administration of bombesin, opioid peptide and NPY, hepatic blood flow is altered by central CRF and TRH, hepatic proliferation is regulated by central  $\beta$ -endorphin and TRH, and central CRF and TRH interfere experimental liver injury. Among these peptides central TRH is the strongest candidate for playing an important role in hepatic physiological function. Through their use, new knowledge on central and peripheral mechanisms underlying brain regulation of hepatic function will be revealed. Further studies in regard to the physiological relevance of the central action of neuropeptides on specific brain sites should be performed for unraveling the underlying pathways mediating brain liver interaction.

**Acknowledgement** This work was supported by Japan Research Foundation for Clinical Pharmacology, the Akiyama Foundation, Kanoe Memorial Foundation and the Ministry of Education, Culture and Science of Japan (No. 0767554, No. 09670503).

### REFERENCES

- Pavlov I. The work of the digestive glands. (English translation. London: C. Griffin & Co. 1910)
- Selye H. Syndrome produced by diverse nocuous agents. *Nature*, 1932;138:32
- Brooks FP. Central neural control of acid secretion. In: C.F. Code, eds. Handbook of physiology. Washington, D.C.: American Physiological Society, 1967: 805
- Krieger DT. Brain peptides: what, where and why? *Science*, 1983;222: 975-985
- Leslie RA. Neuroactive substances in the dorsal vagal complex of the medulla oblongata: Nucleus of the tractus solitarius, area postrema, and dorsal motor nucleus of the vagus. *Neurochem Int*, 1985;7:191-211
- Swanson LW, Sawchenko PE. Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei. *Ann Rev Neurosci*, 1983;6: 269-324
- Tache Y, Vale W, Rivier J, Brown M. Brain regulation of gastric secretion: influence of neuropeptides. *Proc Nat Acad Sci USA*, 1980;77:5515-5519
- Tache Y, Vale W, Brown M. Thyrotropin-releasing hormone-CNS action to stimulate gastric acid secretion. *Nature*, 1980;287:149-151
- Lenz HJ, Klapdor R, Hester SE, Webb VJ, Galyean RF, Rivier JE, Brown MR. Inhibition of gastric acid secretion by brain peptides in the dog. Role of the autonomic nervous system and gastrin. *Gastroenterology*, 1986;91:905-912
- Tache Y. Central regulation of gastric acid secretion. In: L.R. Johnson, J. Christensen, E.D. Jacobson and J.H. Walsh, eds. Physiology of the gastrointestinal tract. New York: Raven Press, 1987:911-930
- Skaaring P, Bierring F. On the intrinsic innervation of normal rat liver. Histochemical and scanning electron microscopical studies. *Cell Tiss Res*, 1976; 171(2):141-155
- Sutherland SD. An evaluation of cholinesterase techniques in the study of the intrinsic innervation of the liver. *J Anat*, 1964;98:321-326
- Kohno T, Mori S, Mito M. Cells of origin innervating the liver and their axonal projections with synaptic terminals into the liver parenchyma in rats. *Hokkaido J Med Sci*, 1987;62:933-946
- Manaker S, Rizio G. Autoradiographic localization of thyrotropin-releasing hormone and substance P receptors in the rat dorsal vagal complex. *J Comp Neurol*, 1989;290:516-526
- Rinaman L, Miselis RR. Thyrotropin-releasing hormone-immunoreactive nerve terminals synapse on the dendrites of gastric vagal motoneurons in the rat. *J Comp Neurol*, 1990;294:235-251
- Tache Y, Stephens RL, Ishikawa T. Central nervous system action of TRH to influence gastrointestinal function and ulceration. *Ann NY Acad Sci*, 1989; 553:269-285
- Garrick T, Buack S, Veiseh A, Tache Y. Thyrotropin-releasing hormone (TRH)

- acts centrally to stimulate gastric contractility in rats. *Life Sci*, 1987;40:649-657
- 18 Goto Y, Tache Y. Gastric erosions induced by intracisternal thyrotropin releasing hormone (TRH) in rats. *Peptides*, 1985;6:153-156
  - 19 Maeda-Hagiwara M, Watanabe H. Inhibitory effects of intrahypothalamic injection of calcitonin on TRH stimulated gastric acid secretion in rats. *Jpn J Pharmacol*, 1985;39:173-178
  - 20 Ishikawa T, Yang H, Tache Y. Medullary sites of action of the TRH analogue, RX 77368, for stimulation of gastric acid secretion in the rat. *Gastroenterology*, 1988;95:1470-1476
  - 21 Stephens RL, Ishikawa T, Weiner H, Novin D, Tache Y. TRH analogue, RX 77368, injected into dorsal vagal complex stimulates gastric secretion in rats. *Am J Physiol*, 1988;254:G639-G643
  - 22 Yang H, Ishikawa T, Tache Y. Microinjection of TRH analogs into the raphe pallidus stimulates gastric acid secretion in the rat. *Brain Res*, 1990;531:280-285
  - 23 Carniero JJ, Donald DE. Change in liver blood flow and blood content in dogs during direct and reflex alteration of hepatic sympathetic nerve activity. *Circ Res*, 1977;40:150-158
  - 24 Lauth WW. Hepatic nerves: a review of their functions and effects. *Can J Pharmacol Physiol*, 1980;58:105-123
  - 25 Folkow B, Rubinstein E. Behavioral and autonomic patterns evoked by stimulation of the lateral hypothalamic area in the cat. *Acta Physiol Scand*, 1965;65:292-299
  - 26 Tsybenko VA, Yanchuk PI. Central nervous control of hepatic circulation. *J Auton Nerv Syst*, 1991;33:255-266
  - 27 Koo A, Liang I. Microvascular filling pattern in rat liver sinusoids during vagal stimulation. *J Physiol (Lond)*, 1979;295:191-199
  - 28 Koo A, Liang I. Stimulation and blockade of cholinergic receptors in terminal liver microcirculation in rats. *Am J Physiol*, 1979;236:E728-E732
  - 29 Tamori K, Yoneda M, Nakamura K, I.M. Effect of intracisternal thyrotropin-releasing hormone on hepatic blood flow in rats. *Am J Physiol*, 1998;274:G277-G282
  - 30 Yoneda M, Tamori K, Nakade Y, Takamoto S, Yokohama S, Aso K, Sato Y et al. Thyrotropin-releasing hormone (TRH) in the left dorsal vagal complex (DVC) increases the hepatic blood flow in rats (abst). *Gastroenterology* (in press)
  - 31 Higgins GM, R.M.A. Experimental pathology of the liver-I. Restriction of the liver of the white rat following partial surgical removal. *Arch Pathol*, 1931;12:186-202
  - 32 Bucher NLR. Liver regeneration: an overview. *J Gastroenterol Hepatol*, 1991;6:615-624
  - 33 Iwai M, Shimazu T. Alteration in sympathetic nerve activity during liver regeneration in rats after partial hepatectomy. *J Auton Nerv Syst*, 1992;41:209-214
  - 34 MacManus JP, Braceland BM, Youdale T, Whitfield JF. Adrenergic antagonists, and a possible link between the increase in cyclic adenosine 3', 5'-monophosphate and DNA synthesis during liver regeneration. *J Cell Physiol*, 1973;82:157-164
  - 35 Cruise JL, Knechtle SJ, Bollinger RR, Kuhn C, Michalopoulos G.  $\alpha$ -1 adrenergic effects and liver regeneration. *Hepatology*, 1987;7:1189-1194
  - 36 Kato H, Shimazu T. Effect of autonomic denervation on DNA synthesis during liver regeneration after partial hepatectomy. *Eur J Biochem*, 1983;190:473-478
  - 37 Ohtake M, Sakaguchi T, Yoshida K, Muto T. Hepatic branch vagotomy cansuppress liver regeneration in partially hepatectomized rats. *HPB Surgery*, 1993;6:277-286
  - 38 Kiba T, Tanaka K, Endo O, Inoue S. Role of vagus nerve in increased DNA synthesis after hypothalamic ventromedial lesion in rat liver. *Am J Physiol*, 1992;262:G483-G487
  - 39 Yoneda M, Tamori K, Sato Y, Yokohama S, Nakamura K, Makino I. Central thyrotropin-releasing hormone stimulates hepatic DNA synthesis in rats. *Hepatology*, 1997;26:1203-1208
  - 40 McCann MJ, Hermann GE, Rogers RC. Thyrotropin releasing hormone: effect on identified neurons of the dorsal vagal complex. *J Auton Nerve Syst*, 1989;26:107-112
  - 41 Somiya H, Tonoue T. Neuropeptides as central integrators of autonomic nerve activity: effect of TRH, SRIF, VIP and bombesin on gastric and adrenal nerves. *Regul Pept*, 1984; 9: 47-52
  - 42 Sato Y, Yoneda M, Yokohama S, Tamori K, Nakamura K, Makino I. Protective effect of central thyrotropin releasing hormone (TRH) on CCl4-induced liver damage in rats (abst). *Gastroenterology*, 1996;110:A1312
  - 43 Tache Y, Goto Y, Gunion MW, Vale W, River J, Brown M. Inhibition of gastric acid secretion in rats by intracerebral injection of corticotropin-releasing factor. *Science*, 1983;222:935-937
  - 44 Lenz HJ, Raedler A, Greten H, Vale WW, Rivier JE. Stress-induced gastrointestinal secretory and motor responses in rats are mediated by endogenous corticotropin releasing factor. *Gastroenterology*, 1988;95:1510-1517
  - 45 Gunion MW and Tache Y. Intrahypothalamic microinfusion of corticotropin-releasing factor inhibits gastric acid secretion but increases secretion volume in rats. *Brain Res*, 1987;411:156-161
  - 46 Lenz HJ, Burlage M, Raedler A, Greten H. Central nervous system effects of corticotropin-releasing factor on gastrointestinal transit in the rat. *Gastroenterology*, 1988;94:598-602
  - 47 Monnikes H, Raybould HE, Schmidt B, Tache Y. CRF in the paraventricular nucleus of the hypothalamus stimulates colonic motor activity in fasted rats. *Peptides*, 1993;14:743-747
  - 48 Monnikes H, Schmidt BG, Tebbe J, Bauer C, Tache Y. Microinfusion of corticotropin releasing factor into the locus coeruleus/subcoeruleus nuclei stimulates colonic motor function in rats. *Brain Research*, 1994;644:101-108
  - 49 Nakade Y, Yoneda M, Takamoto S, Yokohama S, Tamori K, Aso K, Sato Y et al. Central corticotropin releasing factor (CRF) decreases the hepatic blood flow in rats (abst). *Gastroenterology* (in press)
  - 50 Yokohama S, Yoneda M, Tamori K, Sato Y, Hasegawa T, Nakamura K, Makino I. Effect of central corticotropin releasing factor (CRF) on carbon tetrachloride (CCl4) induced acute liver injury in rat (abst). *Gastroenterology*, 1997;112:A1201
  - 51 Tatemoto K. Neuropeptide Y: Complete amino acid sequence of brain peptide. *Proc Nat Acad Sci USA*, 1982;79:2585-2589
  - 52 Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y-a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature*, 1982;269:659-660
  - 53 Lundberg JM, Terenius L, Hokfelt T, Goldstein M. High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. *Neurosci Lett*, 1983;42:167-172
  - 54 Allen JM, Gu J, Adrian TE, Polak JM, Bloom SR. Neuropeptide Y in the guinea-pig biliary tract. *Experientia*, 1984;40:765-767
  - 55 De Quidt ME, Emson PC. Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system-2. Immunohistochemical analysis. *Neuroscience*, 1986;18:545-618
  - 56 Hokelt T, Lundberg JM, Tatemoto K, Mutt V, Terenius L, Polak J, Bloom S et al. Neuropeptide Y (NPY) and FMRFamide neuropeptide-like immunoreactivities in catecholamine neurons of the rat medulla oblongata. *Acta Physiol Scand*, 1983;117:315-318
  - 57 Yamazoe M, Shiosaka S, Emson PC and Tohyama M. Distribution of neuropeptide Y in the lower brainstem: an immunohistochemical analysis. *Brain Res*, 1985;355:109-120
  - 58 Gillis RA, Quest JA, Pagani FD, Norman WP. Central centers in the central nervous system for regulating gastrointestinal motility. In: J.D. Wood, eds. *Handbook of Physiology*, 6. The gastrointestinal system Bethesda: *American Physiological Society*, 1989:621-683
  - 59 Reilly FD, McCuskey AP, R.S. M. Intrahepatic distribution of nerves in the rat. *Anat Rec*, 1978;191:55-67
  - 60 Bogach PG, Lyashchenko PS. Changes in bile secretion during hypothalamic stimulation in dogs. In: eds. *Problems of physiology of the hypothalamus*. Moscow: Kiev, 1974:56-64
  - 61 Cucchiaro G, Yamaguchi Y, Mills E, Kuhn CM, Branum GD, Meyers WC. Evaluation of selective liver denervation methods. *Am J Physiol*, 1990;259:G781-785
  - 62 Fritz ME, Brooks FP. Control of bile flow in the cholecystectomized dog. *Am J Physiol*, 1963;204:825-828
  - 63 Moltz JH, McDonald JK. Neuropeptide Y: direct and indirect action on insulin secretion in the rat. *Peptides*, 1985;6:1155-1159
  - 64 Matsuda M, Aono M, Moriga M, Okuma M. Centrally administered NPY stimulated gastric acid and pepsin secretion by a vagally mediated mechanism. *Regul Pept*, 1991;35:31-41
  - 65 Geoghegan JG, Lawson DC, Cheng CA, Opara E, Taylor IL, Pappas TN. Intracerebroventricular neuropeptide Y increases gastric and pancreatic secretion in the dog. *Gastroenterology*, 1993;105:1069-1077
  - 66 Farouk M, Geoghegan JG, Pruthi RS, Thomson HJ, Pappas TN, Meyers WC. Intracerebroventricular neuropeptide Y stimulates bile secretion via a vagal mechanism. *Gut*, 1992;33:1562-1565
  - 67 Yoneda M, Tamasawa N, Takebe K, Tamori K, Yokohama S, Sato Y, Nakamura K et al. Central neuropeptide Y enhances bile secretion through vagal and muscarinic but not nitric oxide pathways in rats. *Peptides*, 1995;16:727-732
  - 68 Yoneda M, Yokohama S, Tamori K, Sato Y, Nakamura K, Makino I. Neuropeptide Y in the dorsal vagal complex stimulates bicarbonate-dependent bile secretion in rats. *Gastroenterology*, 1997;112:1673-1680
  - 69 Bartolome JV, Bartolome MB, Lorber BA, Dileo SJ, Scanberg SM. Effect of central administration of beta-endorphin on brain and liver DNA synthesis in preweanling rats. *Neuroscience*, 1991;40:289-294
  - 70 Yao CZ, MacLellan DG, Thompson JC. Intracerebroventricular administration of bombesin inhibits biliary and gastric secretion in the rat. *J Neurosci Res*, 1989;22:461-463
  - 71 Berbasa NV, Zhou J, Ravi J. Intracisternal (ic) administration of the opioid peptide analogue D-ala-met-enkephalinamide (D-Ala-Met-Enk) modulate bile flow by an opioid receptor-mediated mechanism in the brain (abst). *Gastroenterology*, 1997;112:A1225

# Evolution of gastrointestinal double contrast radiography in China: researches, application and popularization

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**Subject headings** Gastrointestinal diseases/radiotherapy; barium/diagnostic use; contrast media

Gastrointestinal double contrast radiography (DC) is a major procedure for gastrointestinal (GI) diagnoses, even for small and early structural lesions. Based on experiences reported at home and abroad, GI radiologists in China have studied DC in many aspects in the past few decades: including mechanisms of imaging, physical factors influencing appearance of images, better preparation of images barium sulfate (contrast media), substructure of area gastrica, measurement of image density, significance of some phenomena and signs, etc. Great efforts have been dedicated to its clinical application and popularization throughout the country and noticeable achievements have made.

## MECHANICS OF IMAGING<sup>[1-9]</sup>

Barium suspension (BS) and gas are contrast media for DC. Both are fluids. Being influenced by principles of fluid mechanics, most DC images often appeared pleomorphic and changeable.

**Wetting** It is defined as a phenomenon occurring upon the contact of the liquid (e.g. BS) with solid (e.g. GI mucosa). Adoption of a kind of BS which has appropriate wetting as well as high concentration and low viscosity, is an important prerequisite for DC. Chinese radiologists have made significant improvement in the DC quality of home-made barium preparations<sup>[5-9]</sup>.

**Barium collection in recesses** More BS is retained in the concave recesses of the angles formed by any protruding or depressing part of the mural surface. This is induced by the cohesive force of the fluid and the surface phenomenon and has considerable

significance in differentiating protrusion (blurred in outer side) from depression (blurred in inner side) in nature of lesions. (Figure 1).

**Ad-gravitational wall (Ad wall) and Ab-gravitational wall (Ab wall)** The flow, spreading, distribution and stagnation of BS in the air filled sac (such as GI lumen) is much influenced by the effect of gravity. The wall of the enclosed sac may be divided into 3 categories: Ad wall, Ab wall and lateral wall, just like the floor, ceiling and lateral wall of a room. These denominations of the walls are relative and interchangeable, which depends on the body position adopted at the time of examination. For example, the anterior wall of stomach is Ab wall in supine position, but will be changed to Ad wall in prone position. One notable point is that the lesion (e.g. polyp or ulcer) may give similar or entirely different manifestations when it is located on the Ad wall or Ab wall. Cretian phenomena (e.g. hanging droplet) can occur only on Ab wall whereas others (e.g. barium pool) are limited to the Ad wall. These conditions and terms in DC imaging are very helpful in understanding and describing the shape and position of lesions (Figure 2).

## SUB-STRUCTURE OF AREA GASTRICA<sup>[10-13]</sup>

Using a flexible specimen holder and magnifying technique, experimental DC was made on 10 human gastric specimens to investigate the differences in appearance of area gastrica (AG, 2mm-3mm in size) by the authors. The following new finer distinctions were discovered: silkworm-like and petal-like AG occurring in 25%-74%; sub<sup>a2</sup>groove and sub-area of AG; the "tep-over lines" are helpful in discrimination AG of the overlapping Ad wall or Ab wall. The diagnostic significance of such detailed AG study in early gastric carcinoma has been evaluated. (Figures 3-8).

## MEASUREMENT OF IMAGE DENSITY<sup>[1,3,4]</sup>

The image density (E) the figure obtained from a densitometer, i.e., the logarithm of the degree of light attenuation ("blackening") of the part of the film, was examined by our group. E is an important quantification standard for more subtle differentiation between various parts of DC. We

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Received 1998-02-08

measured the E value of different phases in 97 DC cases. It is the first reported series of measurement of E value in DC.

#### PHENOMENA AND SIGNS<sup>[14-34]</sup>

Some DC phenomena and signs have been investigated. The results showed that several of them are extremely valuable for determining the shape, site and nature of lesions (Table 1).

**Vertical plate phenomenon** It is the appearance of BS coating surface which lies in a direction tangential to the X-ray beam. It is similar to the change in transparency of a glass plate when its position is turned from transverse to vertical. The depth of BS in the site of vertical plate is much thicker than other parts. They, therefore, occur as single or multiple dense white lines. The majority of anatomical and pathological structures can be better revealed by this phenomena.

**Overlapping white line** The term is used to denote partially visible white line seen through a relatively shallow barium pool of the ad-gravitational wall. The overlapping white line is produced by linear image of the vertical plate that projects within the field of the Ab wall. We can always trace the existence and nature of such lesions and to determine its Ab wall origin (Figure 6).

**Tide and rock phenomenon** A protrusion (rock) within the shadow of barium pool (water tide) is rather similar to the relation between the ebb and tide of water and a rock in it. A low and small protrusion could only be demonstrated at "shallow water tide" phase (Figure 9).

**Eye-like sign** It is a characteristic feature of polyp (Figures 10-11).

**Foggy droplets sign** This is the characteristic feature

of carcinoma located at Ab wall (Figure 12-19).

**Foggy droplets sign** This is the characteristic appearance of lateral wall carcinoma involving the Ab wall and often accompanied by the foggy droplets sign (Figures 12-19).

#### APPLICATION AND POPULARIZATION<sup>[4,34]</sup>

GI radiologists in China have been giving increasing emphasis on the application and popularization of DC. Seminars have been held each year in Shanghai and other regions since the 80s. Among the radiological techniques to be popularized, DC may be a prominent one in terms of duration and scale of its nation-wide recommendation throughout these years.

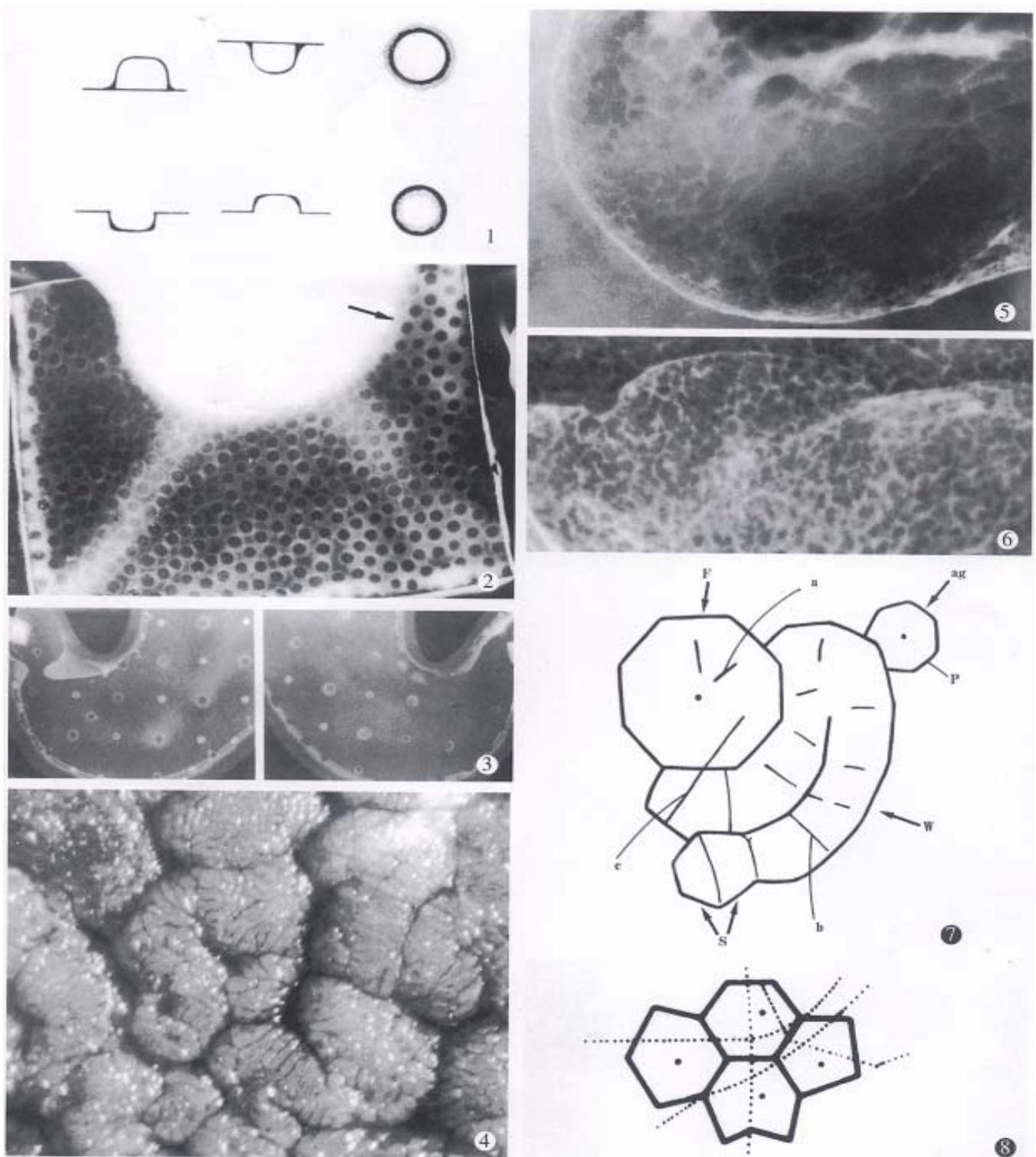
In many regions of China, DC has been accepted as a routine technique for GI barium examination; and is one of the standard of assessment in testing the specialty level of radiological practice.

Preliminary data of inquiry from 315 radiology departments showed that among up to 90 000 DC cases, the detectability of GI structural lesions in DE was about 8% higher than when traditional barium studies were employed. The number of early GI cancer found by DC has been increased markedly.

According to a general survey in China, up to 41% of radiological departments have not yet employed DC; and in more than 20%, DC technology has not attained to a qualified standard. Besides, up to 31% DC cases were misdiagnosed because of unfamiliarity with DC appearances, or were not properly interpreted. This status suggests that more strenuous education of DC should be continued.

**Table 1** Features and frequencies (%) occurring in 200 different lesions of Ab wall<sup>[19]</sup>

	Lesions (n=200)	Involving lateral wall	Foggy droplets	Multiple mural lines	Overlapping white line	Eye- like	Ring
Anterior gastric wall	150	-46 +104					
Carcinoma		-33 +72	30(91) 70(97)	0 70(97)	16(48) 10(14)	0 0	0 0
Polyp	19	-12 +7	0 0	0 0	0 0	3 0	12(90) 0
Ulcer	26	-6 +20	0 0	0 0	0 0	0 0	3(50) 0
Localized colon wall							
Carcinoma	39		38(97)	35(87)	37(95)	0	5(13)
Polyp	8		0	0	5(56)		8(100)
Diverlieulum	3		0	0	0		3(100)



**Figure 1** Diagrammatic drawing shows the different manifestations in protrusion (blurring in outer side) and in depression (blurring in inner side) caused by barium collection in recesses.

**Figure 2** Model DC. The larger round images are protrusions and the smaller ones are depressions. They are all located on Ad wall in A and in Ab wall in B. Note the difference in protrusion (blurring in outer) and in depression (blurring in inner); and the difference of the same lesion in Ad wall (A) or in Ab wall in B.

**Figure 3** Gastric specimen magnified ( $\times 10$ ) with a biomicroscopy shows the substructures of AG.

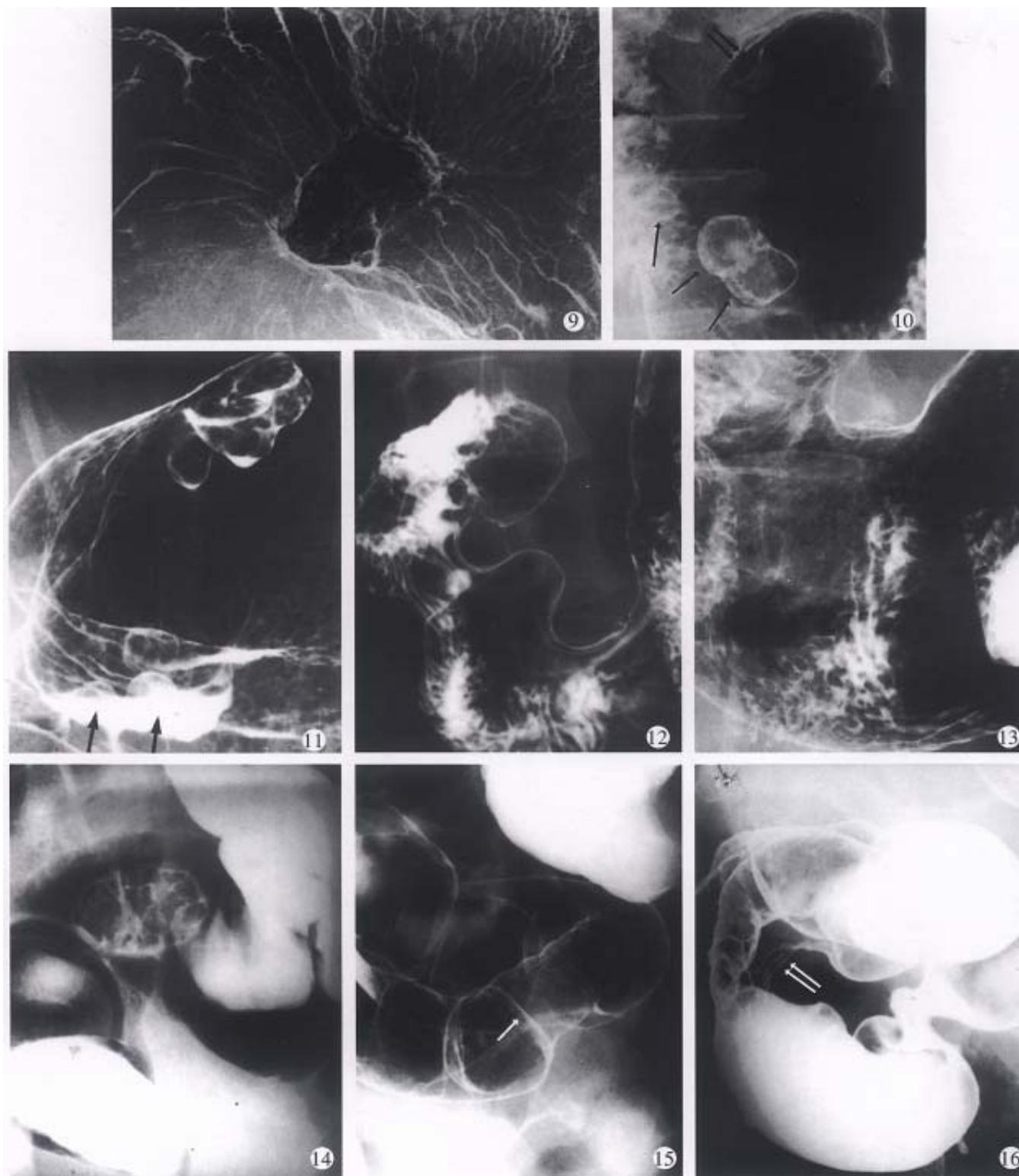
**Figure 4** Area gastrica in clinical DC shows silkworm-like AG, petal-like AG and the step-over lines of two overlapping walls.

**Figure 5** DC of human gastric specimen shows the subtle difference of AG in two overlapping walls (upper 1/3 part) and single unoverlapping wall (lower 2/3 part).

**Figure 6** Diagrammatic representation of AG. W, silkworm-like AG; SA, sub-AG; SV, subgrooves.

**Figure 7** Diagram depicts the step-over lines of AG in two overlapping walls seen in DC film.

**Figure 8** Gastric specimen with barium coated and radiographed by soft X-ray showing the ulcer crater and the grooves of AG surrounding it.



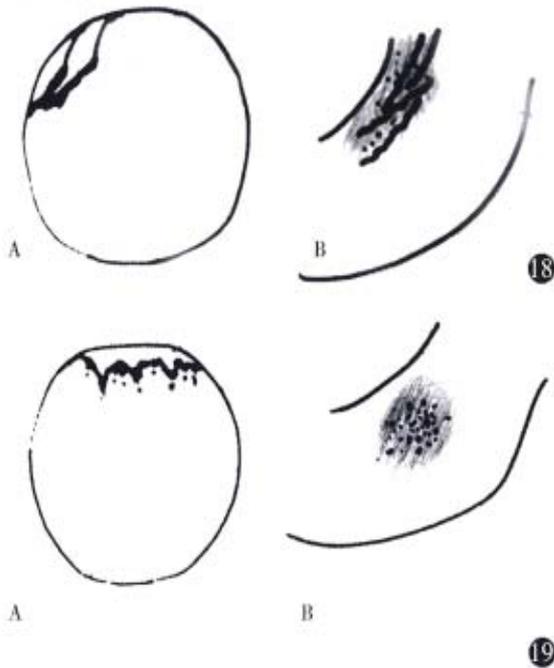
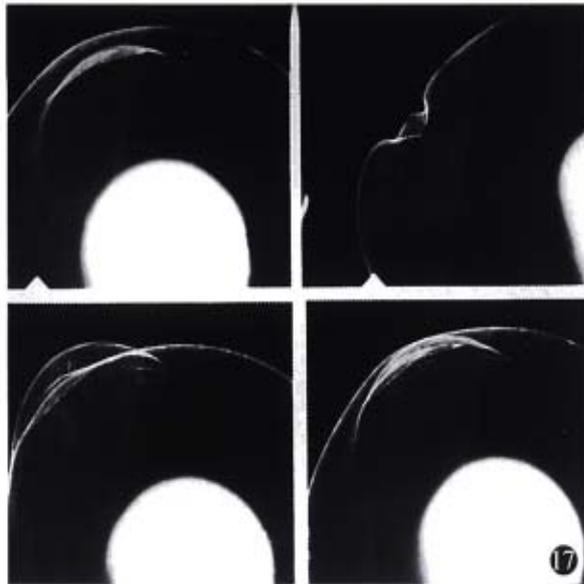
**Figure 9** The same sized small round protrusions located in Ads wall. Note the different appearance in various phase of “tide and rock”. Those are most distinctable in “low water tide” (arrow).

**Figure 10-11** A patient with 6 polyps in gastric antrum. In Fig 10, 3 polyps in Ab wall (anterior wall) appeared as eye-like sign (single arrow), one in lateral wall appeared as bowler hat sign (double arrows). In Figure 11, the 3 polyps in Ab wall and 3 in Ad wall (single arrow) all can be seen in horizontal projection.

**Figure 12-13** Two patients with gastric carcinoma located in lessor curvature involving Ab wall. Both cases in DC appeared as multiple mural lines sign and foggy droplets sign.

**Figure 14** Sigmoid colon carcinoma appeared as foggy droplets sign.

**Figure 15-16** Localized carcinoma of sigmoid colon, two projections in the same case, showing the multiple mural lines sign (double arrows) and the foggy droplets sign (single arrow). Figure 16 the multiple mural lines sign (double arrows) and overlapping white lines (single arrow).



**Figure 17** Model DC, demonstrating the mechanism of multiple mural lines sign. The “infiltration” of the lateral upper wall of the sac made the part of the wall less expansion (right upper picture, horizontal projection) and appeared as multiple lines of rigidifying, roughing and “whiter” in image density in other 3 pictures taken in perpendicular projection.

**Figure 18** Diagrammatic drawing demonstrates the mechanism of multiple mural lines sign on horizontal projection (A) and its appearance on perpendicular projection (B).

**Figure 19** Diagram of foggy droplets sign. More BS have been stagnated on the irregular surface of Ab wall carcinoma (A) and “foggy droplets” appearance was formed (B).

A, Horizontal projection; B, perpendicular projection.

## REFERENCES

- Shang KZ, Yu X, Guo MJ. An experimental study on the mechanism of gastrointestinal double contrast image formation and its clinical application. *Chin J Radiol*, 1985;19(4):197-200
- Shang KZ, Zou Z, Chang TQ *et al*. Physical interpretation and clinical significance of gastrointestinal double contrast radiography. *Shanghai Med J*, 1982; 5(3):154-157
- Shang KZ, Zou Z, and Yu X. Image formation in double contrast roentgenography of the GI tract: experimental and theoretical observation and practice implications. *Chin Med J*, 1985;98(6):391-400
- Shang KZ, Chen JR (ed). Principle and diagnosis of gastrointestinal radiography. Shanghai: *Shanghai Scientific and Technical Publisher*, 1995: 23-88
- Shang KZ, Gou MJ. Experimental investigation and evaluation of several home made barium sulfate preparations for double contrast radiography. *Chin J Radiol*, 1984;18(2):95-98
- Shang KZ. Property, function and application trend of DC barium sulfate preparation. *Chin J Radiol*, 1996;30(11):795-798
- Fan J, Chen XR, Shen TZ *et al*. Experimental researches on flocculation and antiflocculation of double contrast barium sulfate preparation. *J Clin Radiol*, 1984;3(3):125-127
- Chen XR, Mei H, Shen TZ *et al*. Studies on heterogeneity in size and shape of barium sulfate particles for gastric double contrast radiography. *Acta Academiae Medicinae Shanghai*, 1984;11(2):112-116
- Chen XR, Shang KZ. Current status of research studies in barium sulfate preparation for double contrast radiography. *Foreign Med Scien-Clin Radiol*, 1984;7(2):65-68
- Shang KZ, Guo MJ, Ji BQ *et al*. Evaluation on the anatomical and physical basis and the clinical significance of area gastrica. *Chin J Radiol*, 1990;24(3): 182-185
- Li FS, Chang TL, Hu MH *et al*. Roentgenologic investigation of area gastrica. *Chin J Radiol*, 1984;18(1):6-8
- Fan J, Chen XR, Shen TZ *et al*. X-ray appearances of areae gastricae in normal and chronic gastritis. *Chin J Radiol*, 1984;18(1):1-3
- Shang KZ. The anatomy and radiology of the areae gastricae. 1989;23(Suppl): 33-35
- Gao YO, Gao YG (ed). Double contrast radiography of gastrointestinal tract. *Beijing, People's Medical Publishing House*, 1984:32-53
- Liu GN, Xie JX, Fan JD. Hypotonic duodenography. *Chin J Radiol*, 1980;14(3): 135-137
- Chang TL, Li FS. Double contrast enterocolysis of small bowel. *Chin J Radiol*; 17(2):90-92
- Xu JB, Shen MJ, Zhu YT *et al*. Duodenum and jejunum intubation for barium enema of small bowel. *J Clin Radiol*, 1986;5(2):72-74
- Shang KZ, Guo MJ, Ji BQ, Zuo Z. Investigation on characteristic appearance in double contrast radiography of gastric lesion involving anterior wall. *Chin J Dig*, 1990;10(5):271-273
- Shang KZ, Ji BQ, Jio TD *et al*. “Foggy droplets” and “multiple mural lines”: two valuable signs in double contrast radiography (DC) of gastric and colonic carcinoma. *J Pract Radiol*, 1994;10(9):514-518
- Chen XR, Fan J, Shen TZ *et al*. Double contrast radiography of stomach. *J Clin Radiol*, 1982;1(2):115-117
- Chen KM, Chen XR, Shen TZ *et al*. Double contrast radiography of colon after colonofiberscopy. *Chin J Radiol*, 1989;23(6):370-372
- Chen KM, Chen XR. Comparison of colonic double contrast radiography and colonoscopy in patient with intestinal hemorrhage. *Acta Acad Med Shanghai*, 1991;18(6):409-411
- Chen JR. Single and double contrast gastric barium studies. *J Clin Radiol*, 1989;8(7):253-255
- Chen JR. Mass survey of gastric carcinoma by X-ray screening. *J Clin Radiol*, 1984;24(Suppl):1-3
- Chang TQ, Yang CR. Double contrast barium meal for small intestine by retrograd gas insufflation. *J Clin Radiol*, 1990;9(3):132-133
- Chen KM, Chen XR, Shen TZ *et al*. X-ray diagnosis of ulcerative colitis. *Shanghai Med J*, 1988;11(3):178-180
- Xie JX, Liu GN, Fan JD *et al*. Normal patterns of cardiac region in double contrast radiography. *Chin J Radiol*, 1985;19(4):235-238
- Shi ML. X-ray diagnosis of malignant lymphoma in stomach (73 cases analysis). *J Clin Radiol*, 1984;3(1):57-59
- Jiong H, Tang OR. Early gastric carcinoma diagnosed in gastrointestinal barium studies (64 cases report). *Chin J Dig*, 1992;12(3):177-199
- Li SN, Gao YJ. Radiological and pathological investigation of carcinoid in gastrointestinal tract. *Chin J Radiol*, 1981;15(1):8-10
- Chang JR, Chang XP. Double contrast radiographic findings in early gastric carcinoma (depression type) and the relating technicality. *Chin J Radiol*, 1986; 20(Suppl):20-23
- Cheng YD. Clinical application of duodenal double contrast radiography. *J Clin Radiol*, 1987;6(3):136-138
- Shang KZ, Chen JR, Jio TD *et al*. Investigation on characteristic appearances of lesions located in ab-gravitational wall (AW) of stomach and colon in double contrast barium examination. *Chin J Radiol*, 1993;27(7):462-466
- Shang KZ. Interpretation and differentiation of double contrast gastrointestinal studies. *Chin J Radiol*, 1994;28(3):201-203

# Expression of intercellular adhesive molecule-1 in liver cancer tissues and liver cancer metastasis

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**Subject headings** liver cancer; tumor metastasis; intercellular adhesion molecule-1

## Abstract

**AIM** To study the relationship between intercellular adhesive molecule-1 (ICAM-1) and liver cancer metastasis and to search for factors to predict metastasis of liver cancer.

**METHODS** ICAM-1 expression in fresh tissues of normal liver and hepatocellular cancer (HCC) was examined by immunoperoxidase staining. The expression of ICAM-1 in human hepatoma, tumor surrounding tissues and normal livers were semiquantitatively analyzed by Dot immunoblot. Tissue ICAM-1 expression at mRNA level was detected by Northern blot.

**RESULTS** All 6 cases of normal liver samples were negative in anti-ICAM-1 immunohistochemical staining, 80.0% (36/45) of HCC presented various ICAM-1 expression. The number of positive cells was a little higher in large tumors, tumors with intact capsule and metastasis, but there was no significant difference. Two cases with cancer embolus also had high ICAM-1 expression. ICAM-1 concentration in HCC ( $13.43 \pm 0.09$ ) was higher than that in tumor surrounding tissues ( $5.89 \pm 0.17$ ,  $P < 0.01$ ) and normal livers ( $4.27 \pm 0.21$ ,  $P < 0.01$ ). It was also higher in metastasis group ( $20.24 \pm 0.30$ ) than in nonmetastasis group ( $10.23 \pm 0.12$ ,  $P < 0.05$ ). Northern blot analysis revealed that ICAM-1 expression at mRNA level was also higher in HCC and cancer embolus than that in tumor surrounding tissues and normal livers.

**CONCLUSION** Tissue ICAM-1 could indicate the growth and metastasis of HCC, and may be an index that can predict liver cancer metastasis.

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\*Supported with Grant for Leading Specialty by the Shanghai Health Bureau, National Natural Science Foundation No.396707 and China. Medical Board No.93-583

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Received 1998-01-20

## INTRODUCTION

Tumor metastasis is one of the main causes of poor prognosis of hepatocellular carcinoma (HCC)<sup>[1]</sup>. The process of metastasis includes dissociation of tumor cells from primary location; arrest, adhesion and extravasation of metastatic tumor cells. Adhesive molecules on endothelial cells such as selectin cause circulating tumor cells to roll along the surface. Other molecules on tumor cells including intercellular adhesive molecule-1 (ICAM-1), vascular cellular adhesive molecule-1 (VCAM-1) and so on, subsequently stop the cells completely, then extravasate through the blood vessels into selective target tissues. During the metastatic cascade, tumor cells interact with various host cells (endothelial cells, platelets or lymphocyte), and/or extracellular matrix and basement membrane components, the homotypic and heterotypic cell clumps form a multicellular embolus, which can enhance the survival, arrest and invasiveness of tumor cells<sup>[2]</sup>. The metastatic potential of cancer cells is related to the activity of their surface adhesive molecule<sup>[3]</sup>. Recently, it was noticed that ICAM-1 plays an important role in this process. In this study, we investigated the relationship between ICAM-1 expression and the metastasis of HCC.

## MATERIALS AND METHODS

### Patients

The tissues of HCC used for immunohistochemical study were collected from 45 patients (39 males and 6 females, aged 10-82 years with a median of 51.1) who had undergone hepatectomy for HCC. All the cases were diagnosed pathologically. Eighteen of the patients were in early stage (without symptoms and signs of HCC), and 27 in moderate stage (having symptoms and signs of HCC, but no ascites, jaundice and long distance metastasis). Thirty patients had positive AFP ( $\geq 30 \mu\text{g/L}$ ) and 15 had negative AFP. The median diameter of the resected tumor was 6.7 cm (1.5-18), 30 had large tumors ( $> 5 \text{ cm}$ ) and 15 were had small tumors ( $\leq 5 \text{ cm}$ ). Patients with embolus in portal vein and its main branch or with intrahepatic and extrahepatic metastasis nodules belonged to the metastasis group, the other patients were to nonmetastasis group. Six normal livers were from the surrounding noncirrhotic liver tissues of

pathologically proved benign tumors (4 hemangioma, 1 adenoma and 1 cyst).

The tissues used for detection of ICAM-1 were collected from 40 patients with HCC, 20 of them had large tumors and 22 encapsulated: 9 patients were in early stage and 31 in moderate stage. Positive AFP was found in 30 cases. There were 16 patients in the metastasis group, and 24 in nonmetastasis group. The 8 normal liver tissues were collected from the surrounding noncirrhotic tissues of pathologically proved benign liver tumors (5 hemangioma, 1 adenoma and 2 cyst).

### Reagents

Monoclonal antibody of ICAM-1 (1 µg/L) was purchased from R-G Company, Britain; AKP goat anti mouse IgG and biotinylated rabbit anti-mouse IgG were from Sino-American Company, China; TRIzol™ total isolation reagent from Gibco Company, USA; and megaprime™ DNA labeling systems from Amersham Company, UK.

### Methods

**Indirect immunoperoxidase staining** The fresh specimens embedded by O.C.T were frozen in liquid nitrogen, and then stored at -70°C. Serial frozen sections (6 µm) of liver specimens were fixed with acetone. After preincubated with human serum to reduce background, the slides were incubated with the primary antibody (1:50) at 4°C overnight, biotinylated rabbit anti mouse immunoglobulin (1:200) was used at 37°C for 1 h, added with avidin biotin HRP mixture (1:100 v/v), and counterstained with hematoxylin.

**Dot immuno blot** Three hundred mg tissue was homogenized in 1.5 ml suspended buffer solution (0.1 mol/L NaCl, 0.01 mol/L Tris. Cl, pH 7.6, 0.001 mol/L EDTA pH 8.9, 1% Triton-X-100), the protein concentration was determined by Hatree method<sup>[4]</sup>. The supernatant (30 µl) was spotted onto nitrocellulose membrane in a dot blot format. After non-specific blocking with 5% lipid free milk, the blots were incubated with ICAM-1 antibody (1:500) at room temperature for 2 h, and then incubated with AKP conjugated goat anti-mouse IgG (1:200) for 2 h at room temperature, then stained with NBT/BCIP (2:1 v/v). The integrated optical density (IOD) of the blot was measured by MIAS 300 automatic image analyzer. Tissue ICAM 1 = (sample IOD background IOD) × sample area / (µg protein concentration × 1000).

**Northern blot** Total RNA was extracted by TRIzol™ reagent according to the manufacturer's instruction (Gibco Life Technologies Cat No. 15596.026). Total RNA of 20 µg was denatured in

formaldehyde, electrophoresed, and transferred to nitrocellulose membrane. The plasmid containing ICAM-1 probe sequence was kindly provided by Dr. Christian Stratowa (Ernst Boehringer Institute, Vienna, Austria). Northern transfer membranes were prehybridized for 4 h at 42°C with 5×SSPE/5×SSPE/5× Denhardt's solution/1% SDS/100 mg/L Salmon sperm DNA. The 1.8 kb cDNA was labeled with 32P isotope by random primer method (Megaprime™ DNA Labeling Systems, Amersham). Northern blot analysis was performed under stringent washing conditions, 1×SSC 0.1% SDS, 30 min at room temperature and 1×SSC 0.1% SDS, 1 h at 50°C. Autoradiography was carried out at -70°C for 7 days.

**Data analysis** Significance was assessed by Student's *t* test. The results were expressed as  $\bar{x} \pm s$ .

## RESULTS

### Immunohistochemical observation of ICAM-1 in human HCC

By using anti ICAM-1 monoclonal antibody, no ICAM-1 was found in normal liver tissues (Figure 1). ICAM-1 was positive in fresh HCC (Figure 2), mainly on cell membrane, with a rate of 80% (36/45). In this group, 42.6% showed strong positive (++)<sup>aa</sup>, 30%-70% positive cells), others were weak positive (+, 0-30% positive cells). ICAM 1 was also positive in 2 cases with tumor embolus in portal vein (Figure 3).

### Semi-quantitative analysis of human tissue ICAM-1

Dot immuno-blot was employed in semi-quantitative analysis of tissue ICAM-1 (Figure 4). Table 1 demonstrates that ICAM-1 content in HCC was about two-fold higher than that in the tumor surrounding liver tissues and normal controls ( $P < 0.01$ ), no significant difference was found between the latter two.

**Table 1** ICAM-1 expression in different liver tissues [ $(\bar{x} \pm s)/1000$ ]

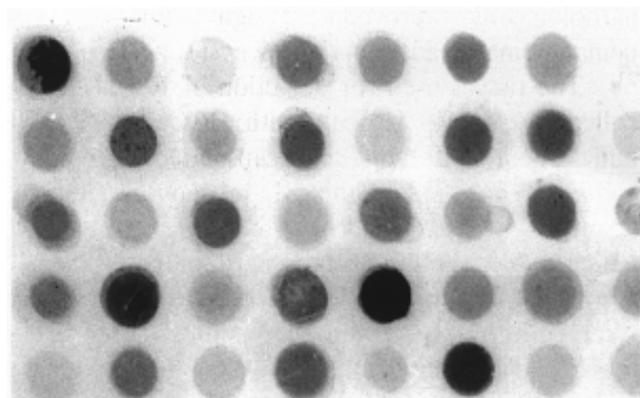
Groups	Cases	ICAM-1
HCC	40	13.43 ± 0.09
Tumor surrounding liver tissues	40	5.89 ± 0.17
Normal liver	8	4.27 ± 0.21

$P < 0.01$ , compared with HCC group

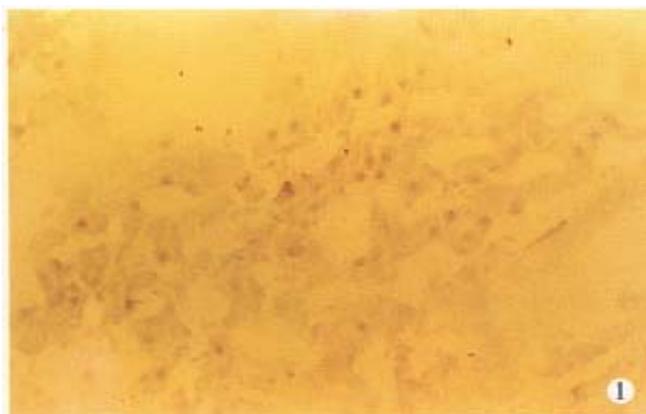
The expression of tissue ICAM 1 was also compared with tumor biological characters (Table 2), ICAM-1 in cancer tissues was only related to tumor metastasis, and there was no relationship with tumor size and capsule formation.

**Table 2 The relationship of ICAM 1 content with some tumor biocharacters ( $\bar{x} \pm s$ )**

Groups	Cases	ICAM-1	P- value
Metastasis	16	20.24±0.30	<0.05
Nonmetastasis	24	10.23±0.12	
Metastasis tumor surrounding liver	16	6.80±0.08	>0.05
Nonmetastasis tumor surrounding liver	21	4.78±0.04	
Intact capsule	22	13.55±0.20	>0.05
Nonintact capsule	15	12.93±0.32	
Large HCC	20	14.45±0.26	>0.05
Small HCC	14	10.96±0.47	



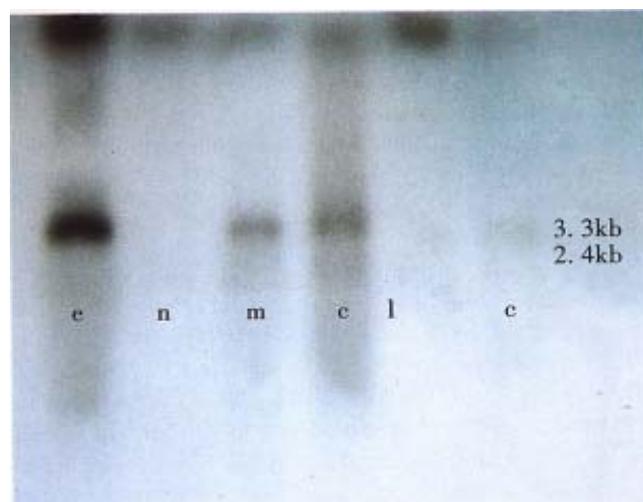
**Figure 4** Tissue ICAM-1 expression by Dot immunoblot.



**Figure 1** ICAM-1 expression in normal liver tissues by immunohistochemical staining.  
**Figure 2** ICAM-1 expression in liver cancer tissues by immunohistochemical staining.  
**Figure 3** ICAM-1 expression in tumor embolus by immunohistochemical staining.

**ICAM-1 expression at transcriptional level**

In this study, special DNA probe was used in Northern blot for detecting ICAM-1 mRNA, two transcripts with 3.3 kb and 2.4 kb in length was defined.  $\beta$ -actin was acted as internal standard. The data obtained from image analysis indicated that ICAM-1 mRNA expression in tumors was higher than that in tumor surrounding liver tissues and normal controls as well as cancer embolus (Figure 5).



**Figure 5** Detection of ICAM-1 mRNA by Northern blot. E: embolus; N; tumor surrounding liver; C: liver cancer; normal liver

**DISCUSSION**

Intercellular adhesive molecule-1 (ICAM-1, CD54), a transmembrane glycoprotein, is a member of the immunoglobulin superfamily and functions as counterreceptor for leukocyte function associated antigen-1 (LFA-1, CD11a/CD18) and complement receptor type 3 (MAC-1, CD11b/CD18)<sup>[5]</sup>. It mediates homotypic and heterotypic cell interaction, not only plays an important role in the tumor genesis, but also can reflect tumor curative

effect<sup>[6]</sup>, and may be a critical factor in the process of bloodborne metastasis of cancer<sup>[7]</sup>. Recently it was found that ICAM-1 plays an important role in tumor recurrence and metastasis. Hung<sup>[8]</sup> *et al* found that anti-ICAM-1Ab can prevent the growth of APH-77 tumors in SCID mice by altering the homing and growth of tumor cells in certain anatomical sites. Most of normal tissues do not express ICAM-1, but ICAM-1 expression has been observed in many tumors, such as renal cancer, uterine cancer, lung cancer, gastric cancer, bladder cancer, etc. Santarosa<sup>[9]</sup> *et al* demonstrated that renal cancer recurrence after operation was related to ICAM-1 expression in cancer tissues and patients with <50% of ICAM-1 positive cells in cancer tissues had prolonged disease-free survival after a median follow-up of 60 months.

In this study, all 6 cases of normal liver sample were negative in anti ICAM-1 immunohistochemical staining, 36/45 HCC presented various ICAM-1 expression. The rate of positive cell was a little higher in large tumors, tumors with intact capsule and tumors with metastasis, but there was no significant difference. It has been noticed that 2 cases with embolus also had high ICAM-1 expression. As indicated in semiquantitative analysis of tissue ICAM-1, ICAM-1 concentration in liver cancer was higher than in tumor surrounding liver tissues and normal livers. Tissue ICAM-1 was higher in metastasis group than that in nonmetastasis group. Northern blot analysis revealed that ICAM-1 expression at mRNA level was also higher in HCC and cancer embolus than in tumor surrounding liver tissues and normal controls. So it was suggested that ICAM-1 might be one of the characters of liver cancer, and could be a diagnostic indicator for metastatic status of liver cancer.

The deduced ICAM-1 amino acid sequence analysis revealed the presence of C<sub>3</sub>/C<sub>4</sub> binding protein consensus sequence between residues 341 and 404, so ICAM-1 may be involved in C<sub>3b</sub> binding. Since C<sub>3b</sub> binding protein generally acts to accelerate the dissociation of active complement complexes, ICAM-1 may help the tumor escape immune destruction, providing an important property needed for successful metastasis. Functioning as a ligand of LFA-1, ICAM-1 can establish the heterotypic adhesion of tumor cells with migratory and invasive leukocytes present in the tumor cells, enabling individual

cell to dissociate from primary tumor and go into circulation<sup>[10]</sup>. There is soluble ICAM-1 (sICAM-1) in serum, which comes from proteolytic cleavage of membrane associate ICAM-1. Since LFA-1 could also be blocked with soluble ICAM-1, sICAM-1 may also block the attachment of cytotoxic T cell and/or NK cells to cancer cells. This could be one of the mechanisms of cancer cells escaping from the immunosurveillance system of the host, and can inhibit lymphocyte mediated cytotoxicity, therefore a favorable environment for tumor cell proliferation would be created<sup>[11]</sup>. Spontaneous regression of human liver tumor was quite rare and the tumors were unusually resistant to various immunotherapy, probably due to the high content of sICAM-1 in those patients.

In our experiment, we also found that there was no statistically significant difference in ICAM-1 expression between tumor surrounding liver tissues and normal livers, and between tumor metastasis group and nonmetastasis group. So we think that ICAM-1 reflects the metastatic ability of cancer cells but not the cancer environment.

In conclusion, tissue ICAM-1 could reflect the growth and metastatic status of HCC and may be a factor to predict liver cancer metastasis.

## REFERENCES

- 1 Zhou XD, Tang ZY, Yu YQ, Yang BH, Lu JZ, Lin ZY *et al.* Recurrence after resection of  $\alpha$ -fetoprotein-positive hepatocellular carcinoma. *J Cancer Res Clin Oncol*, 1994;120(6):369-373
- 2 Yasoshima T, Denne R, Kawaguchi S, Sato N, Okada Y, Ura H *et al.* Establishment and characterization of human gastric carcinoma lines with high metastatic potential in the ability to metastasize in the liver of nude mice. *Jpn J Cancer Res*, 1996;87(2):153-160
- 3 Nasu K, Narahara H, Etoh Y, Kawano Y, Hirota Y, Miyakawa I. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1) and the expression of ICAM-1 mRNA in uterine cancer. *Gynecol Oncol*, 1997;65(2):304-308
- 4 Hartree EF. Determination of protein: A modification of Lowry method that give a linear photometric response. *Ann Biochem*, 1972;48(3):422-427
- 5 Budnik A, Grewe M, Gyufko K, Krutmann J. Analysis of the production of soluble ICAM-1 molecules by human cell. *Exper Hematol*, 1996;24(2):352-359
- 6 Yukihiko S, Masami M, Takashi T, Kashii Y, Miyamoto M, Nishimori H *et al.* Serum concentration of intercellular adhesion molecule-1 in patients with hepatocellular carcinoma is a marker of the disease progression and prognosis. *epatology*, 1995;22(2):525-531
- 7 Regimbald H, Pilarski LM, Longernerker BM, Reddish MA, Zimmermann G, Hugh JC. The breast mucin as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer. *Cancer Res*, 1996;56(8):4244-4229
- 8 Huang YW, Richardson JA, Vitetta ES. Anti-CD54(ICAM-1) has antitumor activity in SCID mice with human. *Cancer Res*, 1995;55(3):610-616
- 9 Santarosa M, Fanaro D, Quai M, Spada A, Sacco C, Talamini *et al.* Expression and release of intercellular adhesion molecule-1 in renal-cancer patients. *Int J Cancer*, 1995;62(3):271-275
- 10 Johnson P, Stade G, Holzmann B, Schwable W, Riethneller G. De novo expression of intercellular adhesion molecule-1 in melanoma correlates with increased risk of metastasis. *Proc Natl Acad Sci USA*, 1989;86(2):641-644
- 11 Pirisi M, Falletti E, Fabris C, Soardo G, Toniutto P, Vitulli D *et al.* Circulating intercellular adhesion molecule-1 (cICAM-1) concentration in liver disease: Relationship with cholestasis and functioning hepatic mass. *Clin Chem*, 1994; 102(5):600-604

# Study of heteroserum-induced rat liver fibrosis model and its mechanism

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**Subject headings** liver cirrhosis; heteroserum; disease models, animal; liver/pathology; mast cell; IgG; complement C3; rats

## Abstract

**AIM** To investigate the morphological changes in the process of heteroserum induced rat liver fibrosis and the mechanism of fibrogenesis of this model.

**METHODS** A model of heteroserum-induced rat liver fibrosis was established by intraperitoneal injection of porcine serum. In addition to the observation of the morphological changes of this model, the infiltration of eosinophils and mast cells were measured quantitatively and the deposition of IgG and complement C3 was detected by immunofluorescence.

**RESULTS** The rat liver fibrosis was induced successfully at the end of the 8th week after the injection of heteroserum. Besides the increase of hepatic stellate cells (HSC) during the process of liver fibrosis, proliferation and activation of primary mesenchyma cells (PMCs) were also found. In the early stage, the infiltration of eosinophils and mast cells was significantly increased and the deposition of IgG and complement C3 was positive in the portal tracts and septa, while gradually reduced after the injection was stopped.

**CONCLUSIONS** This model is suitable for the research on liver fibrogenesis; the pathogenesis of this model may be related with the allergen-induced late phase reaction (LPR) caused by the injection of heteroserum, and the HSCs and the PMCs are important sources of ECM-producing cells.

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\*Supported by the National Natural Science Foundation of China, 39330140 and Major Subject Fund of Shanghai Educational Committee.

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Received 1998-02-18

## INTRODUCTION

There are several kinds of animal model of liver fibrosis in the literature, among which CCl<sub>4</sub>-induced model initiated with marked damage of liver tissues was extensively investigated and applied. There have been a few reports about immune liver fibrosis model without severe injury of hepatocytes because of the complexity of its making and the higher mortality of the animals. In this paper, a kind of immune liver fibrosis model established by intraperitoneal injection of heteroserum was reported in this paper, and the morphological characteristics and the pathogenesis were studied.

## MATERIALS AND METHODS

The model was established according to the method of Paronetto's<sup>[1]</sup>. Forty Wistar female rats weighing about 100g and fed with common stuff and water were randomly divided into 5 groups. In each group, 5 rats were injected with 0.5ml porcine serum intraperitoneally twice a week for 10 weeks, and 3 rats with sterile physiological saline in stead. Three groups of rats were killed at the end of the 3rd, 8th and 10th week, and the other two groups at the end of the 15th and 20th week respectively. The liver tissue was regularly fixed, embedded and sliced for light or electron microscopy, and serial paraffin sections were stained with H.E., von Gieson and toluidine blue. IgG and complement C<sub>3</sub> were detected by immunofluorescence in the frozen sections. Rabbit antibody against human C<sub>3</sub> was purchased from Dako Co. Rabbit antibody against rat IgG and FITC labeled goat antibody against rabbit IgG were prepared in our department. Eosinophils and mast cells in the liver tissue were counted and statistically analyzed with rank-sum test.

## RESULTS

### *Morphological changes*

During the whole period of the experiment no rats died and all the experimental rats advanced into liver fibrosis after injection of porcine serum for 8 weeks. Observed with the naked eye, the liver surface appeared normal until the end of the 8th week, and presented tiny particle-like changes, the tissue became hard afterwards, and the hardness increased progressively.

Microscopically, at the end of the 3rd week of the injection, one rat remained almost normal. The portal tracts enlarged mildly with extracellular matrix (ECM) and proliferated mesenchymal cells and small bile ducts without canal were visible in 4/5 rats although there existed the structure of the liver lobules. Most of the interstitial cells were enlarged and spindle in shape (Figure 1). The increased ECM and cells were also observed around the hepatic veins and central veins. In some areas, the early cellular septa consisting of the increased interstitial cells were found and inserted into the parenchyma. In one of the rats, the lobules were almost completely separated by the septa. At this stage, the liver cells had no obvious damage except the apoptosis of individual cells near the septa. Under the electron microscope, the sorts of mesenchymal cells in septa were different, some of which were typical fibroblasts, and some appeared as primitive mesenchymal cells (PMCs) with a high ratio of nucleus to plasm and a large elliptic nucleus containing 2.4 nucleolus and a few of cell organelles (Figure 2). Hepatic stellate cells (HSCs) of perisinusoid close to the portal tracts or septa were elongated with less or none lipid droplets and increased and dilated endoplasmic reticula, which was often near the mesenchymal cells (Figure 3).

At the end of the 8th week, the structure of liver lobules was undisriminated in all of 5 experiment rats, and markedly enlarged portal tracts with connective tissues were distinctly exhibited in VG stain. The septa containing many mesenchymal cells and collagen fibrils (so-called cellular fibrous septa) were extended into parenchyma, connected with adjacent portal tracts or hepatic veins and separated the lobules. The number of mesenchymal cells increased apparently in the portal tracts and septa, and PMCs were relatively less in these areas. Hepatic cells were swollen with vacuoles in cytoplasm, and some of which trapped in septa. Elcotronmicroscopically, among the mesenchymal cells in or around the portal tracts or septa, PMCs were less in number while HSCs increased as compared with those at the end of the 3rd week. The transitional cells between PMCs and fibroblasts were also seen with increased and dilated endoplasmic reticula and slightly leaner nucleus with small wrinkled?nuclear membrane (Figure 4). However, myocyte-like structure as macula densa or dense body was not seen in these mesenchymal cells. At the end of the 10th week, the amount of ECMs in portal tracts and septa increased obviously, but the number of mesenchymal cells decreased. As a result, the fibrous septa mainly composed of ECMs were

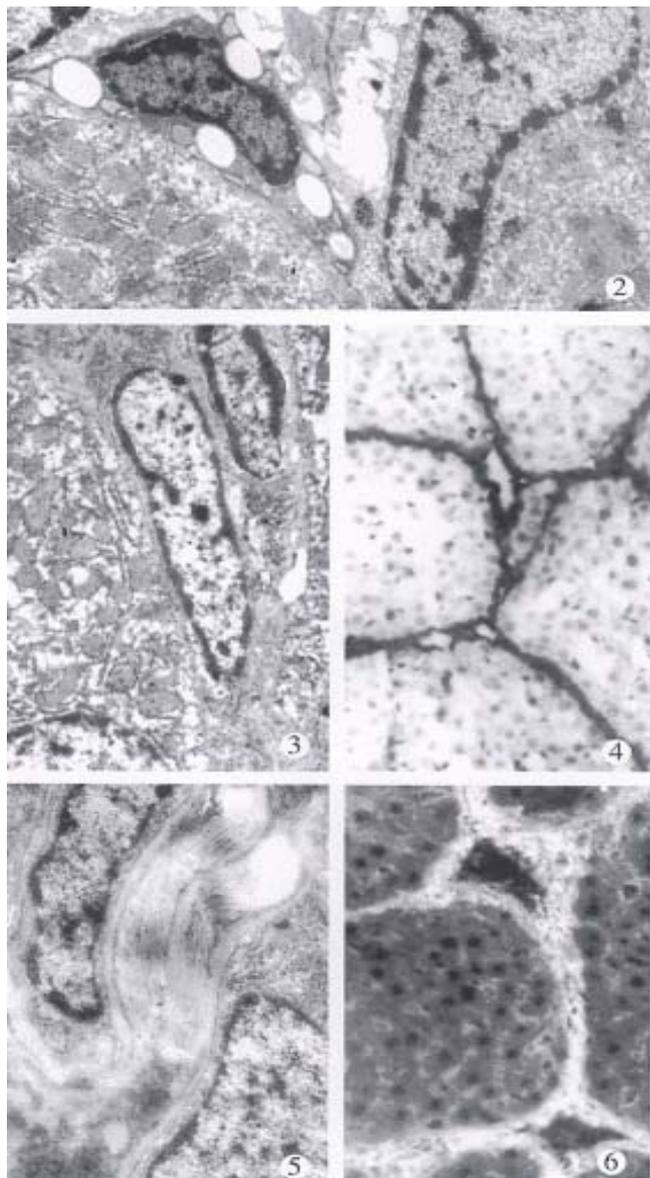
formed and revealed strong VG stain (Figure 5). The cells in the fibrous septa were fibroblasts and fibrocytes with leggy and deeply colored nucleus. At the end of the 15th week, i.e. 5 weeks after the serum injection, the liver structure was similar to that in the 10th week, but the number of mesenchymal cells declined progressively, and some of fibrous septa were thin or even diminished, and faded in VG stain. Under the EM, lipid droplets in HSCs increased again, although a large amount of bunched and intersected collagen in septa remained the same. In addition, some blood vessel walls thickened and became homogeneous in portal tracts and septa, but necrosis and infiltration of neutrophils were never discerned throughout the experiment.

#### *Changes of eosinophils and mast cells*

Dramatic changes were found in the number of eosinophils and mast cells in portal tracts and septa. The average of their counts in the experimental groups was higher than that of the control groups (Table 1). Infiltration of eosinophils was also shown in the portal tracts and some of early septa at the end of the 3rd week (Figure 1), and peaked at the end of the 8th week. However, it gradually decreased from the end of the 10th week, and was seldom seen after the intermission of the injection. Mast cells stained with toluidine blue were seen around blood vessels in the larger portal tracts in the normal rats, but were scarce in the smaller portal tracts. After injection of porcine serum, the number of mast cells in the smaller portal tracts and septa also increased in different stages of the experiment with the same trend of count changes as that of eosinophils although there was no significant difference between various stages.



**Figure 1** At the end of the 3rd week, the structure of liver lobules was defined well, the portal tracts were enlarged mildly with increased ECM and proliferated mesenchymal cells. The infiltration of eosinophils was visible in these areas. HE×200



**Figure 2** PMC with larger elliptic nucleus and HSC nearby at the end of the 3rd week. EM×5000

**Figure 3** The activated HSC enlarged with organelles and less lipid droplets at the end of the 3rd week. EM×4000

**Figure 4** A large quantity of collagen fibrils around the PMC and the fibroblast. EM×8000

**Figure 5** The fibrous septa inserted into and circled the parenchyma at the end of the 10th week. VG×40

**Figure 6** IgG was diffusely deposited in the septa and portal tracts at the end of the 15th week. Immunofluorescence×100

**Table 1** Average count of eosinophils and mast cells in different groups

Groups	Eosinophil	Mast cell
Control	1.17±0.80	1.24±0.31
3rd week	18.35±7.74 <sup>a</sup>	2.82±0.72 <sup>a</sup>
8th week	22.72±11.76 <sup>b</sup>	7.30±2.72 <sup>b</sup>
10th week	8.10±2.16 <sup>a</sup>	6.20±1.05 <sup>a</sup>
15th week	6.60±3.96 <sup>b</sup>	2.94±0.71 <sup>a</sup>
20th week	3.30±1.18 <sup>b</sup>	1.85±0.52 <sup>a</sup>

<sup>a</sup>P<0.01, <sup>b</sup>P<0.05, vs control group.

**Detection of IgG and complement C3 in liver tissue**

IgG and complement C3 in the normal liver tissue detected with immunofluorescence was lightly distributed along the sinusoids and blood vessels. At the end of the 3rd week, besides the deposition as that in the control rats, linear or spotty positive reaction of IgG and C3 were also detected in the portal tracts and early formed septa. At the end of the 8th week the degree of their positive reaction was enhanced and the reaction looked like threads and strands in the same area, and was also strongly positive in the wall of sinusoid and blood vessels. At the end of the 10th and 15th week, the distribution of IgG and C3 was diffused in the septa and portal tracts (Figure 6), and weakened obviously at the end of the 20th week.

**DISCUSSION**

The different modes of immune rat liver fibrotic model induced with human or bovine albumin intravenously have been reported<sup>[2,3]</sup>. After a large dose of injection, focal necrosis and fibrosis were found, leading to allergic shock and a high mortality of about 50%. If a small dose was administered for 16 weeks, different degrees of fibrosis formed in 80% with a mortality of 20%-30%, although the mortality could be reduced when the mixture of albumin and prostaglandin E2 were added. Therefore, those models were not ideal for their complicated procedures, severe damage of liver tissue and high mortality of animal. In our experiment, liver fibrosis formed in all the rats after continuous injection of porcine serum intraperitoneally for 8 weeks, and no rat died during the experiment. In addition, morphology of liver tissue was distinct without severe injury of hepatic cells, therefore, it facilitates in situ study and is superior to the the CCl4-induced fibrosis model with severe distortion of liver structure. As a result, our model is more suitable for research on liver fibrogenesis with the advantages of less expenses, short duration and higher success rate and simplicity of pathological changes. Our model can reveal clearly the three stages in the process of the experiment: the cellular septa formation, cellular-fibrous septa and fibrous septa. As for the source of ECM-producing cells, it has been considered that HSCs are the most important cells. This was further confirmed in our model by the appearance of proliferation and activation of HSCs with abundant collagen fibrils around. At the same time, PMS, the immature mesenchymal cells mostly located in the portal tracts and early septa, were considered as another source of ECM-producing cells, increased and developed into fibroblasts. It is suggested that in porcine serum induced liver fibrosis model, ECM-producing cells may both originate from PMCs and HSCs, and synthesize and excrete ECM,

sequentially accumulate and finally result in liver fibrosis. However, in this observation the myofibroblast, the another sort of ECM-related cells characterized with the myocyte-like structure such as macula densa or dense body in cytoplasm, was not found, which differed from the CCl<sub>4</sub>-induced model but was similar to that of Bhunchet<sup>[4]</sup>.

Pathogenesis of heteroserum-induced immune liver fibrosis has not been clarified. Wang BN and Zhu QG and their coworkers<sup>[2,3]</sup> reported that IgG and complement C3 were detected in the liver tissue with obvious fall of C3 in serum, and they considered the mechanism of their model as type III allergic reaction due to the immune complex deposition. In our experiment, there was no typical type III allergic reactive pathological changes such as fibrinoid necrosis and infiltration of neutrophils in connective tissues or blood vessels although the deposition of IgG and C3 was seen in the portal tracts and septa and the walls of blood vessels were thickened. The role of immune complex in this model was still needed to be confirmed in the future. It was noticed that the appearance of the large number of eosinophils infiltrated in the portal tracts in the early stage was exactly the same as what happened in the late phase reaction (LPR) in type I allergic response. Eosinophils infiltration was considered to be intimately associated with chronic inflammations with fibrosis such as sclerotic mediastinitis, sclerotic cholangitis, idiopathic pulmonary fibrosis and so on<sup>[5]</sup>. It was reported that a number of eosinophils existed in bleomycin-induced pulmonary fibrosis and the high level of

TGF- $\beta_1$  was expressed in the tissue with in situ hybridization and immunohistochemical techniques<sup>[6]</sup>. Upregulation of expression of TGF- $\beta_1$  was proved to be closely related with tissue repair and fibrosis<sup>[7,8]</sup>. All these phenomena suggest the hypothesis of this experimental liver fibrogenesis that LPR is initiated by heteroserum and mediated by mast cells to attract a number of eosinophils, which release active mediators such as TGF- $\beta_1$  to activate and proliferate PMCs and HSCs, and to increase the synthesis of ECM and eventually result in excessive accumulation of ECM in the tissue.

**Acknowledgments** We wish to thank Mr. Chao HK for animal feeding and Miss Zhuang L and Gu YH for their technical work. We are also grateful to Prof. Liang ZL for polishing the English language in this paper.

## REFERENCES

- 1 Paronetto F, Popper H. Chronic liver injury induced by immunologic reaction: cirrhosis following immunization with heterologous sera. *Am J Pathol*, 1966; 49(6):1087-1101
- 2 Wang BE, Wang ZF, Yin WY, Huang AF, Li JJ. Study of experimental model of immune liver fibrosis. *Natl Med J China*, 1989;69(9):503-505
- 3 Zhu QG, Fang BW, Hu QG, Li JN, Feng QL. Investigation of bovine albumin-induced liver fibrosis model with immune damage. *Chin J Pathol*, 1993;22(2):121-122
- 4 Bhunchet E, Wake K. Role of mesenchymal cell populations in porcine serum-induced rat liver fibrosis. *Hepatology*, 1992;16(6):1452-1473
- 5 Noguchi H *et al.* Tissue eosinophilia and eosinophil degranulation in syndromes associated with fibrosis. *Am J Pathol*, 1992;140(2):521-528
- 6 Zhang K, Flanders KC, Phan SH. Cellular localization of transformation growth factor  $\beta$  expression in Blomycin-induced pulmonary fibrosis. *Am J Pathol*, 1995;147(2):352-361
- 7 Nakatsukasa H, Nagy P, Evarto RP, Hsia CC, Marsden E, Thorgerirsson SS. Cellular distribution of transforming growth factor- $\beta_1$  and procollagen types I, III and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J Clin Invest*, 1990;85(6):1833-1842
- 8 Yoshioka K, Takemoza T, Muzakami K, Okada M, Hino S, Miyamoto H *et al.* Transforming growth factor  $\beta$  protein and mRNA in glomeruli in normal and diseased human kidneys. *Lab Invest*, 1993;68(2):154-163

# Transduction of human hepatocellular carcinoma cells with human $\gamma$ -interferon gene via retroviral vector \*

QIAN Shu-Bing and CHEN Shi-Shu

**Subject headings** Carcinoma,hepatocellular/therapy; interferon II/genetics;retroviridae;immunotherapy,adoptive

## Abstract

**AIM** To investigate the therapeutic potential of gamma interferon (IFN- $\gamma$ ) genemodified human hepatocellular carcinoma (HCC) cells.

**METHODS** The IFN- $\gamma$  gene was introduced retrovirally into four HCC cell lines. Secreted IFN- $\gamma$  activity was assessed using bioassay. The expression of MHC molecules was detected by FACS. Tumorigenicity was analysed by tumor formation in nude mice.

**RESULTS** Four IFN- $\gamma$  gene transduced HCC cell lines secreted different amounts of IFN- $\gamma$ , as in the same case of five clones derived from one HCC cell line. Transduction with IFN- $\gamma$  caused significant increase in the expression of major histocompatibility complex (MHC) antigens on HCC cells. The expression of HLA class I was increased by 2-3 times in terms of mean fluorescence intensities, while for class II expression, the percentage of positive cells augmented from <10% to >50%. When equal amount of tumor cells were injected into nude mice, the tumor igenicity some transduced cells decreased dramantically.

**CONCLUSION** IFN- $\gamma$  gene transduction can convert weakly imunogenic HCC cells to activate antitumor immune response, and further pave the way for the future use of such gene modified tumor cells as a modality for the cancer immunotherapy.

## INTRODUCTION

The past several years have seen an explosive growth in cancer immunotherapy using cytokine genetransduced tumor cell vaccines. This strategy seeks to locally alter the immunological environment of the tumor cell so as to enhance either antigen presentation of tumor-specific antigens to the immune system or both the activation of tumor-specific lymphocytes and nonspecific immunity. Many cytokine genes have been introduced into tumor cells with varying effects on both tumorigenicity and immunogenicity<sup>[1,2]</sup>. The success of cytokinesecreting tumor vaccines in murine models of cancer has led to the initiation of clinical trials in patients<sup>[3]</sup>. IFN- $\gamma$  is a pleiotropic cytokine produced by activated T-lymphocytes, which can influence the outcome of an immune response in several distinct ways<sup>[4]</sup>. An important property of IFN- $\gamma$  is the ability to up-regulate the expression of major histocompatibility complex (MHC) molecules, which play a central role in immune response. An increase in immunogenicity after MHC class I up-regulation by IFN- $\gamma$  is thought to be due to improved presentation of tumor-specific antigens to CD8+ CTLs<sup>[5]</sup>. We successfully transduced IFN- $\gamma$  into four HCC cell lines with retroviral vector results showed a significant up-regulation of surface MHC molecules. Moreover, transduced HCC cells decreased in tumor growth. The increased immunogenicity and decreased tumorigenicity might reflect the immunotherapeutic potential of such IFN- $\gamma$  gene transduced HCC vaccines.

## MATERIALS AND METHODS

### Cell lines

The following human hepatocellular carcinoma cell line were used: QGY7701, SMMC7721, BEL7404 and HHCL. All the cell lines were purchased from Bank of Cell, Institute of Cell Biology, Chinese Academy of Sciences (Shanghai). HCC cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100U/mL), streptomycin (100  $\mu$ g/mL) and 2mM-glutamine. The monolayer was propagated by trypsinization as required.

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Presented at the First International Symposium on Gene Therapy, Beijing, July 7-10, 1997

\*Supported by the National "863" High Tech Project Foundation No.863-102-16-05 and the National Natural Science Foundation of China, No.39470792.

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Received 1997-10-28

### **Construct of retroviruses and IFN- $\gamma$ transduction**

Human full-length IFN- $\gamma$  cDNA encoding a leading peptide and IFN- $\gamma$  was cloned into pLXSN retroviral vector (provided by Dr. AD Miller), generating recombinant construct pL (IFN- $\gamma$ )SN. The inserted IFN- $\gamma$  gene was driven by the long terminal repeat (LTR), of moloney murine leukemia virus (MLV) while the neomycin phosphotransferase gene (Neo-R) was driven by both the LTR and the simian virus early promoter (SV40). The vector was introduced into amphotropic packaging cell line PA317. Transfected cells were selected in 0.4mg/mL G418 (Gibco) and resistant colonies were isolated and amplified. Viral titers of the retroviral supernatant ranged from  $5 \times 10^4$  to  $2 \times 10^6$  colony forming units (CFU)/mL, when assayed for their ability to transfer neomycin-resistance to NIH3T3 cells.

HCC cells ( $0.5-1 \times 10^6$ ) were cultured overnight in T80 flasks (Nunc). Retroviral supernatants supplemented with 8mg/L polybrene (Sigma) were added to each flask to obtain a ratio of about 1MOI/cell. The following day, cells were fully selected by the neomycin analogue, geneticin G418. The concentration of G418 used for the selection ranged from 0.5 to 1g/L (active dose), depending on the sensitivity of each cell to the toxic effects of G418. Approximately, cells were maintained in selecting medium for 2 weeks. Colonies that survived the G418 selection were isolated and expanded.

### **IFN- $\gamma$ assay**

The amount of IFN- $\gamma$  produced by transduced cells was determined by the standard cytopathic inhibition assay. Briefly,  $5 \times 10^5$  cells were incubated for 24 hours, then supernatants were harvested, centrifuged and aliquoted as test samples. Human fibroblasts were cultured with serial dilutions of test samples or standard IFN- $\gamma$  (Boehringer Mannheim). The cells were challenged with vesicular stomatitis virus (VSV) and cultured overnight. The IFN- $\gamma$  titer was calculated as the reciprocal of the dilution that protected 50% of the monolayer cells from the cytopathic effect of the virus.

### **Flow cytometric analysis**

Flow cytometry was performed for quantitative analysis of surface MHC class I and II expression. One million cells were harvested by trypsinization, washed twice in phosphate balance solution (PBS) containing 2% FCS and 0.1% sodium azide (Sigma), and incubated for 30min at 4°C with saturating amounts of monoclonal antibodies (anti-HLA class I and anti-HLA class II, Dept. Immunology, Beijing Medical University). After

twice washing, cells were incubated with a  $1 \times 50$  dilution of fluorescence isothiocyanate-conjugated goat anti-mouse IgG (Huamei Co., Shanghai) for 30min at 4°C in the dark. The cells were washed twice, fixed with PBS containing 0.2% formaldehyde and examined by an FACScan flow cytometer (Becton Dickinson) for the percentage of positive cells and mean fluorescence intensities.

### **Tumor formation in nude mice**

Female athymic nude BALB/c mice of 4-5 weeks (purchased from Animal Center, Chinese Academy of Sciences, Shanghai) were housed under pathogen free conditions. Six to eight animal in a group were used. Cells ( $3 \times 10^6$  in 200 $\mu$ L) were injected into the right flank of the mice. The tumors were measured by a caliper in two dimensions and the volume were calculated using the formula ( $\text{width}^2 \times \text{length} / 2$ ). At the end of 45 days, the tumors were removed and weighed.

## **RESULTS**

### **Production of IFN- $\gamma$ from transduced clones**

The tumor cell lines were successfully infected with recombinant retrovirus containing IFN- $\gamma$  cDNA. G418-resistant colonies were isolated and expanded to cell lines for further analysis. According to the number of selected clone, each transduced cell line was designated as QGY7701.3A, SMMC7721.2C, BEL7404.6A and HHCL.5D, respectively. Five different clones derived from the transduced HHCL were designated following the same rule. Southern blot analysis was made and showed that the intact gene of interest was present in the transduced cell lines in the form of provirus. Northern blot analysis further indicated that the correct mRNA species were transcribed (data not shown). Secretion of IFN- $\gamma$  by HCC cells transduced with the retroviral vectors was determined by an appropriate bioassay. As shown in Table 1, transduced cell lines derived from different HCC cell lines produced different amounts of IFN- $\gamma$ , as the same case of five clones derived from one parental HHCL.

### **Enhancement of the MHC molecule expression after IFN- $\gamma$ gene transduction**

All the four HCC cell lines used in this study were screened for cell surface expression of HLA molecules by flow cytometry as described above. As shown in Table 1, all the cell lines expressed HLA class I molecules, however the expression of HLA class II molecules was very low. Transduction with the IFN- $\gamma$  gene resulted in significant increases in the expression of HLA molecules. The expression of HLA class I molecules was increased 2-3 times in terms of

fluorescence intensities, while for class II molecules, the percentage of positive cells was augmented from <10% to >50%. There was no correlation between the degree of increase in HLA expression and the amounts of IFN- $\gamma$  production, even when five transduced clones derived from one parental cell line HHCL were compared. But it is fairly clear that the cells with lower expression of HLA class I molecules, such as QGY7701 and BEL7404, have stronger response to IFN- $\gamma$  transduction in inducing the expression of HLA molecules, and thus resulting in more increase in the intensities of such molecules (Table 1).

### Tumor growth in nude mice

Live parental  $3 \times 10^6$  or transduced HCC cells were injected s.c. into the thigh region of nude mice, and tumor growth was measured weekly. The

tumorigenicity was different in each parental HCC cell. The tumor growth of QGY7701 was more rapid than HHCL. Injection of the former cells resulted in palpable tumor within 7 days, while the latter formed discernible tumor after 14 days. However, the tumor formation of respective IFN- $\gamma$  transduced HCC cells was also different. Transduced QGY7701.3A showed no distinguishable decrease in its tumor growth as compared with that of parental cell line, whereas transduced HHCL.5D reduced its tumorigenicity dramatically (Table 2). In addition to the much lower tumor incidence (1/7), there was a significant decrease in both the size and the weight of the tumor ( $P < 0.01$ ). The other two transduced HCC cells also inhibited the tumor growth, although in different degrees. It is notable that the reduction in tumor growth following IFN- $\gamma$  transduction was related to the original tumor formation potential of its parental HCC cell line.

**Table 1 Secretion of IFN- $\gamma$  and expression of HLA antigens by transduced hepatocellular carcinoma cells in culture**

Cell lines	IFN- $\gamma$ Production (U/ $5 \times 10^5$ cells/24h)	Control* MCN(% positive) $\Delta\Delta$	HLA class I * * MCN (% positive)	HLA class II $\Delta$ MCN (% positive)
QGY7701	0	18(6)	159(99)	26(11)
QGY7701.3A	75	21(5)	306(100)	28(77)
SMMC7721	0	9(2)	200(100)	13(12)
SMMC7721.2C	150	21(3)	348(100)	14(58)
BEL7704	0	20(7)	34(93)	21(8)
BEL7404.6A	75	22(5)	117(100)	29(47)
HHCL	0	10(3)	232(100)	12(10)
HHCL.2C	100	22(6)	340(100)	37(56)
HHCL.2D	25	14(6)	440(100)	12(68)
HHCL.5C	100	11(4)	401(100)	19(83)
HHCL.6C	75	15(3)	550(100)	10(44)
HHCL.5D	200	10(3)	341(100)	10(40)

\*Cells were stained with fluorescence isothiocyanatic-conjugated goat anti-mouse IgG (FITC-IgG) as a control.

\*\*Cells were stained with anti-HLA class I McAb following with FITC-IgG.

$\Delta$ Cells were stained with anti-HLA class II McAb following with FITC-IgG.

$\Delta\Delta$ Mean fluorescence channel number (MCN) and percentages of positive cells (in parentheses).

**Table 2 Formation of tumors by parental and transduced hepatocellular carcinoma cells in nude mice**

Cell	Incidence	Volume (cm <sup>3</sup> )	Weight (g)
QGY7701	8/8	5.58 $\pm$ 0.90	1.62 $\pm$ 0.36
QGY7701.3A	8/8	5.16 $\pm$ 1.16	1.60 $\pm$ 0.72
SMMC7721	7/7	3.87 $\pm$ 0.24	0.55 $\pm$ 0.12S
MMC7721.2C	5/6	1.26 $\pm$ 0.32 <sup>a</sup>	0.28 $\pm$ 0.08 <sup>a</sup>
BEL7404	8/8	4.32 $\pm$ 0.28	0.78 $\pm$ 0.14
BEL7404.6A	8/8	3.35 $\pm$ 0.21	0.50 $\pm$ 0.12
HHCL	8/8	2.04 $\pm$ 0.32	0.35 $\pm$ 0.18
HHCL.5D	1/7	0.15 <sup>b</sup>	0.02 <sup>b</sup>

All the values are measured at 6 weeks after injection with equal amounts of cells ( $3 \times 10^6$  each), and presented as the average value  $\pm$  the standard error of the mean

<sup>a</sup> $P < 0.05$ , compared with SMMC7721 value by Student's *t* test

<sup>b</sup> $P < 0.01$ , compared with HHCL value by Student's *t* test

## DISCUSSION

In this study human hepatocellular carcinoma cell line were the genes for human IFN- $\gamma$  were transduced successfully. Four different HCC cells transduced with the IFN- $\gamma$  gene produced varying levels of IFN- $\gamma$ , and such difference is also existed among the five clones derived from one parental HCC cell line (HHCL). Since the distinguished feature of retroviral vector is its integration into the host genomic DNA in the form of provirus<sup>[5]</sup>, the expression difference might be due to the random integration resulting in varying efficiency of gene expression derived by interior promoter.

It has been repeatedly demonstrated that IFN- $\gamma$

can exert significant antitumor effects via either direct antiproliferative effects on the tumor or indirectly through the host immune system, including enhancement of MHC class I and II expression, activation of macrophage and natural killer cells, generation of cytotoxic T-lymphocytes and induction of tumor associated antigen<sup>[4]</sup>. MHC class I and II molecules play a central role in cellular immunity and tumor surveillance. Recent studies have demonstrated that the loss of MHC class I expression was associated with tumor progression or metastasis<sup>[7]</sup>. Enhancing MHC class I expression by tumor cells may promote antitumor response against them. Our results in MHC as consistent with most others in melanoma and RCC<sup>[8-10]</sup>. The cell surface expression of both HLA class I and II molecules was increased in HCC cells transduced with the IFN- $\gamma$  gene. Considering the original display of HLA class I, but not class II in parental HCC cells, it is not surprising that following IFN- $\gamma$  transduction, HLA class I expression was significantly increased in terms of mean fluorescence intensities, while for class II, in terms of percentage of positive cells. There was no definite correlation between the magnitude of the increase in the expression of HLA molecules and the amount of the IFN- $\gamma$  secreted by transduced HCC cells. However, the magnitude of the increase in the expression of HLA class I in mean fluorescence intensities appeared to be greater in transduced HCC cells with lower expression of such molecules in its parental cells. This suggests that the augmentation of MHC expression is associated with both the effects of IFN- $\gamma$  and the potential of expression of such molecules.

Most animal studies have showed that IFN- $\gamma$  secretion by tumor cells results in reduced tumorigenicity. However, when the tumorigenicity of human tumor cells was examined in nude mice, several elements must be considered. Nude mice is a T cell deficient strain, but other components of the immune system may still exist. In addition, human IFN- $\gamma$  secreted by transduced HCC cells have little effect on mouse immune system. Our results obtained from nude mice injected with parental or

transduced HCC cells were different. That the tumor growth of the transduced QGY7701.3A in nude mice was not significantly different from that of the parental untransduced cell lines was surprising. But it should be noted that QGY7701 was of the highest tumorigenicity among the four HCC cell lines. In contrast to QGY7701, HHCL, which was of the lowest tumorigenicity, showed dramatic decrease in tumor growth in nude mice following transduction with IFN- $\gamma$  gene. The observation of such difference might reflect either the heterogeneity of the cells with which we worked or the limited effects of IFN- $\gamma$  for tumor cells of high tumorigenicity.

In summary, the data obtained in this study indicated that IFN- $\gamma$  gene modified HCC cells might be useful in the treatment of human cancers, especially in inducing specific immune responses. However, the heterogeneity of tumor cells should be considered in establishing effective tumor vaccine, thus a more potential tumor vaccine can be selected and an increased antitumor immunity induced *in vivo* can be obtained. This study has laid ground for the future use of cytokine gene modified tumor cells as a modality for the cancer immunotherapy.

## REFERENCES

- 1 Gansbacher B, Zier K, Daniels B, Cronin K, Mannerji R, Gilboa E. Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med*, 1990;172(5):1227-1224
- 2 Giboa E, Lysterly HK, Vieweg J, Satio S. Cytokine gene therapy in tumor. *Semin Cancer Biol*, 1994;5(3):409-417
- 3 Special report. Human gene marker/therapy clinical protocols. *Hum Gene Ther*, 1996;7(4):567-588
- 4 Sigel JP. Effects of interferon-gamma on the activation of human T-lymphocytes. *Cell Immunol*, 1988;111(3):461-472
- 5 Restifo NP, Spiess PJ, Karp SE, Mule JJ, Rosenberg SA. A nonimmunogenic sarcoma transduced with the cDNA for interferon  $\gamma$  elicits CD8+T cells against the wild type tumor: correlation with antigen presentation capability. *J Exp Med*, 1992;175(6):1423-1431
- 6 Crystal RG. Transfer of genes to humans: early lessons and obstacles to success. *Science*, 1995;270(4):404-414
- 7 Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today*, 1995;16(7):487-497
- 8 Nayak SK, McCallister T, Han LJ, Gangavalli R, Barber S, Dilloman RO. Transduction of human renal carcinoma cells with human  $\gamma$ -interferon gene via retroviral vector. *Cancer Gene Ther*, 1996;3(3):143-150
- 9 Gansbacher B, Bannerji R, Dannids B, Zier K, Cronin K, Gilboa E. Retroviral vector mediated  $\gamma$  interferon gene transfer into tumor cells generates potent and long lasting antitumor immunity. *Cancer Res*, 1990;50(14):7820-7825
- 10 Ogasawara M, Rosenberg SA. Enhanced HLA molecules and stimulation of autologous human tumor infiltrating lymphocytes following transduction of melanoma cells with  $\gamma$ -interferon genes. *Cancer Res*, 1993;53(7):3561-3568

# Animal experiments and clinical application of CT during percutaneous splenoportography

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**Subject headings** portography; tomography, X-ray computed; animals, laboratory; liver neoplasms/radiography

## Abstract

**AIM** To introduce computed tomography during percutaneous splenoportography (CTSP), a new method for determining hepatic diseases.

**METHODS** Ten hybrid dogs and 20 patients with primary hepatic cancer (PHC) were included in the study. Each dog was examined by CT, CTAP (computed tomography during arterial portography) and CTSP to compare the enhanced degrees of the liver. The 20 PHC patients were examined by CTSP and the appearance of PHC was compared with their pathological results to evaluate the diagnostic significance of CTSP.

**RESULTS** The animal experiments showed that both CTAP and CTSP could obviously enhance the liver ( $P < 0.01$ ), but there was no significant difference in the enhanced results between the two methods ( $P > 0.05$ ). On the CTSP images in the 20 patients, the density of the livers was increased to 168-192Hu, whereas the density of the cancers remained as low as that on the images of CT scans ( $< 58\text{Hu}$ ). The CTSP findings were consistent with the surgical ones from spaceoccupying lesions. Its diagnostic value was obviously superior to that of general enhanced CT and ultrasonic examination. However, it was difficult for CTSP to show nodules less than 1cm in size located on the surface of the liver or the hepatic portal zone.

**CONCLUSIONS** Like CTAP, CTSP is also a sensitive method for showing occupants in the liver. But the equipments and the procedures for CTSP are simpler than for CTAP. Therefore, it is an alternative procedure in clinical practice.

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Received 1998-01-31

## INTRODUCTION

Computed tomography during arterial portography (CTAP) established by Hisa in 1980 is the most sensitive method for showing occupants in the liver<sup>[1-3]</sup>. Unfortunately, CTAP is not convenient for arterial catheterization, which is necessary before CT examination. Based on CTAP, we used CT during splenoportography (CTSP) to show occupants in the liver. CTSP does not require complicated instruments and is easy to operate. And it has been proved to be sensitive and safe for clinical application<sup>[4-5]</sup>.

## MATERIALS AND METHODS

### Animal experiments

**Animals and instruments** Ten hybrid dogs were supplied by the Animal Experimental Unit of Nanfang Hospital. The instruments used in this experiment were: 800mA remote controlled gastrointestinal machine (Daojin, Japan), 5F Cobna catheters, somaton plus whole body CT machine (Siemens Corporation, German), syringes of MCT310-2 model (Medrad Corporation, USA), and 20G trocar (Terumo Corporation, Italy).

**Procedures** Firstly, plain CT scanning was performed in all of the dogs from the top of the diaphragm to the inferior margin of the liver. Each layer was and distance between two neighbouring layers was 10mm. Then, the animals were examined by CTAP. Using Seldinger technology, a catheter was inserted from the left femoral artery to the anterior mesenteric artery (Figure 1). Four hours later, 76% compound meglumine diatrizoate (1.5ml/kg body weight) was injected through the catheter at a rate of 1.5ml/sec and the animals were examined by continuous dynamic scanning. Seven days after CTAP, CTSP was performed. In this procedure, the point of a 20G trocar was inserted into the relatively larger veins or the splenic parenchyma (Figure 2). The same quantity of compound meglumine diatrizoate was injected at the same rate as CTAP. The animals were examined as in the second step.

### Clinical application

**General data** Seventeen men and three women with PHC were examined by CTSP. Nine of the 20 patients were operated on after CTSP to remove the tumors who were proved pathologically to have primary hepatocellular carcinomas. Seven patients

had postoperative recurrence of hepatocellular carcinomas. Diagnosis of PHC in 4 patients was made according to the criteria formulated in the National Consensus Conference on Prevention and Therapy of Hepatic Carcinoma in 1977.

**Instruments** The same trocar, syringes and CT scan machine were used as for the animal experiments.

**Examination methods** The position for insertion of a 20G trocar was determined by CT. The puncture point for CT was generally on the middle or posterior axillary line of the 8th to 11th intercostal space. The depth of the needle was determined by CT image. When the needle was inserted, the patients were scanned once again. When proper position, depth and direction of the needle were achieved (Figure 3), a total volume of 60ml-80ml 60% angiografin (SCHERING Corporation, Germany) was injected at a rate of 1ml/sec. Twenty seconds later, continuous dynamic scanning was started. When the scanning was finished, the trocar was quickly pulled out and the puncture point was pressed for 4-5 minutes and covered with gauze. If necessary, the puncture site should be scanned to get 1-3 images to observe whether bleeding happens.

## RESULTS

### *Animal experiments*

The CT values of the dog livers in plain CT scan, CTAP and CTSP were  $70.5\text{Hu} \pm 8.7\text{Hu}$ ,  $209\text{Hu} \pm 23.9\text{Hu}$  and  $212\text{Hu} \pm 28.2\text{Hu}$ , respectively. CTAP and CTSP could markedly enhance the liver ( $P < 0.01$ ), with no significant difference ( $P > 0.05$ ) (Figures 4). To observe the liver, kidney and spleen, two of the ten dogs were killed each time immediately, 3, 5, 7 or 10 days after CTSP examination. No abnormality was seen in the livers and kidneys of all the dogs. The surface of the spleens was smooth, without haematoma. In the spleens, dotted bleeding sites about  $2\text{mm} \times 2\text{mm}$  appeared where the needle was inserted. Those bleeding sites were substituted by connective tissues later (Figures 5-7).

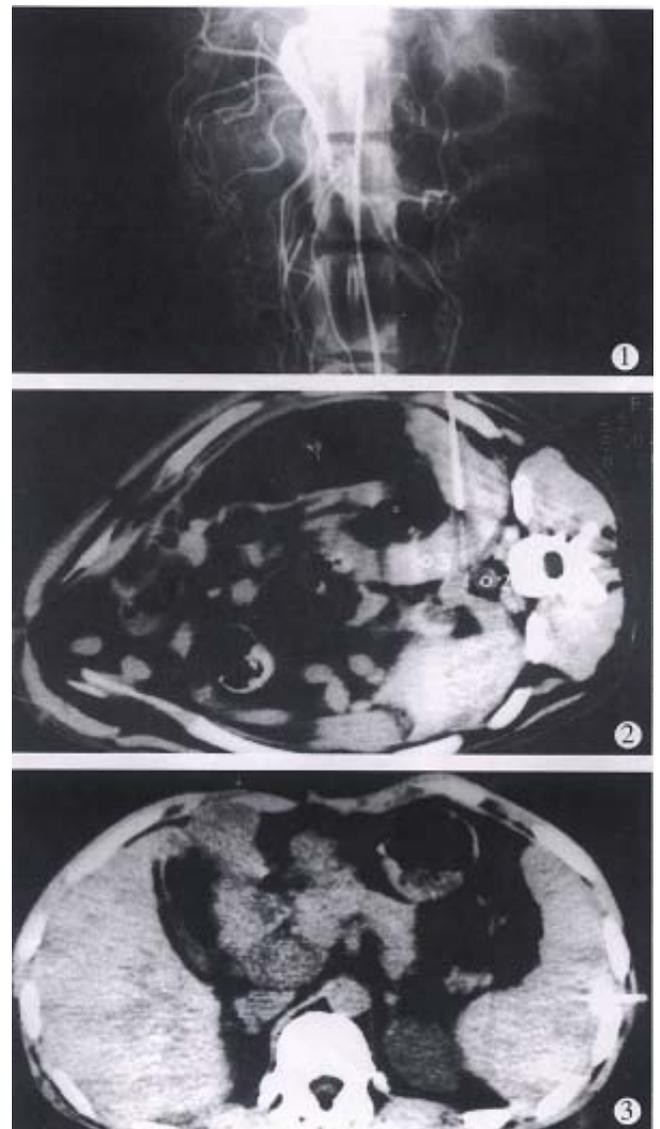
### *Clinical application*

The appearance of PHC in CTSP. In the 20 patients with PHC, 58 foci with low density were found by CTSP. After the contrast medium was injected, the CT values of normal livers could reach  $168\text{Hu}$ - $192\text{Hu}$ , whereas the values of hepatic cancers were no more than  $58\text{Hu}$ . The carcinomas showed similar density in CTSP and plain CT scan. The neoplasms showed clear borders (Figures 8a-c, 9a-c and 10a-d).

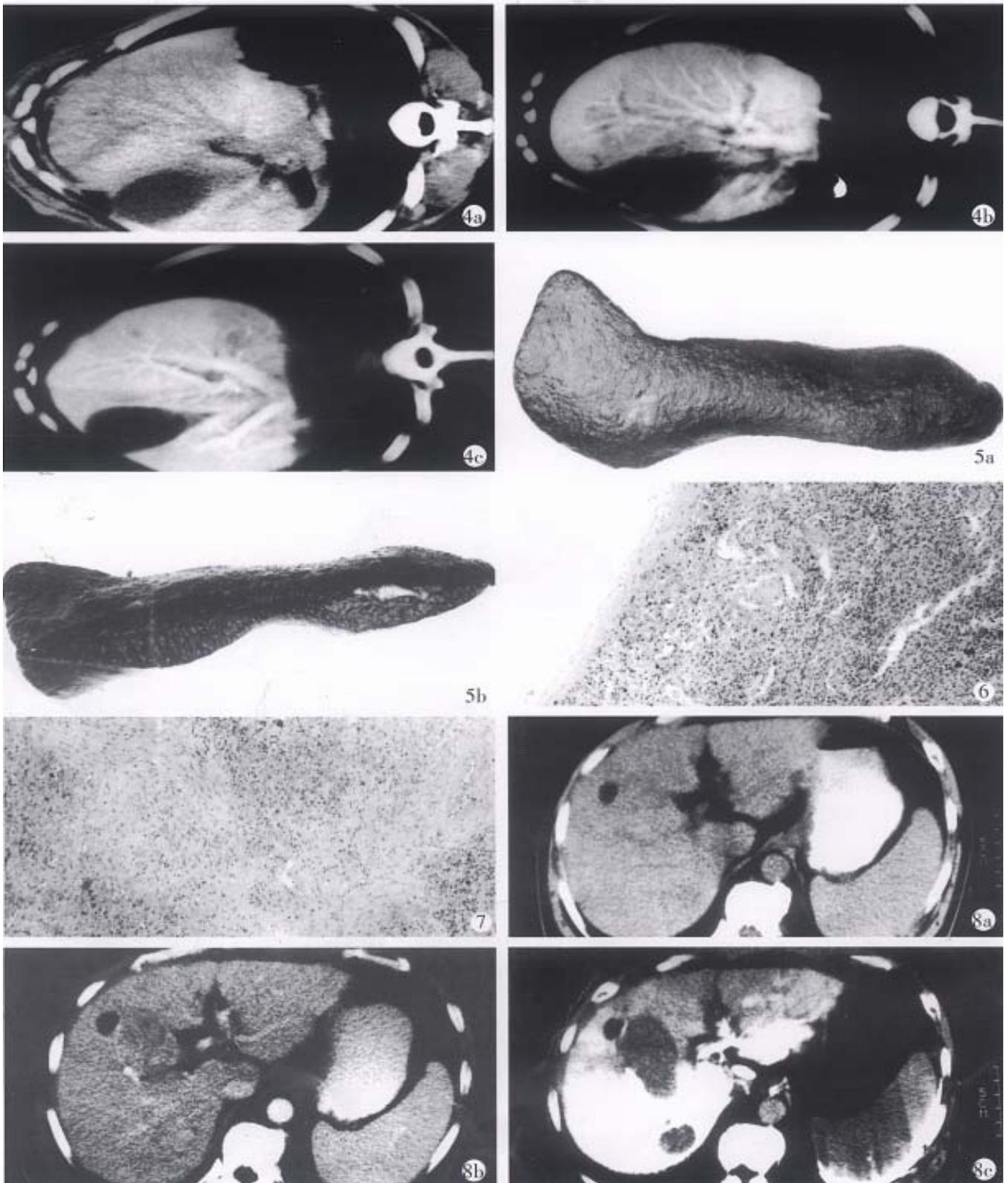
The density of the spleen was not high after injection of the contrast medium, because most of the contrast medium was quickly excreted.

Sometimes, block-shaped contrast medium could be seen remaining in the spleen (Figure 11). In three patients, image of high density could be observed beneath the capsule of the spleen, which may be resulted from reflux of the contrast medium there.

The postoperative reaction of the patients. The patients did not feel obviously uncomfortable when the contrast medium was injected at a rate of 1ml/sec. They felt slight local swelling and pain at the beginning when the injection was rapid (2ml/sec-3ml/sec). The pain was alleviated 10-20 minutes later. Three patients felt more severe pain because the drug was injected into the pleural cavity. The contrast medium was spontaneously absorbed one week later. No bleeding occurred.



**Figure 1** Anterior mesenteric arteriography in a dog. The point of the catheter is in the stem of the anterior mesenteric artery.  
**Figure 2** The point entered the right place of the dog's spleen.  
**Figure 3** The point entered the right place of the patient's spleen.



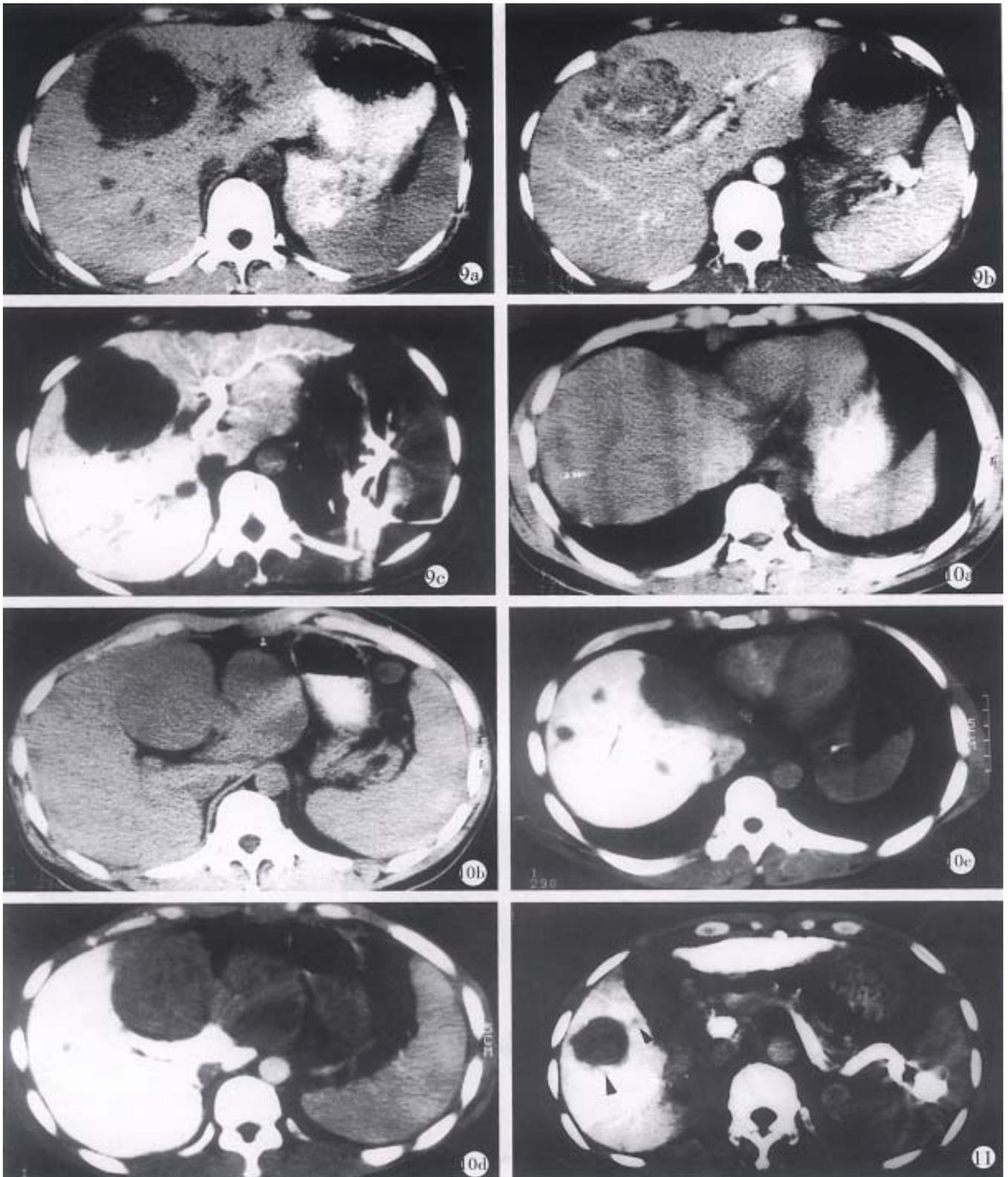
**Figure 4a-c** The CT images of the same dog's liver. a. plain CT scan; b. CTAP; c. CTSP.

**Figure 5** The spleens of the dogs eviscerated 5 days after CTSP. a. The surface of the spleen is smooth and b. there is no bleeding foci in the longitudinally dissected spleen.

**Figure 6** The splenic tissue of one dog killed immediately after CTSP. HE stain  $3.3\times 10$ .

**Figure 7** The spleen of a dog killed 10 days after CTSP. A little proliferation of connective tissue could be seen at the point where the catheter entered the spleen. HE stain  $3.3\times 10$ .

**Figures 8** PHC of a 59-year-old male patient. a. plain CT scan: only a small cyst was seen in the anterior part of the right lobe of the liver. b. enhanced CT: except the cyst, the carcinoma could not be shown clearly. c. CTSP: two carcinomas were clearly shown behind the cyst, and proved to be primary hepatocyte carcinoma.



**Figure 9** Primary hepatocyte carcinoma of a 30- year-old female patient. a. plain CT scan; b. enhanced CT; c. CTSP. Though they all could show carcinoma, CTSP not only could show it more clearly than the other two, but also show metastases to the right of the inferior vena cava.

**Figure 10** Postoperative recurrence and hepatic metastases of primary hepatocyte carcinoma of a 44-year-old female patient. a, b, plain CT scans; c, d, CTSP.

**Figure 11** The contrast medium remained in the spleen after CTSP. The splenic veins show high density. The recurrent cancer in the liver and the child focus in the portal vein (the left posterior arrow) are both shown clearly.

## DISCUSSION

The liver was supplied with blood by the portal vein and the hepatic arteries, but by the portal vein, whereas the hepatic carcinoma was mainly supplied by the hepatic arteries. In CTSP, the contrast medium injected into the spleen reached the liver through the portal vein, therefore, the density of the normal liver tissue obviously increased and that of the hepatic carcinoma tissue remained unchanged. Animal experiments showed that CTSP could have the same enhancement effects as CTAP did, which provided a good radiological basis for CTSP to show occupants in the liver.

CTSP has been shown to be a very sensitive method for showing both primary and metastatic foci of PHC. In 9 patients, CTSP was as sensitive as operative exploration in finding the foci of 1cm in size in the liver. In the other three patients, CTSP could not find nodules <1cm on the surface of the liver or metastatic foci <1cm in the hepatic portal zone that were found during operation. Compared with ultrasonic examination, CTSP could find more foci. Ultrasonic examination could find only 5 of 15 foci  $\leq$  1.5cm found by CTSP in 12 patients. CTSP was even superior to ultrasonic examination in showing neoplasms located on the diaphragmatic surface of the liver. In four patients examined by enhanced CT and CTSP, CTSP showed seven foci  $\leq$  1.5cm, whereas enhanced CT found only three foci unclearly. In general, according to our limited experiences, CTSP could show PHC with high sensitivity. Further work is necessary for qualitative

diagnosis of PHC by CTSP.

CTSP is sensitive in showing occupants of as small as 5mm in size in the liver. Therefore, CTSP should be done when primary or metastatic occupants were suspected in the liver that could not be shown clearly by other examinations. For patients with occluded portal veins, CTSP is not acceptable, because the contrast medium is difficult to pass through the portal veins.

CTSP is a microtraumatic examination. Though early observation in animal experiments could find bleeding foci at the splenic puncture sites, their diameters were less than 2 mm and substituted by connective tissues later, without spleen rupture. No bleeding in the abdominal cavity and spleen rupture occurred either in our clinical application. The vein intima remained smooth and no portal veins were obstructed, no injuries were seen in the livers and the kidneys. Therefore, CTSP could be safely used in clinical practice.

## REFERENCES

- 1 Matsui O, Takahima T, Kadoya M. Dynamic computed tomography during arterial portography: the most sensitive examination for small hepatocellular carcinoma. *J Comp Assist Tomography*, 1985;9(1):19-24
- 2 Matsui O, Takashima T, Kadoya M. Liver metastases from colorectal cancers detection with CT during arterial portography. *Radiology*, 1987;165(1):65-70
- 3 Takayasu K, Moriyama N, Muramatsu Y. The diagnosis of small hepatocellular carcinoma: efficacy of various imaging procedures in 100 patients. *AJR*, 1990;155(1):49-54
- 4 Zhang XL, Chang RM, Cheng GX. Discussion of primary hepatic carcinoma by CT during percutaneous splenoportography. *J Image Intervent Radiol*, 1996; 5(6):339-341
- 5 Zhang XL, Chang RM, Zou CJ. Early diagnosis of primary hepatic carcinoma by liver enhancing CT during percutaneous splenoportography. *J Med Coll PLA*, 1996;11(4):309-313

# Relationship between expression of laminin and pathological features in human colorectal carcinoma \*

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**Subject headings** rectal neoplasms/pathology; colonic neoplasms/pathology; laminin/metabolism; immunohistochemistry

## Abstract

**AIM** To study the expression and significance of laminin in human colorectal carcinoma.

**METHODS** Using the monoclonal antibody to laminin and streptavidin-peroxidase immunohistochemical method, the expression of laminin in 63 cases of human colorectal carcinoma tissues was determined.

**RESULTS** In normal margin intestinal mucosa adjacent to carcinoma, laminin was largely restricted to basement membrane in continuous linear pattern. In contrast, human colorectal carcinomas exhibited a progressive loss of an intact basement membrane that was correlated with decreasing differentiation degree. Well and moderately differentiated tumors exhibited a thin basement membrane with intermittent disruptions, and poorly differentiated tumors exhibited no areas of intact basement membrane. An association was found between lack of basement membrane laminin immunohistochemical staining in colorectal carcinoma and poorly differentiated tumor ( $P < 0.01$ ).

**CONCLUSION** Immunohistochemical staining for laminin could provide a very useful index for the determination of the differentiation degree of colorectal carcinoma.

## INTRODUCTION

Normal epithelial tissues secrete, assemble, and adhere to well-defined basement membranes. Loss of a well-defined basement membrane is one of the features of neoplastic proliferation for epithelial-derived tumors<sup>[1]</sup>. Laminin, a major glycoprotein of basement membrane which has been shown to regulate a variety of biological phenomena including cell attachment, growth, morphology and cell migration by specific high affinity receptors, plays an important role in the interaction of tumor cells with the basement membrane, the differentiation, invasion and metastasis of tumors<sup>[2,3]</sup>. Immunohistological studies, by using antibodies specific for laminin and other basement membrane components, such as type-IV collagen, have revealed disruption or loss of basement membrane staining in a number of malignant tumors, including breast, pancreatic, bladder, prostatic and colorectal carcinomas<sup>[4]</sup>. In this study, basement membrane protein laminin was assessed by streptavidin-peroxidase immunohistochemical method to facilitate the understanding of the relationship between laminin expression in human colorectal carcinoma and the pathological features of tumor

## MATERIALS AND METHODS

### Materials

Sixty-three cases of human colorectal carcinoma specimens were obtained from the Department of Pathology, China Medical University. Thirty-three were male and 30 were female with ages ranging from 26 to 72 years. The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Five  $\mu\text{m}$  paraffin sections were cut and used for HE staining and immunohistochemistry.

### Methods

Laminin expression was determined by using streptavidin-peroxidase (SP) immunohistochemical method with anti-laminin monoclonal antibody (SIGMA, 1:1000; SP kit ZYMED). Both negative and positive controls were set. The staining was scored according to both intensity and continuity of the basement membrane. They were scored as negative(-), fragment pattern(+), interrupted linear pattern(++), and continuous linear pattern(+++). Negative and fragment patterns were

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\*Project supported by the National Natural Science Foundation of China, No. 3904005.

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Received 1997-11-26 Revised 1998-01-10

classified as discontinuity and the other two patterns as continuity. Statistical analyses were conducted by  $\chi^2$  test. Results were considered significantly different when  $P$  value was less than 0.05.

## RESULTS

### Tumor grading and laminin expression

Normal large intestinal mucosa adjacent to carcinoma exhibited an organized glandular epithelium overlying a well-defined continuous linear basement membrane that stained for laminin (+++), (Figure 1). Well and moderately differentiated colorectal carcinomas differed from normal colon in which periglandular basement membrane staining was thin and intermittently discontinuous (++ to +++), (Figure 2). Poorly differentiated tumors consisted of solid nonglandular islets of cells. Patches of laminin staining was scattered throughout the tumor. Nonpolarized cytoplasmic laminin staining without a surrounding basement membrane (- to +) (Figure 3) was found sometimes in the nests of cells in these tumors.

Relationship between tumor grade and basement membrane laminin staining pattern in the tumors is shown in Table 1. Of the well and moderately differentiated cases, 80% and 57.1% showed continuous basement membrane laminin staining respectively, compared with 14.6% of poorly differentiated ones. When the staining patterns for poorly differentiated tumors were compared statistically with well and moderately differentiated tumors, a significant difference was found ( $\chi^2: P < 0.01$ ). Hence, discontinuous basement membrane laminin staining is associated with poor differentiation of tumors.

**Table 1 Tumor grading and laminin staining patterns**

Tumor grade	n	Laminin staining pattern	
		++ to +++	- to +
Well differentiated	20	16(80.0%)	4(20.0%) <sup>a</sup>
Moderately differentiated	21	12(47.1%)	9(42.9%) <sup>a</sup>
Poorly differentiated	22	3(13.6%)	19(86.4%)

<sup>a</sup> $P < 0.01$  vs poorly differentiated tumor.

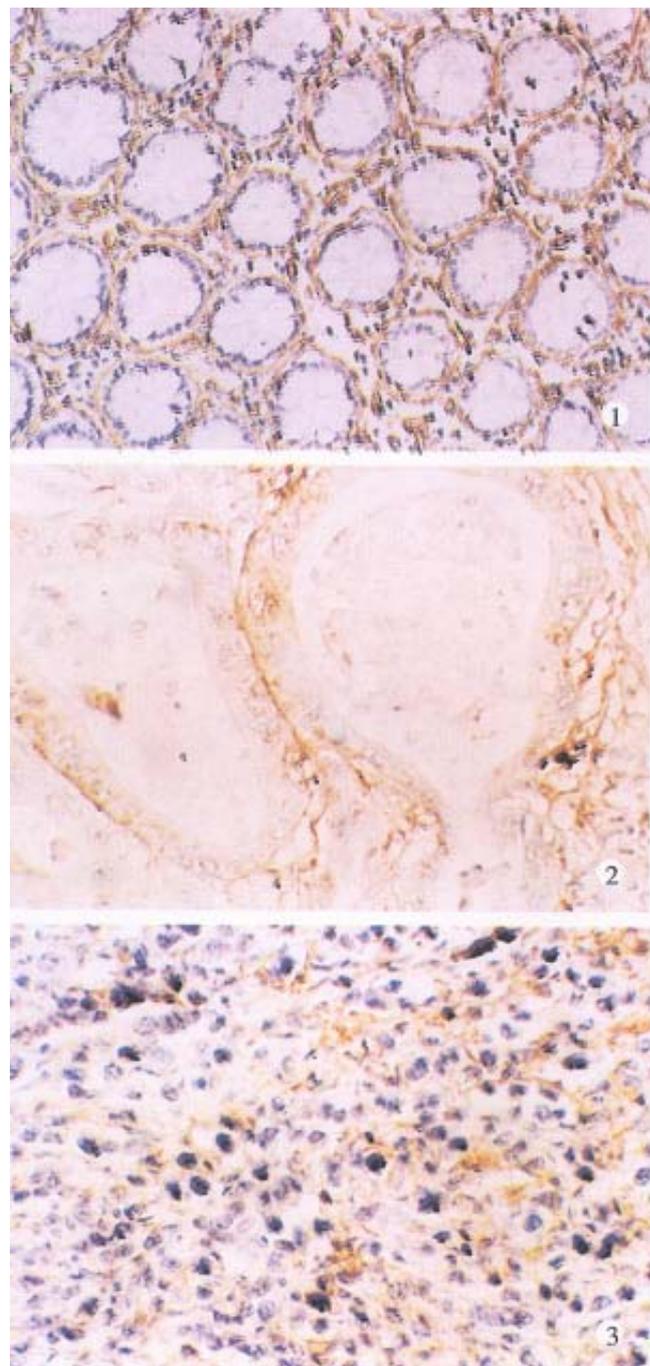
### Tumor stage and laminin expression

Comparison between basement membrane laminin staining pattern in tumor and tumor stage is given in Table 2. When regional lymph nodes were involved (Dukes's stage C), there was no linear staining of basement membrane laminin in 13/21 (61%) cases of primary tumors, whereas the majority (23/42) of stages A and B tumors showed continuous basement membrane laminin staining. However, there was no statistical significance ( $\chi^2: P > 0.05$ ).

**Table 2 Tumor stage and laminin staining pattern**

Tumor grade	n	Laminin staining pattern	
		++ to +++	- to +
Dukes's stage A	36	20(45 <sup>a</sup> ±6%)	16(44.4%)
Dukes's stage B	6	3(50.0%)	3(50.0%) <sup>a</sup>
Dukes's stage C	21	8(38.1%)	13(61.9%)

<sup>a</sup> $P > 0.05$  vs Dukes's stage A tumor.



**Figure 1** Expression of laminin in normal large intestinal mucosa. SP×100

**Figure 2** Expression of laminin in well differentiated colorectal carcinoma. SP×200

**Figure 3** Expression of laminin in poorly differentiated colorectal carcinoma. SP×200

### Tumor invasion depth and laminin expression

With the increasing maximum depth of tumor invasion, discontinuous basement membrane laminin staining became more prominent in tumors (Table 3), but without statistical significance ( $\chi^2$ :  $P>0.05$ ).

**Table 3 Tumor invasion depth and laminin staining pattern**

Depth of invasion	n	Laminin staining pattern	
		++ to +++	- to +
Mucosa and submucosa	4	3(75.0%)	1(25.0%)
Muscularis	32	17(53.1%)	15(46.9%) <sup>a</sup>
Serosa and subserosa	27	11(40.7%)	16(59.3%) <sup>a</sup>

<sup>a</sup> $P>0.05$  vs tumor not extended beyond the submucosa.

### DISCUSSION

Human colorectal carcinomas are comprised, in part, of malignant cells of varying degrees of differentiation from well to poorly differentiated. At present, tumor histology is the primary criterion used to determine the degree of differentiation of a given tumor. It is becoming increasingly apparent, however that the morphological characteristics of tumors that relate to their degree of differentiation result from alterations in the biological properties of the cells<sup>[5]</sup>. Moreover, these biological properties probably contribute to the clinical behavior associated with tumors of a particular differentiation state. For example, poorly differentiated colorectal carcinomas are considered, in general, to be more aggressive and to offer a worse progress than well and moderately differentiated tumors<sup>[6]</sup>.

Laminin, the major basement membrane component, was determined by using immunohistochemical method. We demonstrated a defined, continuous basement membrane underlying normal colonic epithelium. In contrast, human colorectal carcinomas exhibited a progressive loss of an intact basement membrane that was correlated with decreasing differentiation degree. Well and moderately differentiated tumors showed a thin basement membrane with intermittent disruptions, and poorly differentiated tumors exhibited no areas of intact basement membrane. An association was found between lack of basement membrane laminin immunohistochemical staining in colorectal carcinoma and poorly differentiated ( $P<0.01$ ).

We also found an association between discontinuous basement membrane laminin

immunostaining in colorectal carcinomas and more advanced tumor stage; and with increasing depth of invasion, tumors more frequently show discontinuous basement membrane laminin staining. An important underlying factor may be the basement membrane laminin discontinuity in tumor is associated with poor tumor differentiation, which itself is linked with a more invasive phenotype.

There is an evidence for a number of mechanisms explaining basement membrane discontinuity in malignant tumors. Daneker *et al*<sup>[7]</sup> reported that in poorly differentiated colon carcinoma cells, newly synthesized laminin was secreted more slowly than in well differentiated cells. Basement membrane degradation by tumor-derived proteinases may also be important. Proteinase specific for the basement membrane component type-IV collagen has been identified in invasive tumors. Increased amount of the degradative enzymes cathepsin B, urokinase-type plasminogen activator and  $\beta$ -glucuronidase has been demonstrated in colorectal carcinomas<sup>[4,8-9]</sup>.

Up to now, the genetic and biochemical factors that under the tumor differentiation and other biological properties are poorly understood. Studies aimed at defining these factors would not only contribute to an understanding of differentiation at molecular and cellular level but also would provide new reagents and criteria for a more precise determination of the degree of tumor differentiation, thus facilitate the understanding of the biological behaviors of tumors. The results of this study confirmed that immunohistochemical staining of laminin could provide a very useful index for the determination of the differentiation degree of human colorectal carcinoma.

### REFERENCES

- 1 Grover A, Andrews G, Adamson ED. Role of laminin in epithelium formation by F9 aggregates. *J Cell Biol*, 1983;97(1):137-144
- 2 Terranova VP, Hujanen ES, Martin GR. Basement membrane and the invasive activity of metastatic tumor cells. *J Natl Cancer Inst*, 1986;77(2):311-316
- 3 Liotta LA. Tumor invasion and metastasis: role of the extracellular matrix: Rhoads memorial award lecture. *Cancer Res*, 1980;46(1):1-7
- 4 Liotta LA. Cancer cell invasion and metastasis. *Scientific Am*, 1992;266(2):34-41
- 5 Wewer UM, Tarabozetti G, Sobel ME, Albrechtsen R, Litta LA. Role of laminin receptor in tumor cell migration. *Cancer Res*, 1987;47(21):5691-5698
- 6 Klimplifinger M, Beham A, Denk H. Pathologic findings in colorectal cancers and discussion of their significance for tumor therapy according to stage. *Wien Klin Wochenschr*, 1987;99(2):488-493
- 7 Daneker Jr GW, Mercurio AM, Wolf LGB, Salem RR, Bagli DJ, Steele Jr GD. Laminin expression in colorectal carcinomas varying in degree of differentiation. *Arch Surg*, 1987;122(12):1470-1474
- 8 Boyd D, Florent G, Kim P, Brattain M. Determination of the levels of urokinase and its receptor in human colon carcinoma cell lines. *Cancer Res*, 1988;48(11):3112-3116
- 9 Feng Shu, Song Jindan. Study on the relationship between invasiveness and  $\beta$ -glucuronidase secreted by human colorectal carcinoma cell line. *Chin J Cell Biol*, 1996;18(4):138-140

# Peripheral mechanism of inhibitory effect of centrally administrated histamine on gastric acid secretion

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**Subject headings** gastric acid/secretion; histamine; stomach/physiology; somatostatin; acetylcholine M receptor; rats

## Abstract

**AIM** To study the peripheral mechanism of the inhibitory effect of intra-third ventricular administration (icv) of histamine (HA) on gastric acid secretion in rats.

**METHODS** Gastric acid was continuously washed with 37°C saline by a perfusion pump in male adrenalectomized SD rats. Drugs were injected intravenously (iv) by a syringe pump and their effect on pentagastrin-induced (10 µg · kg · h, iv) gastric acid secretion was observed.

**RESULTS** The inhibitory effect of HA (1 µg, icv) on gastric acid secretion was blocked by subdiaphragmatic vagotomy, and pretreatment with atropine (0.005 mg · kg · h, iv). Pretreatment with somatostatin antagonist, cyclo-[7-aminoheptanoyl-Phe-D-Trp-Lys-Thr(Bzl)], (2 µg · 4 µg · kg · 100 min, iv) could also block the inhibitory effect of HA on gastric acid secretion in a dose dependent manner.

**CONCLUSION** The inhibitory effect of centrally administrated HA on gastric acid secretion may be mediated by vagi, acetylcholine M receptor and somatostatin.

## INTRODUCTION

It has been reported by our laboratory that intra-third-ventricular administration (icv) of histamine (HA) or 2-pyridylethylamine (PEA), a H<sub>1</sub>-receptor agonist, inhibits gastric acid secretion induced by intravenous (iv) pentagastrin (G-5) in rats. The inhibitory effect of HA or PEA on gastric acid secretion was mediated in turn by corticotropin-releasing factor (CRF) and β-endorphin in central nervous system and abolished by subdiaphragmatic vagotomy (SV)<sup>[1-3]</sup>. The aim of the present study was designed to determine the peripheral mechanism of inhibitory effect of centrally administrated histamine on gastric acid secretion.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley (SD) rats weighing between 200g-300g were used. The animals were deprived of food for 24 hours, but were allowed free access to water prior to anesthesia.

### Animal models

The rats were anesthetized with a single intraperitoneal injection of pentobarbital (50mg/kg). Intra-third-ventricular implantation and acute gastric lumen perfusion were carried out as described previously by our laboratory<sup>[1]</sup>. Gastric perfusion samples were collected every 10 minutes and were titrated by 0.01mol/L NaOH to neuter. Total acid output per 10 minute was calculated. The anus temperature of rats was kept at 37°C by electric light during the experiment. Sufficient pentobarbital was given subcutaneously before G-5 was injected.

It has been reported by our laboratory<sup>[2]</sup> that adrenal gland was associated with stimulating effect of icv PEA on gastric acid secretion in SV rats. To remove this effect, the following experiments were done in adrenalectomized rats.

After gastric acid secretion was kept at base level (0.8-3.5 µmol/10min) for 20 minutes, G-5 was injected iv by a syringe pump to increase gastric acid secretion. After the gastric acid secretion was increased and kept stable for 30 minutes, other experimental drugs were given. HA was administered by a syringe pump in a silicon tube, which was equally long and connected with the

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\*Supported by the Doctoral Program Fund for Institutions of Higher Education. This subject was awarded the first prize of outstanding thesis by the Guangdong Association of Physiological Sciences.

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Received 1998-01-31

implanted cannula. The injected volume of HA solution or vehicle was 5  $\mu$ l. The acute SV was performed as described previously<sup>[4]</sup>.

G-5 and HA were purchased from Shanghai Dongfeng Biochemistry Reagent Factory, Chinese Academy of Sciences. Cyclo-[7-aminoheptanoyl-Phe-D-Trp-Lys-Thr (Bzl)] (c-PTLT), from Sigma Company, U.S.A. and atropine sulfate, from Guangzhou Qiaoguang Pharmaceutical Factory.

**Data analysis**

Experimental data was expressed in % change of total acid output, which was calculated as follows: % change of total acid output=(E2-E1)/E1 $\times$ 100%, in which E1 represents total acid output per 10 minutes before experimental drugs were given (it is the mean of total acid output of 30 minutes before experimental drugs were given); E2, the total acid output (TAO) per 10 minutes after experimental drugs were given; “+”, the TAO increase and “-”, TAO decrease. Significance was assessed by Student’s paired *t* test. The results were expressed in  $\bar{x}\pm s_x$ .

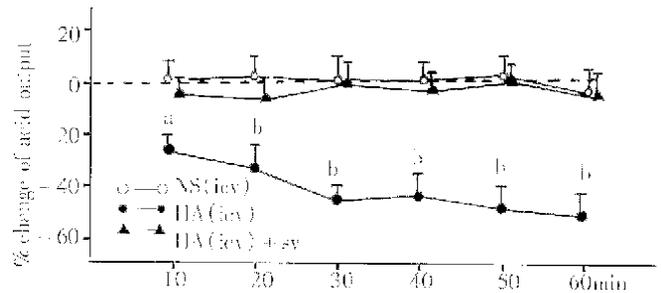
**RESULTS**

All the experiments were done on the basis of iv injection of G-5 in a dosage of 10  $\mu$ g  $\cdot$  kg  $\cdot$  h. HA, injected icv in the dosage of 1  $\mu$ g/5  $\mu$ l (*n*=9), decreased significantly the TAO (the maximum reached 44%). When HA group was compared with NS group (5  $\mu$ l, *n*=9), *P* values were less than 0.05, 0.01 or 0.001 at 10min, 20min, 30min, 40min, 50min and 60min (Figure 1). In SV rats (*n*=8), icv injection of HA in the same dosage had no significant effect on the TAO (Figure 1).

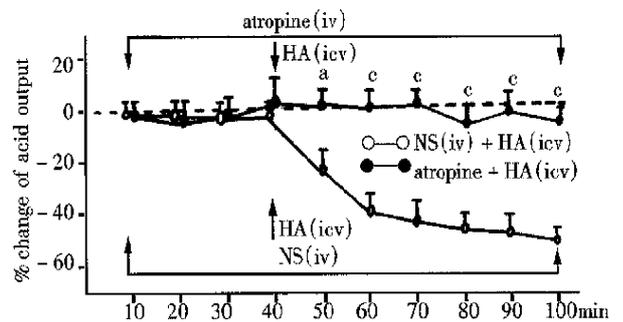
HA (icv 1  $\mu$ g/5  $\mu$ l, *n*=8) was injected 40 minutes after the atropine (iv, 0.05mg  $\cdot$  kg  $\cdot$  100min) was administered, the total acid output, had no significant change (Figure 2) compared with that before HA. If HA (icv, 1  $\mu$ g/5  $\mu$ l, *n*=9) was injected 40 minutes after iv NS was given, the total acid output, decreased significantly (the maximum to -44%) compared with that before HA. Compared with NS group, atropine group had significant changes at 50min, 60min, 70min, 80min, 90min and 100min (*P*<0.05, 0.01 or 0.001), (Figure 2).

There were no significant changes (*P*>0.05) in the total acid output at 10min, 20min, 30min, 40min, 50min, 60min, 70min, 80min, 90min, and 100min with pretreatment of c-PTLT (4  $\mu$ g  $\cdot$  kg  $\cdot$  100min, iv). These results suggest c-PTLT in the dosage of 4  $\mu$ g  $\cdot$  kg  $\cdot$  100min had no significant effect on gastric acid secretion induced by iv G-5. The following experiments were divided into three groups according to the difference of c-PTLT dosage: group 1, 4  $\mu$ g  $\cdot$  kg  $\cdot$  100min; group 2, 3  $\mu$ g  $\cdot$  kg

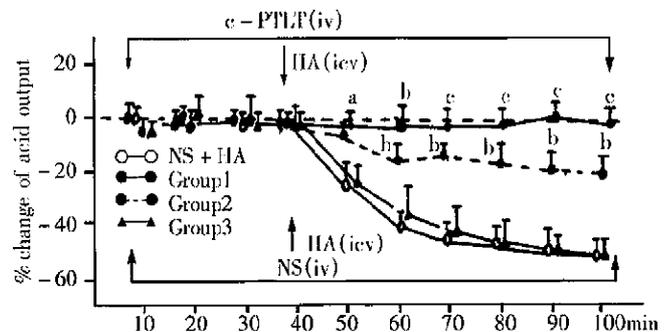
$\cdot$  100min; and group 3, 2  $\mu$ g  $\cdot$  kg  $\cdot$  100min. In group 1 (*n*=9), 40 minutes after c-PTLT was given HA (icv, 1  $\mu$ g/5  $\mu$ l), had no significant effect on the TAO (Figure 3), suggesting the inhibitory effect of HA (icv) on gastric acid secretion was blocked. In the control group (iv NS), HA (icv, 1  $\mu$ g/5  $\mu$ l), injected 40 minutes after NS was injected, could still inhibit gastric acid secretion. Compared between the two groups, *P* values were less than 0.01 or 0.001 at 60min, 70min, 80min, 90min and 100min.



**Figure 1** Effects of intra-third-ventricular injection of histamine (HA) on gastric acid output induced by G-5 in adrenalectomized and subdiaphragmatic vagotomized (SV) rats.



**Figure 2** Effects of iv administration of atropine sulfate on icv histamine-induced inhibition of gastric acid output in adrenalectomized rats.



**Figure 3** Effects of iv administration of c-PTLT on icv histamine-induced inhibition of gastric acid output in adrenalectomized rats. <sup>a</sup>*P*<0.05 compared with NS group; <sup>b</sup>*P*<0.01; <sup>c</sup>*P*<0.001.

In group 2 (n=9), the inhibitory effect of HA (icv, 1  $\mu\text{g}/5 \mu\text{l}$ ) on gastric acid secretion was partly blocked (Figure 3). Compared with the control group, *P* values were less than 0.05, 0.01 or 0.001 at 50min, 60min, 70min, 80min, 90min and 100min.

In group 3 (n=8), the inhibitory effect of HA (icv, 1  $\mu\text{g}/5 \mu\text{l}$ ) on gastric acid secretion was not changed. Compared with the control group, *P* values were more than 0.05.

## DISCUSSION

The present study demonstrated that HA has central inhibitory effect on gastric acid secretion in adrenalectomized rats. This result is consistent with the results reported by Wang, Sun and Li<sup>[1-3]</sup>. The central inhibitory effect of HA on gastric acid secretion could be blocked by pretreatment with either SV, or atropine and c-PTLT, an antagonist of somatostatin, suggesting that the central inhibitory effect of HA on gastric acid secretion may be mediated by vagi, acetylcholine M receptor and somatostatin.

It is D cell that synthesizes and secretes somatostatin in mammals. D cell is located near G cell in gastric antrum, and along gastric gland, particularly near the parietal cell in oxyntic mucosa. Somatostatin, mediated by its receptor in the membrane of parietal cells, inhibits gastric acid secretion induced by gastrin and acetylcholine. In addition, somatostatin inhibits the gastrin secretion in the basal condition or the gastrin secretion induced by feeding, acetylcholine and bombesin. In stomach, somatostatin inhibits the histamine secretion in the basal condition or induced by gastrin. The c-PTLT, as an artificial antagonist of somatostatin, completely blocked the inhibitory effects of exogenous somatostatin on growth hormone, insulin, and glucagon release. The efficiency of c-PTLT was demonstrated by the almost complete and sustained reversal of acid inhibition after exogenous infusion of somatostatin. Therefore, c-PTLT should have effectively reversed inhibition by endogenously released somatostatin throughout the period of the intraduodenal fat perfusion<sup>[5]</sup>. In the present study, c-PTLT, dose-dependently inhibited central inhibitory effect of HA on gastric acid secretion, which might be mediated by somatostatin in the periphery. The blocked central inhibitory effect of HA on gastric acid secretion by SV suggests that this effect was accomplished by the vagi. That vagi was involved in the control of somatostatin secretion was proved by some early studies. Vagotomy reduced the somatostatin responses to feeding during the first 30-min period following the ingestion of the meal. Atropine sulfate in the dosage of 0.02 mg  $\cdot$  kg  $\cdot$  h, iv decreased the somatostatin responses to the meal, while the dosage of 0.05mg  $\cdot$  kg  $\cdot$  h blocked such responses<sup>[6]</sup>. This suggests that vagus and

cholinergic mechanisms play important roles in the control of somatostatin secretion responses to the meal. After vagotomy, atropine sulfate still decreased the somatostatin secretion<sup>[6]</sup>, indicating that local factors are involved in the control of somatostatin secretion. CRF, injected icv, increased blood level of somatostatin. This effect was blocked by vagotomy or pretreatment with atropine<sup>[7]</sup>. Li *et al*<sup>[3]</sup> in our laboratory reported that the central inhibitory effect of PEA on gastric acid secretion was blocked by antiserum of CRF, suggesting that the effect of PEA is mediated by CRF in the central nervous system. According to this report and the present study, vagi or cholinergic mechanisms are involved in the control of somatostatin secretion. Holst<sup>[8]</sup> reported that vagus stimulation or atropine mediated by GRP (gastrin-releasing polypeptide)-containing fibers stimulated somatostatin secretion in the isolated perfused porcine antrum. Schubert<sup>[9]</sup> reported that methacholine exerted dual inhibitory and stimulatory effects on somatostatin cells of mucosal segments from the fundus and antrum of rat or the isolated lumenally perfused mouse stomach. There are some contradictory reports about somatostatin secretion of vagal control. For example, somatostatin secretion was decreased by vagus stimulation and this effect was abolished by atropine 10-9M<sup>[10]</sup>. As there are lots of fibers in vagi, the above contradictory reports may be related with too much fibers stimulation when vagi was stimulated electrically. In summary, the mechanism that somatostatin secretion is controlled by vagi still remains unclear and more studies are needed.

## REFERENCES

- 1 Wang ZL, Lu GQ. Effect of intra ventricular administration of histamine and its receptor agonists on pentagastrin-induced gastric acid secretion. *Acta Physiologica Sinica*. 1992;44(3):261-268
- 2 Sun CG, Wang ZL, Wang TZ, Lu GQ. Mechanism of effect of intra ventricular administration of histamine H-1-receptor agonists on gastric acid secretion in rats. *Acta Physiologica Sinica*. 1993;45(6):581-586
- 3 Li MY, Wang ZL, Lu GQ. Central mechanism of inhibitory effect of histamine H-1-receptor agonists PEA on gastric acid secretion. *Acta Physiologica Sinica*. 1995;47(3):259-263
- 4 Tache Y, Goto Y, Gunion MW, Rivier J, Debas H. Inhibition of gastric acid secretion in rats and in dogs by corticotropin-releasing factor. *Gastroenterol Jpn*. 1984;86:281-286
- 5 Fung L, Pokol-Daniel S, Greenberg GR. Cholecystokinin type A receptors mediate intestinal fat-induced inhibition of acid secretion through somatostatin-14 in dogs. *Endocrinology*. 1994;134(6):2376-2382
- 6 Woussen-Colle MC, Laliou C, Simoens C, De-Graef J. Effect of vagotomy and atropine on plasma somatostatin responses to a meal in conscious dogs. *Regul Pept*. 1988;21(1,2):29-36
- 7 Smedh U, Uvnas-moberg K. Intracerebroventricularly administered corticotropin-releasing factor releases somatostatin through a cholinergic, vagal pathway in freely fed rats. *Acta Physiol Scand*. 1994;151(2):241-248
- 8 Holst JJ, Skak-Nielsen T, Orskov C, Seier-Poulsen S. Vagal Control of the release of somatostatin, vasoactive intestinal polypeptide, gastrin-releasing peptide, and HCl from porcine non-antral stomach. *Scand J Gastroenterol*. 1992;27(8):677-685
- 9 Schubert ML, Hightower J. Functionally distinct muscarinic receptors on gastric somatostatin cells. *Am J Physiol*. 1990;258(Gastrointest. Liver Physiol.21):G982-987
- 10 Madaus S, Bender H, Schusdziarra V, Kehe K, Munzert G, Weber G *et al*. Vagally induced release of gastrin, somatostatin and bombesin-like immunoreactivity from perfused rat stomach. Effect of stimulation frequency and cholinergic mechanisms. *Regul Pept*. 1990;30(3):179-192

# A clinical evaluation of serum concentrations of intercellular adhesion molecule-1 in patients with gastric cancer

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**Subject headings** stomach neoplasms/immunology; intercellular adhesion molecule-1/blood; lymphatic metastasis; intercellular adhesion molecule-1/analysis; enzyme-linked immunosorbent assay

## Abstract

**AIM** To investigate the correlation between the serum soluble intercellular adhesion molecule-1 (sICAM-1) and the clinicopathologic features and to evaluate the possible prognostic significance of sICAM-1 concentration in gastric cancer.

**METHODS** Thirty-four patients with gastric cancer were prospectively included and evaluated. Venous blood samples were collected before the surgery. Sera were obtained by centrifugation, and store at  $-30^{\circ}\text{C}$  until assay. The control group consisted of 20 healthy volunteers. Serum concentrations of ICAM-1 were measured with the quantitative sandwich enzyme immunoassay technic. Differences between the two groups were analyzed by Student's *t* test.  $\bar{x}+2s$  of normal control sICAM-1 was taken as upper limit to calculate the positive rates.

**RESULTS** The mean value of serum ICAM-1 in patients with gastric cancer was  $367.7 \mu\text{g/L} \pm 104.7 \mu\text{g/L}$  and that of control group was  $236.9 \mu\text{g/L} \pm 74.3 \mu\text{g/L}$ , and the difference was significant ( $P < 0.001$ ). The patients with tumor size of  $\geq 5\text{cm}$  had significantly higher serum concentrations of sICAM-1 than those with smaller ones ( $406.7 \mu\text{g/L} \pm 90.2 \mu\text{g/L}$  vs  $319.9 \mu\text{g/L} \pm 105.3 \mu\text{g/L}$ ,  $P < 0.01$ ). Compared with stages I-II gastric cancer patients, patients with more advanced clinical stage (III-IV) had higher levels of sICAM-1 ( $397.1 \mu\text{g/L} \pm 102.4 \mu\text{g/L}$  vs  $306.0 \mu\text{g/L} \pm 82.3 \mu\text{g/L}$ ,  $P < 0.05$ ). Difference was significant

statistically in sICAM-1 levels between patients with positive lymph node status and those without lymph node involvement ( $403.6 \mu\text{g/L} \pm 99.7 \mu\text{g/L}$  vs  $302.7 \mu\text{g/L} \pm 81.4 \mu\text{g/L}$ ,  $P < 0.01$ ). No relation was observed between the level of sICAM-1 and grade of histological differentiation in the patients with gastric cancer.

**CONCLUSION** Serum sICAM-1 concentration may be a valuable parameter for predicting the prognosis and degree of the gastric cancer.

## INTRODUCTION

Cell adhesion is essential for establishing and maintaining a normal immune defense system. Intercellular adhesion molecule-1 (ICAM-1; CD54), a 80-110KD cell surface glycoprotein and a member of the immunoglobulin supergene family, functions as a ligand for the leucocyte integrin adhesion receptors CD11a/CD18 (LFA-1) and CD11b/CD18 (mac-1). ICAM-1 expresses in various cell types and plays an important role in immunemediated mechanisms, especially in the process of antigen presentation and recognition, lymphocyte cytotoxicity, lymphocyte recruitment and targeting. It has been reported that the upregulated expression of ICAM-1 on cell surfaces occurred in a variety of diseases, including autoimmune diseases, endocrine diseases, and some cancers<sup>[1,2]</sup>. A soluble form of ICAM-1 (sICAM-1) lacking cytoplasmic tail and transmembrane region has also been found<sup>[2]</sup>. sICAM-1 can compete with membranous ICAM-1 to bind LFA-1, so that it can block leukocyte LFA-1 and prevent effective recognition and lysis of target cells by effector leukocyte. This phenomenon represents an important mechanism for tumor escaped from immune surveillance<sup>[3,4]</sup>. The sICAM-1 levels and its prognostic significance for various malignant diseases have been evaluated<sup>[5-8]</sup>. In this study, we determined the concentrations of sICAM-1 antigen in the sera of patients with gastric cancer to reveal the possible relationship between sICAM-1 and clinical pathological parameters such as histologic type, size of tumors and so on.

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Received 1997-11-10 Revised 1998-01-04

## MATERIAL AND METHODS

### Patients and specimens

A group of 34 patients (24 men and 10 women) with gastric cancer were investigated. Their ages ranged from 35 to 74 years with a mean of 54. Eleven patients were in stages I and II, and 23 in stages III and IV. Blood samples were obtained from patients before the initial treatment. Samples collected from 20 healthy volunteers served as controls. All blood samples were processed immediately for centrifugation, and the sera were stored at  $-30^{\circ}\text{C}$  until the sICAM-1 assay.

### Measurement of sICAM-1

Serum sICAM-1 concentrations were determined with sICAM-1 ELISA (Biosource International, USA). Briefly, serum samples were diluted at 1:100 and applied to prepared polystyrene microcells precoated with mouse monoclonal antibody to human ICAM-1, a horseradish peroxidase conjugated anti-ICAM-1 was then added. Test wells are incubated to allow any ICAM-1 to be bound by antibodies on the microtiter plate. The walls were then washed and stabilized chromogen was added to the walls producing a blue color in the presence of peroxidase enzyme. The color reaction was then stopped by the addition of 1mol/L  $\text{H}_2\text{SO}_4$  and changed from blue to yellow. Absorbances for samples and sICAM-1 standards were determined on a spectrophotometer using 450nm. Average absorbance values for each set of duplicate samples or standards were within 15% of the mean. sICAM-1 concentrations in serum sample were determined by comparing the mean absorbance of duplicate samples with the standard curve for each assay.

### Statistical analysis

The Student's *t* test was used for statistical significance of differences between groups.  $P < 0.05$  was considered to be significant.

## RESULT

The mean serum sICAM-1 levels in healthy volunteers and patients with gastric cancer were  $236.9 \mu\text{g/L} \pm 74.3 \mu\text{g/L}$ ,  $367.7 \mu\text{g/L} \pm 104.7 \mu\text{g/L}$ , respectively; and serum sICAM-1 levels in 58.8% of patients exceeded  $385.5 \mu\text{g/L}$  ( $\bar{x} + 2s$  of normal controls), the difference being significant between patients with gastric cancer and healthy controls.

The relationship between serum sICAM-1 levels and clinicopathological features of gastric cancer patients was also observed. Patients with tumor size  $\geq 5\text{cm}$  showed remarkable elevated levels of sICAM-1 than those with tumor size  $< 5\text{cm}$  ( $P <$

$0.01$ ). Patients with advanced gastric cancer (stage III-IV) had higher serum levels of sICAM-1 ( $P < 0.05$ ) than early-stage gastric cancer patients (stage I-II). sICAM-1 levels in patients with lymph node metastasis were significantly higher than in those without lymph node invasion ( $P < 0.01$ ). No correlation was found between sICAM-1 levels and grade of differentiation in the observed gastric cancer patients. The positive rates of each group were calculated with  $\bar{x} + 2s$  of normal control sICAM-1 as a limit. The results are shown in Table 1.

**Table 1 Correlation between soluble ICAM-1 concentrations and clinical histopathology in gastric cancer**

Factors	<i>n</i>	ICAM-1 ( $\bar{x} \pm s$ , $\mu\text{g/L}$ )	Positive rate %( <i>n</i> )
Tumor size			
$\geq 5\text{cm}$	19	$406.7 \pm 90.2^a$	73.3 (14/19)
$< 5\text{cm}$	15	$319.9 \pm 105.3$	40.0 (6/15)
Lymph node invasion			
Positive	21	$403.6 \pm 99.7^a$	71.4 (15/21)
Negative	13	$302.7 \pm 81.4$	38.5 (5/13)
Grade of differentiation			
Differentiated	12	$320.5 \pm 93.2$	33.3 (4/12)
Poorly differentiated	22	$393.9 \pm 103.1$	72.7 (16/22)
Clinical staging			
I-II	11	$306.0 \pm 82.3$	36.4 (4/11)
III-IV	23	$397.1 \pm 102.4^b$	69.6 (16/23)

<sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$ , vs the control.

## DISCUSSION

A previous studies indicated that ICAM-1 was expressed on tumor cells in 12 of 28 cases of gastric carcinoma<sup>[9]</sup>. Expression of ICAM-1 by tumor cells facilitated immune recognition and cytotoxicity<sup>[10,11]</sup>. However, ICAM-1 also represents an escape mechanism in certain cancers. sICAM-1, after shedding from tumor cells or originating from other cells especially mononuclear cells, may block the attachment of cytotoxic T lymphocyte (CTL) cells and/or NK cells to tumor cells, since LFA-1 on immune cells could be blocked with sICAM-1<sup>[3]</sup>. Recently, Becker *et al* found that cytolysis of melanoma cells mediated by CTL in an MHC-restricted manner, could be completely blocked in the presence of sICAM-1, the concentration of sICAM-1 for blocking cytotoxicity was  $950 \mu\text{g/L}$ <sup>[4]</sup>. Because the serum sICAM-1 concentration may reflect the level of sICAM-1 in tumor microenvironment in vivo, an increase in the circulating level of ICAM-1 may reflect unfavourable prognosis. It has been found that patients with malignant melanoma, having high levels of sICAM-1, had a statistically significantly shorter time to first relapse and lower overall survival<sup>[7]</sup>. In this study, we have found that serum

sICAM-1 levels were significantly increased in patients with gastric cancer, which were above the normal range in 20 of 34 gastric cancer patients. It is entirely possible that concentration of sICAM-1 in gastric tumor microenvironment may be high enough to block the cytotoxicity mediated by immune effector cells. To further confirm this hypothesis, we will establish a method to assess the sICAM-1 level in gastric carcinoma tissues.

In this study, we observed the correlation between the level of sICAM-1 and the clinical parameters in patients with gastric carcinoma. Patients with tumor size of  $\geq 5$ cm had higher level of sICAM-1 than those with small ones. This results indicated that tumor load may be correlated with the level of sICAM-1. In other words, the gastric tumor cells were probably the major source of sICAM-1. This was confirmed by the study of Hyodo *et al.* They found that the elevated serum levels of sICAM-1 in a patient with hepatocellular carcinoma decreased after treatment with transcatheter arterial embolization (TAE) in a same pattern of change with the AFP levels. As AFP is produced and shed by hepatocellular carcinoma cells, it could be interpreted that sICAM-1 in HCC patients is released from tumor cells<sup>[8]</sup>. Our study also showed that sICAM-1 levels were correlated with both clinical staging and lymph node involvement. Patients with lymph node invasion and advanced clinical stage of tumors had significantly higher serum concentrations of sICAM-1. Lymph node status, tumor size and clinical stage are significant prognostic indicators, therefore,

sICAM-1 may be a valuable predictor for gastric cancer clinically.

In summary, elevated levels of sICAM-1 exist in gastric cancer as in some other types of tumors. Though serum level of sICAM-1 can not be served as a specific parameter for gastric cancer, it is no doubt that the measurement of the ICAM-1 level may provide a convenient means to obtain a general indication of gastric cancer.

## REFERENCES

- 1 Springer TA. Adhesion receptors of the immune system. *Nature*, 1990;346(6283):425-434
- 2 Rothlein R, Mainolfi EA, Czajkowski M, Marlin SD. A form of circulating ICAM-1 in human serum. *J Immunol*, 1991; 147(11):3788-3793
- 3 Becker JC, Dummer R, Hartmann AA, Burg G, Schmidt RE. Shedding of ICAM-1 from human melanoma cell lines induced by IFN- $\gamma$  and tumor necrosis factor- $\alpha$ : functional consequences on cell-mediated cytotoxicity. *J Immunol*, 1991;147(12):4398-4401
- 4 Becker JC, Christian T, Schmidt RE, Brocker EB. Soluble intercellular adhesion molecule-1 inhibits MHC-restricted specific T cell/tumor interaction. *J Immunol*, 1993;151(12):7224-7232
- 5 Nasu K, Narahara H, Etoh Y, Kawano Y, Hirota Y, Miyakawa. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1) and the expression of ICAM-1 mRNA in uterine cervical cancer. *Gynecol Oncol*, 1997;65(2):304-308
- 6 Tsujisaki M, Imai K, Hirata H, Hanzawa Y, Masuya J, Nakano T *et al.* Detection of circulating intercellular adhesion molecule-1 antigen in malignant diseases. *Clin Exp Immunol*, 1991;85(1):3-8
- 7 Harning R, Mainolfi E, Bystryjn JC, Henn M, Merluzzi VJ, Rothlein R. Serum levels of circulating intercellular adhesion molecule 1 in human malignant melanoma. *Cancer Res*, 1991;51(18):5003-5005
- 8 Hyodo I, Jinno K, Tanimizu M, Hosokawa Y, Nishikawa Y, Akiyama M *et al.* Detection of circulating intercellular adhesion molecule-1 in hepatocellular carcinoma. *Int J Cancer*, 1993;55(5):775-779<sup>9a</sup>
- 9 Nasu R, Mizuno M, Kiso T. Immunohistochemical analysis of intercellular adhesion molecule 1 expression in human gastric adenoma and adenocarcinoma. *Virchows Arch*, 1997;430(4):279-283
- 10 Anichini A, Mortarini R, Alberti S, Mantovani A, Parmiani G. Enhanced lysis of melanoma clones by autologous tumor infiltrating lymphocyte clones after tumor ICAM-1 up regulation: correlation with T cell receptor engagement. *Int J Cancer*, 1993;53(6):994-999
- 11 Liu YZ, Zhang L, Yu YS, Wan Y, Guo M. Cytokine regulations on expression of intercellular adhesion molecule-1 (ICAM-1) in human hepatoma cell line and ins susceptibility to lysis by anti-CD3 monoclonal antibody-activated (CD3AK) cells. *Shanghai J Immunol*, 1997;17(2):74-77

# Expression of bcl-2 protein in gastric carcinoma and its significance \*

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**Subject headings** stomach neoplasms/pathology; bcl-2 protein; gene expression; immunohistochemical; lymphatic metastasis

## Abstract

**AIM** To further study the role of bcl-2 protein expression in gastric carcinogenesis and tumor progression.

**METHODS** Using immunohistochemical staining, the bcl-2 protein expression in 50 cases of gastric carcinoma and its relation to clinical status and pathomorphological parameters were observed.

**RESULTS** Forty-one (82%) cases were positive for bcl-2 protein staining which was located in the cytoplasm and nuclear membrane of tumor cells. The rate of bcl-2 protein expression was not correlated with the patient, sex, tumor size, lymph node status or clinical stages ( $P>0.05$ ). It was strongly associated with intestinal-type tumors and poorly differentiated tumors ( $P<0.05$  and  $P<0.01$ ).

**CONCLUSION** Aberrant bcl-2 protein expression appears to be specifically associated with development of intestinal-type gastric carcinoma, bcl-2 protein expression might play an important role in the early development/promotion and phenotypic differentiation of gastric carcinomas, but not in tumor progression.

## INTRODUCTION

Recently, emphasis has been placed on the role of apoptosis and its regulation in tissue homeostasis and carcinogenesis. To determine whether bcl-2 plays a role in the gastric carcinogenic sequence, an immunohistochemical study of bcl-2 expression in gastric carcinoma and its relation to clinical status, pathomorphological parameters was carried out.

## MATERIALS AND METHODS

### *Histological specimens*

Fifty cases of surgically resected gastric carcinomas (male 34, female 16; mean age 56.3 years) were extracted from the files of the Department of Pathology, Southwest Hospital, Third Military Medical University. All blocks were fixed in 10% formalin and embedded in paraffin. Serial sections were cut from each block in 4  $\mu$ m, stained with hematoxylin and eosin and confirmed pathologically.

### *Immunohistochemical methods*

Immunohistochemical staining for bcl-2 protein was performed using SP technique with the following procedure: ① slides were diparaffinized in two changes of xylene for 10 minutes each and then were hydrated in decreasing concentrations of ethanol and rinsed in phosphate-buffered saline. Endogenous peroxidase was blocked by 3% H<sub>2</sub>O<sub>2</sub> in methanol for 5 minutes, and then incubated for 10 minutes at room temperature in normal goat serum (1:20). ② slides were incubated with a 1:50 dilution of the primary rabbit antihuman bcl-2 monoclonal antibody (Santa Cruz, USA) for 30 minutes at 37°C. A biotin-streptavidin detection system was employed with diaminobenzidine as the chromogen. ③ slides were washed twice with phosphate-buffered saline and incubated with the linking reagent (biotinylated anti-immunoglobulin) for 10 minutes at 37°C. After rinsing in phosphate-buffered saline, the slides were incubated with the peroxidase-conjugated streptavidin label for 10 minutes at 37°C, and incubated with diaminobenzidine and H<sub>2</sub>O<sub>2</sub> for 10 minutes in the dark, the sections were then counterstained with hematoxylin. With each batch of test samples, a positive control consisting of a tissue section from tonsil was evaluated. In addition, a negative control was prepared for each

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\*Key project of the 9th 5-year plan for Medicine and Health of Army, No.96Z047.

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Received 1998-01-20

sample using an irrelevant antibody of the same isotype as the primary antibody.

The immunostaining of bcl-2 was visually classified into four groups by observing 1 000 tumor cells in areas of the sections: no staining present in any of tumor cells (-); slight staining in most of the tumor cells or less than 25% tumor cells with strong staining (+); 26%-50% tumor cells with strong staining (++); and strong staining in more than 51% tumor cells (+++). The classification was done by two senior pathologists who did not know the clinicopathological data.

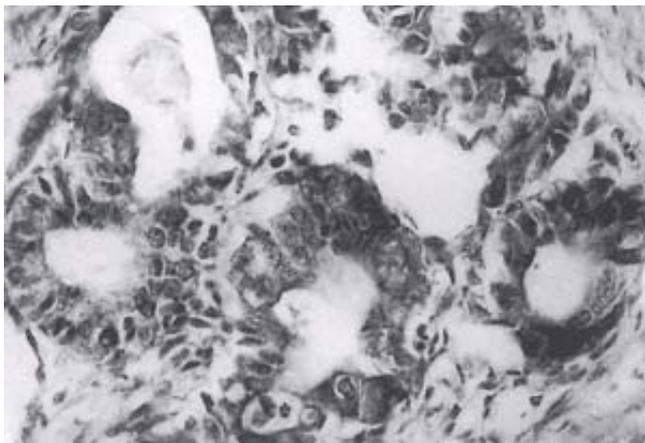
### Statistics

Analysis of data was accomplished using Chi square test. *P* values less than 0.05 were considered to be statistically significant.

## RESULTS

### Expression of bcl-2 protein in gastric carcinoma

Forty-one (82%) of the fifty gastric carcinomas showed immunoreactivity for bcl protein in gastric carcinoma cells. Expression of varied: + in bcl-2 protein, 6(12%), ++ in 20(40%) and +++ in 15 (30%). The bcl-2 protein immunoreactivity appeared brown or dark brown, which was located on the cytoplasm and nuclear membrane of tumor cells (Figure 1). Some of the mature lymphocytes infiltrating in the stroma of gastric carcinomas had bcl-2 protein expression with a strong staining intensity.



**Figure 1** Immunoreactivity of bcl-2 was detected in the cytoplasm of gastric carcinoma cells. SP  $\times 200$

### Correlation between bcl-2 protein expression and clinicopathological parameters of gastric carcinomas

Correlations between bcl-2 protein expression and

clinical pathological data of gastric carcinoma are illustrated in Table 1. The rate of bcl-2 protein expression was not correlated with patient age, sex, tumor size, lymph node status and clinical stages ( $P > 0.05$ ). The immunoreactivity of bcl-2 was significantly associated with morphologic phenotype and grades of differentiation of gastric carcinoma. Twenty-six (92.86%) of 28 gastric carcinomas of intestinal morphologic phenotype were immunoreactive versus 15 (68.16%) of 22 diffuse tumors ( $P < 0.05$ ). Twenty-one (95.45%) of 22 poorly differentiated gastric carcinomas were immunoreactive versus 11 (61.11%) of 18 well and moderately differentiated carcinomas ( $P < 0.01$ ).

**Table 1** Correlation between bcl-2 protein expression and clinicopathological parameters of gastric carcinomas

	<i>n</i>	bcl-2 protein expression				Positive rate (%)
		-	+	++	+++	
<b>Age (a)</b>						
$\leq 59$	36	7	4	15	10	80.56
$\geq 60$	14	2	2	5	5	85.71
<b>Sex</b>						
M	34	7	2	13	12	79.41
F	16	2	4	7	3	87.50
<b>Type</b>						
Intestinal	28	2	3	13	10	92.86 <sup>a</sup>
Diffuse	22	7	3	7	5	68.18
<b>Grade of differentiation</b>						
Well/moderate	18	7	1	5	5	61.11
Poor	22	1	4	12	5	95.45 <sup>b</sup>
Mucoid	10	1	1	3	5	90.00
<b>Tumor size</b>						
$< 5$ cm	31	8	4	11	8	74.19
$\geq 5$ cm	19	1	2	9	7	94.74
<b>Lymph-node metastasis</b>						
Negative	21	4	2	9	6	80.95
Positive	29	5	4	11	9	82.76
<b>Serosal invasion</b>						
Absent	25	7	3	9	6	72.00
Present	25	2	3	11	9	92.00
<b>Clinical stages</b>						
I and II	32	6	5	12	9	81.25
III and IV	18	3	1	8	6	83.33

<sup>a</sup> $\chi^2=5.082$ ,  $P < 0.05$ , vs diffuse-type gastric carcinoma; <sup>b</sup> $\chi^2=7.298$ ,  $P < 0.01$ , vs well/moderately differentiated gastric carcinoma.

## DISCUSSION

The bcl-2 proto-oncogene is initially identified in human follicular and diffuse B-cell lymphomas characterized by the reciprocal translocation<sup>[1]</sup>. The product of the proto-oncogene bcl-2 is a 26-kDa protein that blocks apoptosis. bcl-2 protein is localized in the mitochondria, endoplasmic reticulum and nuclear envelope membranes. More recently, several normal and malignant tissues other than hematolymphoid cells have been shown to express bcl-2, including the lung, breast, prostate, stomach, small bowel and colon<sup>[2-5]</sup>.

The relation of bcl-2 protein expression to clinicopathological parameters of tumors was still unclear. Joensuu *et al*<sup>[6]</sup> demonstrated that bcl-2 expression was correlated with the age of patients, which was more common in aged than in young patients. Silviastriini *et al*<sup>[7]</sup> found that bcl-2 protein expression was related to the tumor size. A significantly higher fraction of bcl-2 positive cells was observed in small tumors than in large tumors. Sierra *et al*<sup>[8]</sup> found that bcl-2 protein was more frequently expressed in tumors with early metastasis than in lymph-node negative tumors. Our study showed that bcl-2 protein expression was associated with morphologic phenotype and grades of differentiation of gastric carcinomas. The difference in the bcl-2 protein expression in the intestinal and diffuse types demonstrated that aberrant bcl-2 protein expression was preferentially associated with development of intestinal-type gastric carcinoma, indicating again the different biologic mechanisms involved in the development of these two histologic subtypes. The difference in the bcl-2 protein expression between poorly differentiated and well/moderately-differentiated gastric carcinomas demonstrated that aberrant bcl-2

protein expression was associated with differentiation or growth speed of gastric carcinomas. There was no significant relationship between bcl-2 protein expression and tumor size, lymph-node metastasis, serosal invasion or clinical stages. Therefore, bcl-2 protein expression might play an important role in the early development and phenotypic differentiation of gastric carcinomas, but not so in tumor progression.

## REFERENCES

- 1 Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*, 1992;80(4):879-886
- 2 Higashiyama M, Doi O, Kodama K, Yokouchi H, Tateishi R. High prevalence of bcl-2 oncoprotein expression in small cell lung cancer. *Anticancer Res*, 1995;15(2):503-505
- 3 Lauwers GY, Scott GV, Karpeh MS. Immunohistochemical evaluation of bcl-2 protein expression in gastric adenocarcinomas. *Cancer*, 1995;75(9):2209-2213
- 4 Alderson LM, Castleberg RL, Harsh IV GR, Louis DN, Henson JW. Human gliomas with wild-type p53 express bcl-2. *Cancer Res*, 1995;55(5):999-1001
- 5 Qi-Long LU, Richard Poulson, Leslie Wong, Couliamos AD. Bcl-2 expression in adult and embryonic non-haematopoietic tissues. *J Pathol*, 1993;169(4):431-438
- 6 Joensuu H, Pylkkanen L, Toikkanen S. bcl-2 protein expression and long-term survival in breast cancer. *Am J Pathol*, 1994;145(5):1191-1198
- 7 Silviastriini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M *et al*. The bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph-node negative breast cancer patients. *J Natl Cancer Inst*, 1994;86(7):499-504
- 8 Sierra A, Lloveras B, Castellsague X, Moreno L, Ramirez MG, Fabra A. Bcl-2 expression is associated with lymph node metastasis in human ductal breast carcinoma. *Int J Cancer*, 1995;60(1):54-60

# Telomeric associations of chromosomes in patients with esophageal squamous cell carcinomas

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**Subject headings** esophageal neoplasms; carcinomas, squamous cell; chromosome; lymphocytes; telomeric DNA

## Abstract

**AIM** To investigate the role of telomeric association in the development of esophageal cancer.

**METHODS** Using chromosome R banding technique, telomeric association of chromosome in peripheral blood lymphocytes from 16 untreated patients with esophageal squamous cell carcinoma were observed and 16 healthy adults served as controls.

**RESULTS** The telomeric association frequencies of cell and chromosomes were significantly higher than those of controls ( $\chi^2=9.56$ ,  $P<0.01$ ), but its distribution on the chromosome showed no significant difference ( $\chi^2=1.01$ ,  $P>0.05$ ) between the two groups.

**CONCLUSION** Chromosomal instability can be initiated by telomeric associations, and sequential chromosome analysis can aid the understanding of the tumor occurrence and progression.

## INTRODUCTION

Telomeres are genetic elements located at the end of all eukaryotic chromosomes and are essential for normal cell viability. One of their basic functions is to protect themselves. DNA at the terminal end of all chromosomes has a special structure to avoid binding to the end of DNA from other chromosomes, thus preventing end-to-end fusion or telomeric association (TAs). Telomeric DNA consists of terminal repeat arrays in the 3' strand (TTAGGG)<sub>n</sub> and has been isolated from telomeres in human. Recent studies have suggested that abnormal telomeric behavior plays a key role in cancer development. Fitzgerald<sup>[1]</sup> reported a case of B-cell leukemia and found chromosomal translocation resulting from TAs. The phenomenon has also been observed in tumor cells from a malignant histiocytoma<sup>[2]</sup> and a case of pre-T-cell acute lymphoblastic leukemia<sup>[3]</sup>, three cases of cardiac myxoma<sup>[4]</sup>, and two cases of renal tumor<sup>[5]</sup>. In the present report, we observed the telomeres in the peripheral blood lymphocytes from 16 untreated patients with esophageal cancer and 16 controls. So as to explore the relationship between abnormal telomeric behavior and chromosomal instability.

## MATERIALS AND METHODS

### Samples

Sixteen patients (12 men, 4 women) with esophageal squamous cell carcinoma admitted to the Institute of Oncology, Yanling County, Sichuan Province, aged 42 to 60 years were selected before the start of any kind of therapy. Sixteen healthy adults (12 men and 4 women) with no history of cancer; genetic diseases aged 35-65 years and radiation exposure served as controls.

### Methods

Whole blood lymphocytes from all individuals were cultured in 199 medium (pH 7.5) containing 5% fetal calf serum, phytohemagglutinin and antibiotics. The cells were grown in the dark at 37°C for 96 hours. At 94 hours, colcemid was added to a final concentration of 0.1ng/L. After hypotonic treatment with KCl and fixation in methanol: acetic (3:1), the slides were prepared by air drying. R banding of chromosomes were obtained according to the technique of GAO Chung-

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Received 1997-11-25

Sheng<sup>[7]</sup> with slight modification. For each individual, 50 metaphases were studied under a same microscope to record the frequency of TAs and the distribution on chromosomes.

## RESULTS

Data on the frequency of TAs per cell in esophageal cancer patients and normal control are shown in Table 1.

**Table 1 Cellular telomeric association frequencies in patients with esophageal cancer and controls**

	Cells observed	Telomeric associations	
		No. of cells	Frequencies(%)
Patients	800	115	14.38
Controls	800	75	9.38

The patients had a TAs frequency of 14.38%, which was significantly higher than that observed in normal controls (9.38%) ( $\chi^2=9.56, P<0.01$ ).

Comparison of distribution of TAs on chromosomes between the patients and controls is shown in Table 2. The frequency of TAs per chromosome was increased as compared with the controls ( $P<0.01$ ). Statistical analysis showed that the distribution of TAs in two groups was nonrandom. In the patients, there was a high frequency in groups E and B, and a low frequency in groups D and G+Y ( $P<0.01$ ).

Proportion analysis of telomeric associations indicated that TAs distribution on chromosome was not different between the two groups ( $P>0.05$ ).

**Table 2 Distribution of TAs on each chromosome in peripheral blood lymphocyte from patients and controls**

Chromosome groups	Chromosomes observed	Patients			Control		
		No. of TAs	Frequencies (%)	Percent (%)	No. of TAs	Frequencies (%)	Percent (%)
A	4 800	42	0.88	16.2	23	0.48	15.3
B	3 200	50	1.56	19.3	30	0.94	20.0
C+X	12 200	72	0.59	27.8	57	0.47	38.0
D	4 800	16	0.33	6.2	6	0.13	4.0
E	4 800	51	1.06	19.7	18	0.38	12.0
F	3 200	14	0.44	5.4	9	0.28	16.0
G+Y	3 800	14	0.37	5.4	7	0.18	4.7
Total	36 800	259	0.70	100	150	0.41	100

## DISCUSSION

Recently, the study on the relationship between abnormal telomeric behavior and mechanism of canceration has become a 'hot spot' in the field of molecular genetics. TAs of human chromosome is a rare phenomenon, which has been observed mostly in metaphase cell of a pathologic nature. Since the 1990s, it has been found that telomeric lengths are

evidently shortened in human colorectal carcinoma, Wilms' tumor; giant cell tumor of bone, breast cancer, lung cancer, etc. while loss of telomeric sequences would lead to chromosomal instability. Because telomere integrity is critical for the normal replication of chromosomes in mitosis, telomeric reduction may lead to chromosomal dysfunction and manifest cytogenetically as TAs. Sawyer<sup>[8]</sup> reported TAs evolving to ring chromosomes in a Pleomorphic xanthoastrocytoma. TAs between chromosomes 15pter and 20qter, and between chromosome 1q and 22qter, evolved in a stepwise fashion to ring chromosome 20 and 22. Thus, TAs is one of the mechanisms that can initiate chromosomal instability by generating subclones with unstable chromosome intermediates and result in ring chromosomes and subsequent chromosome loss. Adamson<sup>[9]</sup> postulated that absent telomere sequence causes chromosome loss and instability, and that it may cause bridge break-fusion cycles, leading to partial chromosome deletion/duplication. In the early tumorigenesis, the telomere repeat sequence (TTAGGG)<sub>n</sub> is often shortened in tumor cells, which may trigger the activation of telomerase to elongate telomere sequence. The shortened telomeric sequence was often shown to be TAs. TAs not only causes nondisjunction but also trigger further structural changes, which may contribute to the complexity of karyotypes of solid tumors and could be one of mechanisms of oncogene activation and/or tumor-suppressor gene disruption.

We had reported genetic instability in patients with esophageal cancer. However, it is necessary to prove whether there is a relationship between chromosomal instability and TAs. In our experiment cellular TAs rate was 14.38% in patients and 9.3% in controls, compared with the report in literature<sup>[10]</sup>, the frequency of TAs was decreased slightly, which may be the difference of susceptibility of tumor and chromosome banding techniques.

To our knowledge, this is the first report on TAs in esophageal cancer. It is not known whether the biologic changes resulting in TAs have any causal role in the carcinogenic process, or are of any importance in tumor cell progression. Molecular studies should increase our understanding of mechanisms involved in telomeric rearrangements and the relationship between chromosome change and pathogenesis and disease course in esophageal cancer.

## REFERENCES

- 1 Fitzgerald PH, Morris CM. Telomeric association in B-cell lymphoid leukemia, *Hum Genet*, 1984;67(4):385-390
- 2 Mandahl N, Heim S, Kristofferson U, Mitelman F, Rooser B, Rydholm A et

- al. Telomeric association in a malignant fibrous histiocytoma. *Hum Genet*, 1985;71(4):321-324
- 3 Morgan R, Jarzhabek V, Jaffe J. Telomeric fusion in pre-T-cell acute lymphoblastic leukemia. *Hum Genet*, 1986;73(3):260-263
- 4 Dewlap GW, Dahl RJ, Spurbeck JL, Carney JA, Cordon H. Chromosomally abnormal clones and nonrandom telomeric translocation in cardiac myxomas. *Mayo Clin Proc*, 1987;62(7):558-567
- 5 Kovacs G, Muller-Brechlin R, Szucs S. Telomeric association in two human renal tumors. *Cancer Genet Cytogenet*, 1987;28(2):363-366
- 6 Gao CS, Qiu HC, Cheng ZY. A simple, stable method for chromosome R banding. *Heredity and Disease*, 1991;8(2):98
- 7 Herbert S, Schwartz, George A, Schwartz HS, Dahir GA, Butler MG. Telomere reduction in giant cell tumor of bone and with aging. *Cancer Genet Cytogenet*, 1993;71(2):132-138
- 8 Sawyer JR, Thomas EL, Roloson GJ, Chadduck WM, Boop FA. Telomeric association evolving to ring chromosomes in a recurrent pleomorphic xanthoastrocytoma. *Cancer Genet Cytogenet*, 1992;60(2):152-157
- 9 Adamson DJA, King DJ, Haites NE. Significant telomere shortening in childhood leukemia. *Cancer Genet Cytogenet*, 191(2):204-206
- 10 C. Fuserer, R Miro, L Barrions, J. Egozcue. Telomere association of chromosomes induced by aphidicolin in a normal individual. *Hum Genet*, 1990;84(5):424-426

# Detection of bacterial DNA from cholesterol gallstones by nested primers polymerase chain reaction

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**Subject headings** cholelithiasis/microbiology; propionibacterium acnes; staphylococcus aureus; DNA, bacterial; polymerase chain reaction

## Abstract

**AIM** To search for bacterial DNA sequences in cholesterol gallstones with negative bacterial culture. **METHODS** DNA was extracted from cholesterol gallstones in gallbladders and nested primers polymerase chain reaction (NP-PCR) was used to amplify bacterial gene fragments for identifying the existence of bacteria. The samples of bacterial DNA extracted from potentially causative or unrelated living bacteria were amplified in vitro as the standard markers and comparative 16S ribosomal RNA sequence analysis was made for bacterial identification.

**RESULTS** The gallbladder gallstones of 30 patients were analyzed and bacterial DNA was found in 26 patients. Among them, gallstones with cholesterol content between 30%-69% were seen in 5 (5/5) patients, 70%-90% in 11(11/14) patients, and more than 90% in 10(10/11) patients. There was no difference either in cholesterol and water content of gallstones or in harboring bacterial DNA of gallstones. *E. coli*-

related DNA fragments appeared in the stones of 8 (26.67%) patients; propionibacteria type DNA in 7 (23.33%); and harbored bacterial gene fragments in 2 patients, similar to *Streptococcus pyogenes*. A more heterogenous sequence collection was found in 7 (23.33%) patients, which could belong to multiple bacterial infections. Two (6.67%) patients had bacterial DNA with low molecular weight which might be related to some unidentified bacteria.

**CONCLUSION** Most cholesterol gallstones harbor bacterial DNA. It is important to determine whether these microorganisms are innocent bystanders or active participants in cholesterol gallstone formation.

## INTRODUCTION

Although several factors are known to trigger nucleation and/or growth of cholesterol crystals, the pathogenesis of cholesterol gallstones so far is still unclear. Most studies suggested that cholesterol gallstones may originate mainly from the disturbances of cholesterol metabolism, especially involving the balance between biliary pronucleating and antinucleating proteins<sup>[1]</sup>. Unfortunately, the role of bacterial etiology was usually neglected. We searched for bacterial DNA sequence in cholesterol gallbladder gallstones with negative bacterial culture by highly sensitive and specific nested primers polymerase chain reaction (NP-PCR) as the molecular genetic evidence of bacterial colonization of cholesterol gallstones.

## MATERIALS AND METHODS

### Subjects

The gallbladder gallstones were consecutively collected from October to December 1995. Patients with positive aerobic or anaerobic bile cultures were excluded. The group for analysis consisted of 5 men and 25 women with a mean age of 54.5 years, ranging from 27-73 years. All of them had symptomatic gallstone disease and underwent selective surgery. Fresh gallbladder bile samples were obtained by needle puncture aspiration during surgery. All stones had diameters of more than 5mm and stored at -20°C.

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\*Supported by the State Education Commission Research Foundation for Scientists Returning from Abroad (1997)436.

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Received 1998-03-01

### Methods

DNA extraction. DNA (3.1 µg-26.2 µg) was obtained from stone samples by the special purification methods using an UV-240 spectrophotometer (Shimadzu Corporation, Japan). Stone samples of about 300mg were crushed in a 5ml Eppendorf tube with a glass rod and incubated with 600 µl 1% sodium dodecyl sulfate rotating overnight at 20°C. Lithium chloride solution (7mol/L) was added to a final concentration of 1.5mol/L for precipitating the interfering substances in gallstones. Bacterial DNA was extracted from potentially causative or unrelated living bacteria including *E. coli*, *Propionibacterium acnes*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Serratia odorifera*. All of them were ATCC strains. The DNA was then extracted by the conventional purification methods, resuspended in 50 µl TE buffer (Tris/EDTA), and stored at -4°C.

**NP-PCR amplification** According to the literature<sup>[2]</sup>, primers sequences were designed for amplifying bacterial 16S ribosomal RNA gene in vitro, including a generic 5' primer set p1: 5'-AGAGTTTGAT (c/t)(c/a) TGGCTCAG-3', P2: 5'-ACTAC (c/t) (a/c/g) GGGTATCTAA(g/t) CC-3' and a new nested 3' primer P3: 5'-ACCGC(g/t) (a/g)CTGCTGGCAC (Institute of Cellular Biology, Chinese academy of Sciences, Shanghai).

The PCR was done in 0.5ml polypropylene microcentrifuge tube with a PTC-100TM Programmable Thermal Controller (MJ Research, INC., USA). The total incubation volume was 25 µl, containing 100mmol/L "tris"-hydrochloric acid, pH 8.3, 500mmol/L potassium chloride, 15mmol/L magnesium chloride, 100 µmol/L of each dNTP, 0.2 µg-1.0 µg template DNA and 1 unit of Taq DNA polymerase (Sino-American Biotec Engineering, China). The reaction mixture was covered with 1 drop of mineral oil. In the first round of amplification, 0.2pmols each of the outer primers (P1, P2), was used then the reaction tubes were supplemented with 0.2pmols each of the inner primers (P1, P3), and 1 µl amplified DNA and 1 unit of Taq DNA polymerase were used in the second round. The PCR cycle took place at 94°C for 30s, 56°C for 1min, and 72°C for 1min 40s. During the last cycle, the 72°C step was extended to 8min. Thirty-five cycles were carried out for each reaction.

**NP-PCR product comparative analysis** The sequence of 16S ribosomal RNA gene fragments amplified by NP-PCR was approximately 500-base pairs. Lambda DNA/EcoRI+Hind III (Promega Corporation, USA) was used as molecular standard. Five µg

reamplified DNA was applied to a 1.4% agarose gel containing 0.8g/L ethidium bromide and after electrophoresis 4.0V/cm, 40min was visualised with an ultraviolet light source. The reamplified DNA of ATCC bacteria was included in every run. The agarose gels from the NP-PCR procedure were then subjected to computer image system for further bands comparative analysis. If the NP-PCR of the first gallstone (chosen at random) was positive, the rest of the stones of a given patient were usually not analyzed. Otherwise, all gallstones or at least five stones would be analyzed.

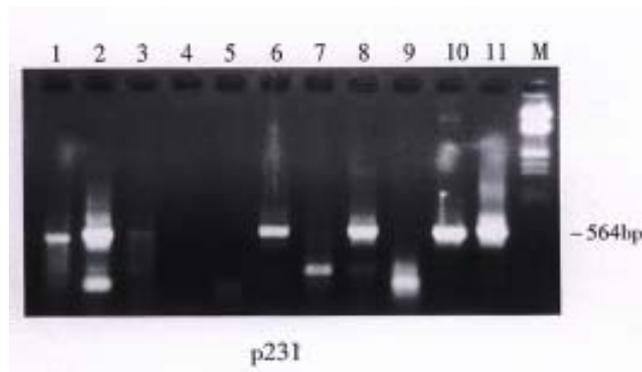
**Cholesterol and water content analysis** DNA isolated from a part of a same gallstone was used for NP-PCR amplification and another part for cholesterol and water content analysis. Accurate 100mg of native gallstone material was dried at 105°C for 3 hours to a constant weight. The cholesterol was extracted by a modified chemical method and underwent photometric test using a 722 Spectrophotometer (Shanghai Third Analysis Instrument Factory). The cholesterol and water content was analyzed according to the manufacturer's instructions.

**Statistical analysis** All values were expressed as  $\bar{x} \pm s$ . The relationship between cholesterol or water content and NP-PCR results were analyzed with Wilcoxon rank sum test.

### RESULTS

The gallstones of 30 patients were analyzed. Bacterial 16S ribosomal RNA gene fragments were amplified in the stones of 26 patients. Amplification was not possible in the remaining 4 patients whose stones had no detectable bacterial DNA (Figure 1). In the 26 patients, the positive gallstones contained cholesterol content between 30%-69% in 5 (5/5) patients, 70%-90% in 11 (11/14) patients, and more than 90% in 10 (10/11) patients. The water content of gallstones changed from 10.3% to 52%. The group with bacterial DNA in the gallstones had water content of  $26.5\% \pm 10.39\%$  while the group without bacterial DNA had water content of  $19.18\% \pm 4.88\%$ . There was no difference either in cholesterol and water content or in harboring bacterial DNA of gallstones ( $P > 0.05$ ) (Tables 1, 2). *E. coli* related DNA fragments were found in the stones of 8 (26.67%) patients. *Propionibacterium*-type DNA was obtained in 7 (23.33%) patients. Stones of 2 (6.67%) patients harbored bacterial gene fragments similar to *Streptococcus pyogenes*. Simultaneously, multiple heterogenous sequence collections were found in 7 (23.33%) patients, which could belong to the mixed infections including *E. coli*,

*Propionibacterium acnes* and *Streptococcus pyogenes* or some other unidentified bacteria such as *Clostridium difficile*. The stones of the other 2 (6.67%) patients had bacterial DNA with lower molecular weight and could be related to some unidentified bacteria.



**Figure 1** Agarose gel electrophoresis comparative analysis of NP-PCR products of bacterial DNA fragments from gallstones. M: Molecular standard, Lambda DNA/EcoR I+ Hind III. Lanes 1, 2, 3, 6, 8 and 10: NP-PCR positive products similar to the amplified products of 11 *P.acnes* 16S rRNA gene; 11: amplified product of *P.acnes* 16S rRNA gene; 7, 9: NP-PCR positive products, molecular weight less than 300bp; 4: negative control; 5: NP-PCR negative product.

**Table 1** The relationship between cholesterol content of gallstones and the rate of gallstones harbor bacterial DNA

Cholesterol content (%)	Harbor bacterial DNA		No harbor bacterial DNA		Total	
	Case	%	Case	%	Case	%
30-69	5	16.67	0	0	5	16.67
70-90	11	36.66	3	10.0	14	46.67
>90	10	33.33	1	33.33	11	36.66

**Table 2** The relationship between water content of gallstones and gallstones harbor bacterial DNA

Group	Case	Water content (%)
Harbor bacterial DNA	26	26.57±10.39
No harbor bacterial DNA	4	19.18±4.88

## DISCUSSION

Few previous studies considered the role of bacteria in the pathogenesis of cholesterol gallstone as there was no direct evidence of bacterial existence, because of the failure in the bacteria culture from cholesterol stones. However, the process in gallbladder gallstone formation may take a longer time, and the embedded bacteria may be destroyed or killed. PCR technique has a high sensitivity and specificity as compared with the bacterial routine culture. Theoretically, PCR can detect a copy gene of a bacterium or cell. The nested primer approach increases the specificity as well as sensitivity of

PCR<sup>[3]</sup>. To prevent artifacts resulting from contamination, gallbladder stones were collected and prepared with stern sterilization, control experiments omitting template DNA were made in parallel, and pre and post-PCR procedures were performed in different rooms. Furthermore, primers were designed according to bacterial genes coding for 16S ribosomal RNA. The structure of these ribosomal RNA genes is highly specific for different bacteria.

Bacteria can invade the biliary tract by ascending from the duodenum and via the hematogenous route from the hepatic portal venous blood<sup>[4]</sup>. Many aerobe, facultative aerobe and anaerobe species such as *E.coli*, *P.vulgaris*, *B.fragilis*, *Clostridium perfringens*, can produce  $\beta$ -glucuronidase that catalyzes the hydrolysis of bilirubin conjugates in bile, leading to increased amounts of unconjugated bilirubin, which precipitates as calcium bilirubinate stones. Bacteria could also disturb gallbladder wall secretion and cause acute or chronic inflammation with resultant precipitation of bile. Bacteria have adhesive properties and can produce glycocalyx or other matrices within which the pigment or cholesterol coalesces. In fact, most of cholesterol gallstones have a pigment nucleus or section. It seems likely that bacterial infection serves as an initiating factor or plays other important roles in the development of gallstones. Besides most bacteria that cause biliary infection can produce palmitate via a phospholipase, and result in precipitation of calcium palmitate. Meanwhile, the reduction of lecithin in bile leads to a lower solubility of bilirubin and promotes banding of bilirubin and calcium. Bacterial phospholipase, on the other hand, degrades lecithin which will reduce the solubility of cholesterol and promote cholesterol nucleation in bile.

*Propionibacterium acnes* is a gram-positive anaerobic rod bacterium which is a normal bacterial colony usually located on the skin and mucosa. The role of *Propionibacterium acnes* in gallstone formation has not been clarified. Up to the present, the possible pathogenic mechanisms are<sup>[5]</sup>: ① This bacterium produces a nonspecific extracellular lipase that catalyses the total hydrolysis of triacylglycerols to free fatty acids and glycerol and that is able to use lecithin as a substrate. ② *Propionibacterium acnes*- also produces a phospholipase C, which is a promoter of cholesterol precipitation<sup>[6]</sup>. ③ *Propionibacterium* can bind to lipids<sup>[7]</sup>, and the binding is increased as lipid hydrophobicity increases. This ability may permit *Propionibacterium acnes* to bind biliary lipid vesicles and degrade them with the extracellular lipolytic enzymes. The nonspecific lipase is a chemotactic

agent for neutrophils, and activation of neutrophils would result in the release of oxygen radicals capable of causing phospholipid peroxidation, which reduces the stability of mixed micellar systems containing cholesterol, lecithin, and cholate. ④ *Propionibacterium acnes* has specific affinity for the calcium containing regions of gallstones and can promote calcium salt precipitation. Once the calcium bilirubinate salt and calcium palmitate salt were formed, the bacterial colony would secrete sufficient lipolytic enzymes to promote cholesterol nucleation.

We found that most cholesterol gallstones harbor bacterial DNA with the highly sensitive, specific and reproducible NP-PCR technique. Bacteria and their metabolites might play an important role in the formation of cholesterol gallstones with different mechanisms. Obviously, the study searching for molecular genetic evidence of bacterial colonization of cholesterol gallstones is

only at the initial stage. A lot of work remains to be done before it can be decided whether these microorganisms have a cause and effect relationship in cholesterol gallstone formation and an infectious etiology can be established. We are now determining heterologous sequence collections with the dideoxy chain termination technique.

## REFERENCES

- 1 Holzbach RT, Busch N. Nucleation and growth of cholesterol crystals. Kinetic determinants in supersaturated native bile. *Gastroenterol Clin North Am*, 1991;20(1):67-84
- 2 Swidsinski A, Ludwig N, Pahlig H *et al.* Molecular genetic evidence of bacterial colonizations of cholesterol gallstones. *Gastroenterology*, 1995;108(3):860-864
- 3 Aurelius E, Johansson B, Skoldenberg B *et al.* Rapid diagnosis of herpes simplex encephalitis by nested polymerase chain reaction assay of cerebrospinal fluid. *Lancet*, 1991;337(8735):189-192
- 4 Wu XT, Xiao LJ. The progress of studies on the role of bacteria in the formation of gallstone. *Foreign Medicine (Surgery)*, 1996;23(5):277-279
- 5 Soloway RD, Growther RS. Bacteria and cholesterol gallstones: molecular biology comes to gallstones pathogenesis. *Gastroenterology*, 1995;108(3):934-936
- 6 Pattinson NR, Willis KE. Effect of phospholipase C on cholesterol solubilization in model bile. *Gastroenterology*, 1991;101(5):1339-1344
- 7 Gibbon EM, Cuniffe WJ, Holland KT. Interaction of propionibacterium acnes with skin lipid in vitro. *J Gen Microbiol*, 1993;139(8):1745-1751

# Effects of octreotide on gallbladder pressure and myoelectric activity of Oddi sphincter in rabbits

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**Subject headings** octreotide/pharmacology; gallbladder/drug effects; oddi sphincter/drug effects; electrophysiology; rabbits; somatostatin; electric stimulation

## Abstract

**AIM** To observe the effect of octreotide (OT) and somatostatin (SS) on gallbladder pressure and myoelectric activity of SO in rabbits.

**METHODS** Male rabbits fasted for 15h-18h and anesthetized with urethane. The mean gallbladder pressure (GP) and myoelectric activity of SO were simultaneously measured with a frog bladder connected to a transducer and a pair of copper electrodes.

**RESULTS** After injection of OT (10  $\mu$ g/kg, iv), the GP decreased in 2min and reached the lowest value in about 60min ( $P < 0.01$ ,  $n = 19$ ), and completely or partially returned to the normal level in 120min. The frequency of myoelectric activity of SO was reduced, even disappeared in 2min ( $P < 0.01$ ,  $n = 19$ ) and returned to normal in about 20min. Injection of SS (10  $\mu$ g/kg, iv) also decreased GP and myoelectric activity of SO ( $P < 0.01$ ,  $n = 7$ ); Before and after injection of OT or SS, injection of CCK-8 (100ng or 200ng) caused similar increase in myoelectric activity of SO and GP ( $P > 0.05$ ). Before and after injection of OT, there were no significant differences in increases of myoelectric activity of SO and GP caused by electric stimulation of dorsal motor nucleus of vagus ( $P > 0.05$ ).

**CONCLUSION** OT and SS decreased GP and myoelectric activity of SO, demonstrating that effects of OT were similar to those of SS. Intravenous injection of OT did not affect the increase of myoelectric activity of SO and GP caused by CCK-8 or electric stimulation of dorsal motor nucleus of vagus.

## INTRODUCTION

Octreotide (OT) is an 8 amino acids synthetic analog of somatostatin (SS). Treatment with OT in acromegaly is effective but leads to gallstone formation. OT injections inhibit gallbladder contraction in acromegalic patients<sup>[1,2]</sup> and normal subjects<sup>[3]</sup>, stimulating human sphincter of Oddi (SO) activity<sup>[4]</sup>, which may impair bile evacuation. However, studies of SS, especially effect of OT on motor function of biliary system in animals are scarce and controversial. The aim of this study was to observe the effects of OT and SS on gallbladder pressure (GP) and myoelectric activity of SO in rabbits.

## MATERIALS AND METHODS

### Preparation of animals

Thirty-eight male rabbits, weighing 2kg-2.5kg were used. They were fasted for 15h-18h with free intake of water and then anesthetized with urethane (1.0g/kg, iv). A cannula was inserted into trachea and the unilateral femoral artery was cathetered for the blood pressure measurement.

### Measurement of GP and myoelectric activity of SO

A frog bladder perfused with saline was placed into the gallbladder and connected to a transducer (TP-200T). A pair of copper electrodes was inserted into subsera of SO. Blood pressure, GP and myoelectric signals of SO were simultaneously measured by a polygrapher (RM-6000, NIHON KHODEN).

### Administration of drugs

OT, cholecystokinin octapeptide (CCK-8) and SS were injected through ear vein. SS and CCK-8 were products of Sigma Chemical Company (St. Louis, U.S.A.) and Peninsula Laboratories (Belmont, U.S.A.) respectively. OT was produced by the Sandoz Pharm Ltd (Besel, Switzerland).

### Electric stimulation of dorsal motor nucleus of vagus (DMV)

The animal's head was fixed in a stereotaxic instrument (I-C model, Jiang Wan, China) A wire electrode was inserted into DMV for stimulation (0.2mA, 10Hz, 0.5ms duration, 1min) according to Messen's method. An indifferent electrode was placed on the skin tissue of skull.

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Received 1998-01-12

**Statistical analysis**

The constant mean GP of each animal was taken as control level (i.e., basic GP served as 0kPa in place of the real value) and the frequency of myoelectric activity of SO was considered as normal value. The percentage of frequency changes=(effect value-normal value) ÷ normal value×100%. All values were expressed as  $\bar{x} \pm s_{\bar{x}}$ . Experimental data were treated statistically by Student's *t* test.

**RESULTS**

**Effect of intravenous injection of OT on GP**

Intravenous injection of OT caused dose-dependent decrease of GP. After injection of OT (10 µg/kg, iv), the GP decreased in 2min (-0.142kPa±0.029kPa, *P*<0.01) and reached the lowest value in 60min (-0.257kPa±0.065kPa, *P*<0.01), and completely or partially returned to the normal level in 120min. Small dose of OT (5 µg/kg, iv), also decreased GP (*P*<0.01) but the effect was slight. No change in GP was found after injection of 1ml of 0.9% saline (*P*>0.05). The differences were significant (*P*<0.01) (Figure 1, Table 1) between the two groups of OT.

**Effect of intravenous injection of OT on myoelectric activity of SO**

After injection of OT (10 µg/kg, iv), the frequency

of myoelectric activity of SO was reduced, even disappeared in 2min (-83.1%±8.0%, *P*<0.01) and returned to normal in about 20min. At the dose of 5 µg/kg of OT, frequency of myoelectric activity of SO also decreased (-54%±6.1%, *P*<0.01). In contrast to OT 10 µg/kg group, the effect is weak (*P*<0.05). Injection of the same volume of 0.9% saline influenced neither GP nor myoelectric activity of SO (Figures 1, 2, Table 2).

**Effect of iv injection of SS on GP and myoelectric activity of SO**

After injection of SS (10 µg/kg, iv) in 7 rabbits, GP and myoelectric activity of SO were decreased. These changes were similar to those in OT (*P*>0.05) (Figure 3, Tables 1, 2).

**Effect of iv OT and SS on changes in GP and myoelectric activity of SO caused by CCK-8**

Ten minutes before and 15 minutes after injection of OT (10 µg/kg, iv), and CCK-8 (100ng) there was a marked increase in GP and myoelectric activity of SO. Before and after injection of SS (10 µg/kg), and CCK-8 (200ng) greatly increased GP and myoelectric activity of SO. These responses to CCK-8 showed no significant differences between pre- and post-injection of OT or SS (*P*>0.05), (Figure 4, Table 3).

**Table 1 Effect of OT and SS on gallbladder pressure**

Groups	n	Changes in gallbladder pressure (basic pressure=0kPa)							
		2	10	20	40	60	80	100	120min
NS	5	-0.024 ±0.020	-0.000 ±0.024	-0.013 ±0.024	-0.013 ±0.032	-0.010 ±0.024	-0.010 ±0.024	-0.012 ±0.024	-0.020 ±0.040
OT (5 µg/kg)	6	-0.074 <sup>a</sup> ±0.012	-0.097 <sup>b</sup> ±0.023	-0.068 ±0.024	-0.135 ±0.050	-0.101 ±0.062	-0.120 ±0.052	-0.068 ±0.038	-0.052 ±0.031
OT2 (10 µg/kg)	19	-0.142 <sup>bc</sup> ±0.020	-0.196 <sup>bd</sup> ±0.052	-0.184 <sup>a</sup> ±0.034	-0.154 <sup>a</sup> ±0.052	-0.257 <sup>a</sup> ±0.065	-0.145 ±0.048	-0.104 ±0.036	-0.040 ±0.042
SS(10 µg/kg)	7	-0.143 <sup>b</sup> ±0.034	-0.122 <sup>a</sup> ±0.037	-0.071 ±0.021	-0.021 ±0.036	0.029 ±0.028			

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs NS group; <sup>c</sup>*P*<0.05, <sup>d</sup>*P*<0.01, vs group 1 OT.

**Table 2 Effect of OT and SS on frequency of myoelectric activity of SO**

Groups	n	Changes in frequency of myoelectric activity of SO (%)							
		2	10	20	40	60	80	100	120min
NS	5	-8.0 ±8.2	-4.0 ±6.5	-2.0 ±5.0	0.0 ±11.0	4.0 ±6.0	-3.0 ±6.0	-1.0 ±8.2	-2.0 ±5.0
OT1 (5 µg/kg)	6	-51.4 <sup>b</sup> ±6.1	-42.3 <sup>a</sup> ±11.9	-18.5 ±9.0	-5.0 ±10.0	-4.0 ±6.0	5.0 ±6.5	-2.0 ±4.5	4.0 ±5.3
OT2 (10 µg/kg)	19	-83.1 <sup>bd</sup> ±8.0	-65.0 <sup>a</sup> ±11.3	-21.5 ±9.3	5.0 ±6.2	3.5 ±9.1	-4.0 ±7.0	-4.0 ±5.0	-3.5 ±8.5
SS (10 µg/kg)	7	-86.7 <sup>b</sup> ±11.0	-78.5 <sup>a</sup> ±14.0	-48.9 ±14.0	-10.0 ±18.8	7.0 ±9.0			

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs NS group; <sup>c</sup>*P*<0.05, <sup>d</sup>*P*<0.01, vs OT 1 group.

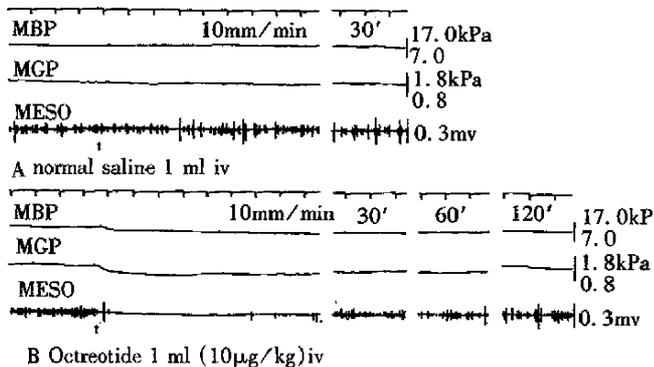
**Table 3 Comparison of effects of iv CCK-8 and electric stimulation of DMV on gallbladder pressure and myoelectric activity of SO after**

Group	n	Changes of frequency of myoelectric activity of SO (%)		Changes in gallbladder pressure (basic pressure = 0kPa)	
		Before	After	Before	After
DMV OT	10	283.8±69.6	285.8±75.8	0.128±0.046	0.123±0.031
CCK-8 (100ng) OT	12	346.8±79.2	275.8±37.7	0.363±0.113	0.321±0.112
CCK-8(200ng) SS	4	485.8±78.9	426.4±59.0	1.795±0.468	1.955±0.340

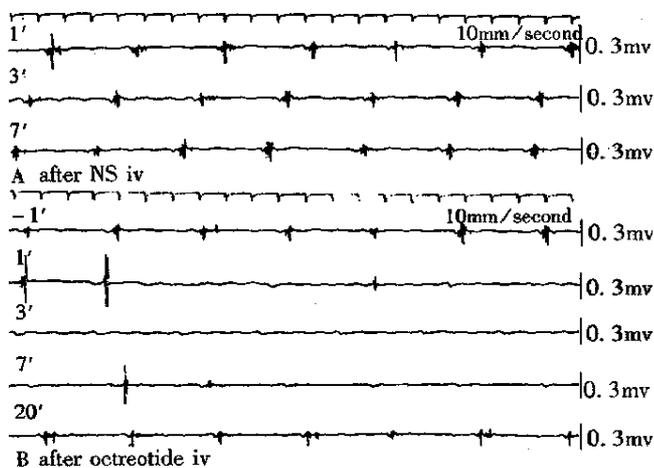
Before, before injection of TO or SS; After, after injection of OT or SS.

**Effects of iv OT on changes of GP and myoelectric activity of SO caused by electric stimulation of DMV**

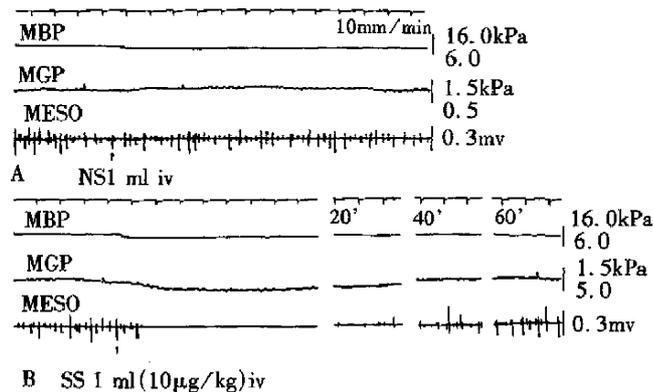
Ten minutes before and after injection of OT (10 µg/kg, iv), electric stimulation of DMV increased GP, frequency and amplitude of myoelectric activity of SO, the effects being similar ( $P>0.05$ ), (Table 3).



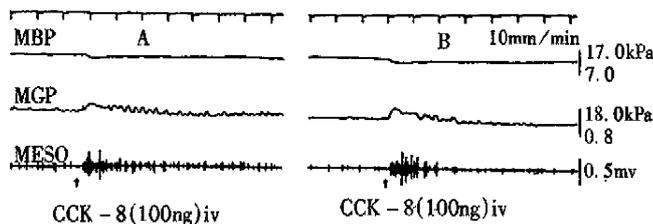
**Figure 1** Effect of iv octreotide on gallbladder pressure and myoelectric activity of SO. A, injection of normal saline; B, injection of OT; MBP, mean blood pressure; MGP, mean gallbladder pressure; MESO, myoelectric activity of SO. ↑, intravenous injection mark.



**Figure 2** Effect of octreotide on myoelectric activity of SO. A, injection of normal saline; B, injection of OT (10 µg/kg, iv)



**Figure 3** Effect of iv SS on gallbladder pressure and myoelectric activity of SO. A, injection of normal saline; B, injection of SS; MBP, mean blood pressure; MGP, mean gallbladder pressure; MESO, myoelectric activity of SO; ↑, iv injection mark.



**Figure 4** Effect of iv CCK-8 on gallbladder pressure and myoelectric activity of SO after injection of octreotide. A, before injection of OT; B, After injection of OT.

**DISCUSSION**

Several studies of SS effect on biliary motility have been reported but the results are different. SS has no effect on contraction of gallbladder smooth muscle strips in guinea pigs, dogs<sup>[5]</sup> and rabbits<sup>[6]</sup>, but inhibits gallbladder motility in human<sup>[7]</sup> and

dogs<sup>[5]</sup> in vivo. SS has been reported to either stimulate or inhibit the SO of dogs<sup>[8,9]</sup>. In the present study, SS decreased both GP and myoelectric activity of SO. The latter response was similar to the inhibitory SS effect on SO in rabbits reported before<sup>[10]</sup>. OT, a long-acting analog of SS, has been found to inhibit human gallbladder motility and to stimulate SO contraction which may impair bile evacuation with a risk of gallstone formation<sup>[1,2,4]</sup>. The animal experiment of OT effect was only conducted on prairie dogs in which OT decreased the motility index of SO, but did not affect the gallbladder pressure<sup>[11]</sup>. In the present study, OT decreased GP and myoelectric activity of SO in rabbits. These results were consistent with those of SS, demonstrating that inhibitory effect of OT is similar to that of SS. The diverse effects of SS and OT on motility of SO and gallbladder may be explained by the species difference.

The motility of gallbladder and SO is regulated by both autonomic nervous system and intestinal hormones. CCK is an important hormone for mediating motility of biliary tract. In the experiments on dogs, SS inhibits contraction of gallbladder strips induced by electric stimulation and decreased GP by CCK-8 in vivo probably through suppressing Ach release by the intrinsic cholinergic neurons, but does not affect contraction of gallbladder strips initiated by Ach or CCK<sup>[5]</sup>. Intravenous injection of SS inhibits contraction of human gallbladder in response to CCK<sup>[7]</sup>. In our study, injection of CCK-8 markedly raised GP and increased myoelectric activity of SO in rabbits. After injection of OT or SS, and CCK-8 resulted in increases of GP and myoelectric activity of SO, indicating that SS and OT do not affect the stimulatory effect of CCK-8 on gallbladder and SO. It is reported that electric stimulation of DMV raises GP and increases myoelectric activity of SO through a cholinergic mechanism of vagal nerve. In this study, before and after injection of OT, electric

stimulation of DMV caused similar increases in GP and myoelectric activity of SO. These results imply that OT did not inhibit the increase in motility of gallbladder and SO caused by DMV stimulation. The mechanisms of OT effect on motor function of biliary tract require further investigations.

In conclusion, OT and SS decrease GP and myoelectric activity of SO, demonstrating that the inhibitory effect of OT is similar to that of SS in rabbits. Before and after injection of OT and SS, and CCK-8 injection or electric stimulation of DMV similar increases are caused in GP and myoelectric activity of SO, suggesting that OT and SS do not affect the increases in motility of gallbladder and SO caused by CCK-8 and electric stimulation of DMV.

## REFERENCES

- 1 Zhu XF, Shi YF, Zhang JX, Dai Q, Harris AG. Prospective study of long-term therapeutic and wide effects on gallbladder function in Chinese acromegalic patients. *Chin J Int Med*, 1991;30(7):405-408
- 2 Stolk MFJ, Van Erpecun KJ, Loppochaar HPF, de Bruin WI, Jansen JBMI, Lamers CBHW *et al.* Postprandial gallbladder motility and hormone release during intermittent and continuous subcutaneous octreotide treatment in acromegaly. *Gut*, 1993;34(6):808-813
- 3 Grimaldi C, Darcourt J, Harris AG, Lebot E, Lapalus F, Delmont, J. Cholescintigraphic study of effect of somatostatin analog, octreotide, on bile secretion and gallbladder emptying in normal subject. *Dig Dis Sci*, 1993;38(9):1718-1721
- 4 Binmoller KF, Dumas R, Harris AG, Dilmont JP. Effect of somatostatin analog octreotide on human sphincter of Oddi. *Dig Dis Sci*, 1992;37(5):775-777
- 5 Milenov K, Rakovska A, Kalfin R, Mantovani P. Effect of somatostatin of canine gallbladder motility. *Neuropeptide*, 1990;17(1):75-80
- 6 Johnson AG, Marshall CE, Wilson IAI. Effect of some drugs and peptide hormones on the responsiveness of rabbits isolated gallbladder to cholecystokinin. *J Physiol*, 1982;332(2):415-425
- 7 Gullo L, Bolondi L, Scarpignato C, Priori P, Casanova P, Labo G. Effect of somatostatin and thyrotropin releasing hormone on cholecystokinin-induced gallbladder emptying. *Dig Dis Sci*, 1986;31(12):1345-1350
- 8 Sievert CE, Potter TJ, Levine AS, Morley JE, Silvis SE, Vennes JA. Effect of bombesin and gastrin-releasing peptide on canine sphincter of Oddi. *Am J Physiol*, 1988;254(3):361-365
- 9 Lin TM, Tust RH. Action of somatostatin on choledochal sphincter, gallbladder and bile flow in rabbits. *Federation Proc*, 1997;36(3):557-558
- 10 Adami GF, Leandri R, Sarles JC. Effect of somatostatin on rabbit Oddi's sphincter in vivo. interrelation of somatostatin and cholecystokinin. *Gastroenterol Clin Biol*, 1986;10(2):108-112
- 11 Ahrendt SA, Ahrendt GH, Lillemore KD, Pitt HA. Effect of octreotide on sphincter of Oddi and gallbladder motility in prairie dogs. *Am J Physiol*, 1992;262(25):G909-G914

# Relationship between enteric microecologic dysbiosis and bacterial translocation in acute necrotizing pancreatitis \*

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**Subject headings** pancreatitis; bacterial translocation; intestines; lipopolysaccharide/blood; amylase/blood; bifidobacterium; lactobacillus

## Abstract

**AIM** To investigate the potential role of intestinal microflora barrier in the pathogenesis of pancreatic infection.

**METHODS** Fifteen dogs were colonized with a strain of *E.coli* JM109 bearing ampicillin resistance plasmid PUC18. The animals were divided into two groups. In experimental group ( $n=8$ ), acute necrotizing pancreatitis (ANP) was induced by injection of 0.5 ml/kg of sodium tarocholate with 3000U/kg trypsin into the pancreatic duct. The control group ( $n=7$ ) underwent laparotomy only. All animals were sacrificed 7 days later. Mucosal and luminal microflora of intestine were analyzed quantitatively, and various organs were harvested for culturing, blood samples were obtained for determination of serum amylase activities and plasma lipopolysaccharide (LPS) concentrations.

**RESULTS** In the experimental group, the number of *E.coli* in the intestine was much higher than those of the controls, while bifidobacterium and lactobacillus were decreased significantly (Jejunum,  $1.75\pm 0.95$  vs  $2.35\pm 0.79$ ,  $P<0.05$ ;  $1.13\pm 0.8$  vs  $1.83\pm 0.64$ ,  $P<0.05$ ; ileum,  $2.89\pm 0.86$  vs  $3.87\pm 1.05$ ,  $P<0.05$ ;  $1.78\pm 0.79$  vs  $3.79\pm 1.11$ ,  $P<0.01$ ; cecum,  $2.70\pm 0.88$  vs  $4.89\pm 0.87$ ,  $P<0.01$ ;  $2.81\pm 0.73$  vs  $3.24\pm 0.84$ ,  $P<0.05$ . Content of Cecum,  $3.06\pm 0.87$  vs  $5.15\pm 1.44$ ,  $P<0.01$ ;  $2.67\pm 0.61$  vs  $4.25\pm 0.81$ ,  $P<0.01$ ), resulting in reversal of bifido-bacterium/*E.coli* ratio as

compared with the control group (jejunum,  $0.51\pm 0.76$  vs  $1.23\pm 0.53$ ,  $P<0.05$ ; ileum,  $0.62\pm 0.68$  vs  $1.16\pm 0.32$ ,  $P<0.05$ ; cecum,  $0.46\pm 0.44$  vs  $1.03\pm 0.64$ ,  $P<0.05$ ). In addition, intestinal bacteria were isolated from organs of all animals in the experimental group, and JM109 was also detected in most cases. Positive blood culture was 75.0% and 62.5% on day 1 and 2 after induction of ANP, respectively, but no bacterium was found in the controls. As compared with the control group, blood LPS levels and serum amylase activities increased 1-3 times and 3-8 times respectively.

**CONCLUSION** Microecological disturbance could occur in ANP, and overgrowth of intestinal gram-negative bacteria may lead to translocation to the pancreas and other organs, becoming the source of pancreatic and peripancreatic infection.

## INTRODUCTION

Secondary pancreatic and peripancreatic infection is a common severe complication in acute necrotizing pancreatitis (ANP) and responsible for 80% of death due to this disease. The pathogenesis of pancreatic infection has not been clear completely. Pathogens isolated from infected pancreas were similar with common intestinal flora, providing indirect evidence of gut origin of pancreatic infection.

The microecological disturbances of intestine might play an important role in the development of pancreatic infection following ANP. The purpose of this study was to determine if indigenous enteric flora were a primary source of pancreatic infection, and to reveal the relationship between enteric microecologic dysbiosis and bacterial translocation in ANP in dogs.

## MATERIALS AND METHODS

Adult mongrel dogs weighing 13kg to 18kg were observed for at least 1 week, prior to the experiment, stools were cultured with eosin methylene blue agar containing ampicillin (100ng/L). Animals without resistant bacteria in stool culture entered the experiment and received

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\*Supported by a grant from the foundation for specialized key scientific projects of the People's Liberation Army.

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Received 1997-12-06

20 000IU gentamicin orally for 2 days to suppress the indigenous enteric flora. *E. coli* JM109 bearing ampicillin-resistance plasmid PUC18 (approximately 10<sup>9</sup> colony-forming units) administered with food. For the rest of the experiment, drinking water was supplemented with 100ng/L ampicillin. Stool samples were cultured with eosin methylene blue agar (supplemented with 100ng/L ampicillin), Colonization was considered established when culture was positive for 3 successive days. Fifteen dogs were then randomly divided into two groups: ANP group ( $n=8$ ) and control group ( $n=7$ ), and laparotomy was performed under general anesthesia (i.v. thiopentalsodium). In ANP group, pancreatitis was induced by injection of 0.5 ml/kg sodium taurocholate with 3 000IU/kg trypsin into the pancreatic duct under pressure of 7.8kPa. The dogs in the control group received laparotomy only.

Before the operation and on days 1, 2, 4 and 7 postoperatively, blood samples were obtained for determination of serum amylase activities (iodium-starch method) and plasma LPS concentrations (LAL test), and blood was cultured for aerobic and anaerobic bacteria on each postoperative day. All dogs were killed on the 7th day after operation. Under strict aseptic conditions, specimens of tissues from mesenteric lymph nodes (MLN), liver, pancreas, spleen, kidney and lung were harvested, weighed, and homogenized. Ten  $\mu$ l of each homogenate was cultured for aerobic and anaerobic bacteria. All bacteria isolated from organs were cultured in luria-bertani (LB) supplemented with 100ng/L ampicillin for 24 hours. Positive germs were initially identified as resistant bacteria. Final identification of those strains was accomplished by confirming the presence of plasmid PUC18. Plasmid DNA was purified by an alkaline lysis method and subjected to restriction digestion with endonuclease EcoR1 (Sigma Corp.) in 37°C water for 1 hour. Ten  $\mu$ l DNA fragments were separated by electrophoresis through horizontal 0.8% agarose gel, stained with ethidium bromide and photographed under ultraviolet lamp in 590nm.

Jejunum, cecum, ileum and content of cecum were harvested, weighed and homogenized in 5ml physiological saline. Homogenate (0.5ml) was serially diluted (10 times), and 10  $\mu$ l dilution was plated on selective media for *E. coli*, enterococci, bacteroids, bifidobacteria and lactobacilli, respectively, and incubated at 37°C for 24-48 hours, aerobically or anaerobically for 48 hours, positive specimens were subcultured and the bacteria identified by standard procedures.

Sections of cecum and pancreas were stained with hematoxylin and eosin and examined under

light microscopy.

Data were analyzed by Student's *t* test, and results were expressed as  $\bar{x}\pm s$ . Differences were considered significant when  $P<0.05$ .

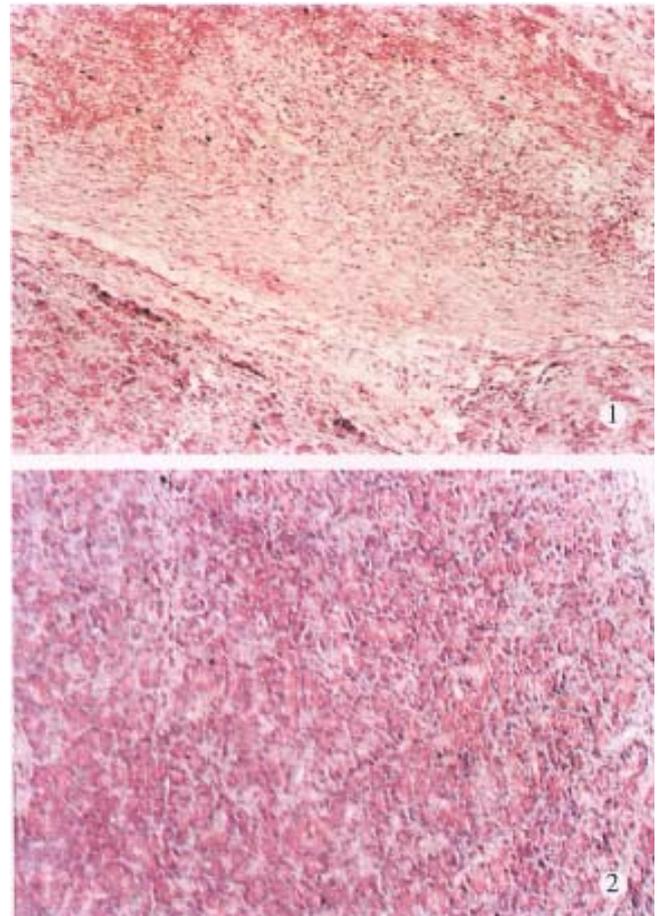
## RESULTS

### *Acute necrotizing pancreatitis*

Laboratory tests showed significant hyperamylasemia on days 1, 2, 4 and 7 after operation in dogs with pancreatitis (Table 1). The pancreas in ANP group appeared enlarged and swollen with visible grey or black areas. Histologic examination revealed severe hemorrhagic necrotizing pancreatitis (Figure 1). In the control group, no abnormalities were found both macroscopically and histologically (Figure 2).

### *Intestinal morphology*

Cecal mucosa were severely damaged in dogs with pancreatitis. The surface epithelium was denuded on the top of the villi, and there was an extensive neutrophilic granulocyte infiltration of the lamina propria. No pathologic changes were noticed in the controls.



**Figure 1** Light micrography showing severe hemorrhage in pancreas of ANP. HE $\times$ 100

**Figure 2** Light micrography of a normal pancreas. HE $\times$ 100

### Intestinal microflora

The population levels of *E.coli* in the mucosa of jejunum, ileum, cecum and in the cecal content were increased significantly in ANP dogs on day 7 postoperatively ( $P<0.05$  or  $P<0.01$ , Table 2), while bifidobacteria and lactobacilli were decreased obviously. The ratio of bifidobacterium/*E.coli* (B/E) was reversed ( $P<0.05$ , Table 3).

### Bacterial translocation

Blood and tissue cultures were negative except for 2 episodes of bacterial translocation to MLN in the control group and were positive in the ANP group, bacterial translocation was found in MLN (100%),

pancreas (87.5%), liver (87.5%), lung (75%), kidney (75%) and spleen (50%). The isolation rate of *E.coli* JM109 was 75% in pancreas, 50% in the liver and lung. Blood positive cultures were seen mainly on the first (75%) and second (62.5%) postoperative day, and JM109 was found in more than 60% of cases.

### LPS concentration

The LPS concentrations in ANP group were elevated significantly as compared with those of the control group in each postoperative day ( $P<0.05$  or  $P<0.01$ , Table 4).

**Table 1 Activity of plasma amylase (U/L)**

Group	Preoperation	d1	d2	d4	d7
Control	796.61±82.41	816.56±57.82	787.26±78.66	807.68±89.56	778.59±80.95
ANP	825.50±82.94	7363.25±1383.26 <sup>b</sup>	7060.75±1135.65 <sup>b</sup>	4590.25±1312.44 <sup>b</sup>	2783.75±893.42 <sup>b</sup>

<sup>b</sup> $P<0.01$ , compared with the control group.

**Table 2 Population levels of mucosal and luminal flora (CFUlogn/g,  $\bar{x}\pm s$ )**

Content	Group	<i>E.coli</i>	Enterococcus	Bacteroid	Bifidobacterium	Lactobacillus
Jejunum	Control	1.91±0.49	1.69±0.79	2.23±0.92	2.35±0.79	1.83±0.64
	ANP	3.42±0.93 <sup>b</sup>	0 <sup>b</sup>	3.75±0.77 <sup>a</sup>	1.75±0.95 <sup>a</sup>	1.13±0.80
Ileum	Control	3.51±0.84	2.05±0.44	3.61±1.06	3.87±1.05	3.79±1.11
	ANP	5.80±1.27 <sup>b</sup>	1.17±0.95 <sup>a</sup>	4.35±0.98 <sup>a</sup>	2.89±0.86 <sup>a</sup>	1.78±0.79b
Cecum	Control	4.74±0.93	2.61±0.77	3.54±0.99	4.89±0.87	3.24±0.84
	ANP	5.88±1.18 <sup>a</sup>	1.27±1.04 <sup>a</sup>	4.01±1.10	2.70±0.88 <sup>b</sup>	2.81±0.73a
Content of cecum	Control	4.86±0.64	3.50±0.85	4.81±0.95	5.15±1.44	4.25±0.81
	ANP	7.43±1.19 <sup>b</sup>	2.27±1.49 <sup>a</sup>	4.72±1.13	3.06±0.89 <sup>b</sup>	2.67±0.61b

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$  compared with the control group.

**Table 3 Ratio of bifidobacterium/*E.coli* (B/E)**

Group	Jejunum	Ileum	Cecum
Control	1.23±0.53	1.16±0.82	1.03±0.64
ANP	0.51±0.76 <sup>a</sup>	0.62±0.68 <sup>a</sup>	0.16±0.44 <sup>a</sup>

<sup>a</sup> $P<0.05$  compared with the control group.

**Table 4 Changes of plasma LPS (Eu/ml)**

Group	d1	d2	d4	d7
Control	0.068±0.005	0.074±0.008	0.064±0.009	0.066±0.007
ANP	0.217±0.085 <sup>b</sup>	0.346±0.127 <sup>b</sup>	0.268±0.054 <sup>b</sup>	0.107±0.064 <sup>a</sup>

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , compared with the control group.

### Plasmid DNA analysis

The strain of ampicillin-resistant *E.coli* was isolated in all dogs with pancreatitis. All ampicillin-resistant *E.coli* isolated from different organs had identical antibiograms and contained plasmid DNA that appeared identical as shown by plasmid electrophoresis profile, indicating that they were

the same strains.

## DISCUSSION

Numerous studies have revealed that intestinal microecologic dystiosis may lead to decreased colonization resistance of the gut, which plays an important role in the pathogenesis of enterogenous infection. Runkel found that gram-negative germs overgrew in cecal mucosa 24-48 hours after onset of pancreatitis, suggesting that microecological disturbance of intestine was an important factor for sepsis following pancreatitis [3]. Kazantsev used plasmid labeled *E.coli* (kanamycin-resistant) to confirm that intestinal bacteria could translocate to pancreas in pancreatitis, but he could not explain the relationship between bacterial translocation and enteric microecologic dysbiosis [4].

The present study showed that the enteric microecologic disturbance did take place following pancreatitis. The population levels of *E.coli* were

increased significantly, while the bifidobacteria and lactobacilli were decreased obviously. So the main manifestation of the disturbance of enteric flora were overgrowth of opportunistic pathogens including aerobic bacteria and facultative anaerobes, and reduction of anaerobic bacteria such as bifidobacteria and lactobacilli, as reported earlier by Gianotti<sup>[5]</sup> *et al.* Blood and organ culture further showed that bacteria translocated to organs and blood in all animals with pancreatitis, and to pancreas in 87.5% of cases, 75% of them were *E.coli* JM109 colonized previously in the gut. These results provided substantial evidence that the gut was the primary source of pancreatic infection, and the translocation of the enteric overgrowing gramnegative germs in the gut, were the main pathogens of pancreatic infection.

The enteric microecologic dysbiosis following ANP might be explained by the overgrowth of gramnegative germs (mainly *E.coli*) and their inhibitory effect on the growth of dominant bacteria in gut such as bifidobacteria, resulting in the decreased colonization resistance and the immunity of host. This disturbance might lead to colonization of potential opportunistic pathogens and increase the chance of bacterial translocation. The intestinal epithelium was also injured by enteric ischemia and ischemia-reperfusion in ANP. In such

circumstances, enteric bacteria which attached to and colonized on the surface of intestinal epithelium, could penetrate the mucosal barrier and translocate to MLN, other organs and blood, and caused infection in the pancreas which was seriously damaged by inflammation, hemorrhage and necrosis. The overgrowth of *E.coli* may also produce a large amount of LPS, becoming the source of endotoxemia following pancreatitis.

In conclusion, our data demonstrated that the enteric microecologic dysbiosis played an important role in the pathogenesis of infection complicating ANP. Taking effective measures to reduce the microecological disturbance and to protect the gut barrier function should be an important principle to prevent infection secondary to acute necrotizing pancreatitis.

## REFERENCES

- 1 Bjornson HS. Pancreatic "abscess": diagnosis and management. *Pancreas*, 1991;6(s1):s31-s36
- 2 Frey CF, Bradley III EL, Beger HG, Rodriguez LF, Larocco MT, Miller TA. Progress in acute pancreatitis. *Surg Gynecol Obstet*, 1988;167(4):282-286
- 3 Runkel NSF, Moody FG, Smith GS. The role of the gut in the development of sepsis in acute pancreatitis. *J Surg Res*, 1991;51(1):18-23
- 4 Kazantsev GB, Hecht DW, Rao R, Fedorak II, Gattuso P, Thompson K. Plasmid labeling confirms bacterial translocation in acute pancreatitis. *Am J Surg*, 1994;167(1):201-207
- 5 Gianotti L, Munda R, Alexander JW, Tchervenkov JI, Babcock GF. Bacterial translocation: a potential source for infection in acute pancreatitis. *Pancreas*, 1993;8(1):551-556

# Effect of *Helicobacter pylori* infection on gastric epithelial proliferation in progression from normal mucosa to gastric carcinoma

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**Subject headings** helicobacter infections; gastric mucosa/microbiology; stomach neoplasms/microbiology; gastric mucosa/pathology

## Abstract

**AIM** To study the effect of *Helicobacter pylori* (*H. pylori*) infection on gastric epithelial proliferation in the progression from normal mucosa to gastric carcinoma.

**METHODS** Gastric biopsy specimens from normal controls ( $n=11$ ), superficial gastritis ( $n=32$ ), atrophic gastritis with intestinal metaplasia ( $n=83$ ), dysplasia ( $n=25$ ) and gastric carcinoma ( $n=10$ ) were studied by immunohistochemical staining of proliferating cell nuclear antigen (PCNA).

**RESULTS** The gastric epithelial proliferation, expressed as PCNA labeling index (LI)%, was progressively increased in successive stages from normal mucosa to gastric carcinoma regardless of *H. pylori* status. There was significant difference in PCNA LI% among all groups ( $P<0.01$ ). The analysis pursuing the effect of *H. pylori* infection on gastric epithelial proliferation in the progression from normal mucosa to gastric carcinoma showed that in superficial gastritis and mild atrophic gastritis groups, PCNA LI% in *H. pylori* positive patients were  $13.14\pm 1.6$  and  $19.68\pm 2.22$  respectively, significantly higher than  $6.95\pm 0.78$  and  $11.34\pm 1.89$  in *H. pylori* negative patients ( $P<0.01$ ); but there was no such difference in other groups ( $P>0.05$ ).

**CONCLUSION** *H. pylori* infection causes increased gastric epithelial proliferation in the stages of superficial and mild atrophic gastritis and may play a part in triggering gastric carcinogenesis.

## INTRODUCTION

Gastric carcinoma is one of the leading causes of malignancy-related death in China, and its etiology has not been fully elucidated. The epidemiological and histopathological studies have shown that *Helicobacter pylori* (*H. pylori*) infection is closely associated with gastric carcinogenesis<sup>[1-5]</sup>. However, the mechanism and the stages in which *H. pylori* participates in the process of gastric carcinogenesis are largely unknown. An increase in epithelial cell proliferation is one of the earliest mucosa changes in the development of gastric cancer, and may serve as a risk indicator for it<sup>[6,7]</sup>. The proliferation nuclear cell antigen (PCNA) expression is a reliable marker for evaluation of cell proliferation<sup>[8,9]</sup>. In this study, we used PCNA as a marker to investigate the effect of *H. pylori* infection on gastric epithelial proliferation in the progression from normal mucosa to gastric carcinoma.

## MATERIALS AND METHODS

### Subjects

Archival gastric biopsy specimens used in this study were randomly selected from those kept in the pathological department of Shanghai Institute of Digestive Diseases between January and December 1996. The specimens were taken from 161 subjects, and the diagnosis was made based on the endoscopic and histological findings. Subjects were assigned to one of the following five study groups according to the histological diagnosis: Group 1, normal gastric mucosa, *H. pylori* negative; group 2, chronic superficial gastritis; group 3, chronic atrophic gastritis with intestinal metaplasia; group 4, dysplasia; and group 5, gastric carcinoma (intestinal type). The atrophy and intestinal metaplasia in gastric mucosa were graded as mild, moderate and severe respectively according to the criteria proposed in Sydney system, and scored 1, 2 and 3. Atrophic gastritis was usually accompanied by intestinal metaplasia, so the scores of atrophy and intestinal metaplasia in each patient were added up, and then further classified as mild for total score equal or less than 2, moderate for scores 3-4, and severe for scores more than 4. The patients with chronic gastritis accompanied by dysplasia all belonged to group 4.

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\*Supported by the Shanghai Municipal Commission of Science and Technology, No.954119023.

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Received 1997-12-18

### PCNA staining

PCNA staining of antral biopsy specimens was performed using immunohistochemical ABC method. Briefly, PCNA staining was proceeded, after deparaffinizing in xylene, clearing in ethanol, rehydrating through graded ethanol and washing in PBS. Endogenous peroxidase was blocked by 0.3% H<sub>2</sub>O<sub>2</sub>, and then washed slightly in PBS. The sections were preincubated with diluted normal goat serum, and then incubated with anti-PCNA antibody (PC10, mouse anti-human, DAKO Company) diluted 1:80 at 4°C overnight. After draining, the sections were incubated with a biotinylated anti-mouse IgG diluted 1:50 for 60min, then incubated with ABC working solution for 30min according to manufacturer's instruction (Sino-American Biotechnology Company). After another washing, the sections were incubated with 3-3'-diaminobenzidine tetrahydrochloride (DAB) solution under microscopic monitoring, and then counterstained with hematoxylin. The stained PCNA positive tissue (gastric polyp) sections served as positive controls. A negative control, where primary antibody was replaced by PBS, was also stained parallelly.

### Analysis of gastric epithelial proliferative activity

PCNA positive cells were counted only in well oriented sections with visible entire gastric pits. A mean number of 10 pits were examined for each specimen, and greater than 500 cells were analysed. Labeling index per cent (LI%) was measured by counting the percentage of the number of PCNA positive cells of the total number of cells.

### Identification of *H. pylori* infection

*H. pylori* was detected under microscopy on the histological sections stained with a modified Giemsa staining method.

### Statistical analysis

LI% was compare among groups, and the significance was analysed using Student' *t* test for unpaired data, *P* values less than 0.05 were considered statistically significant.

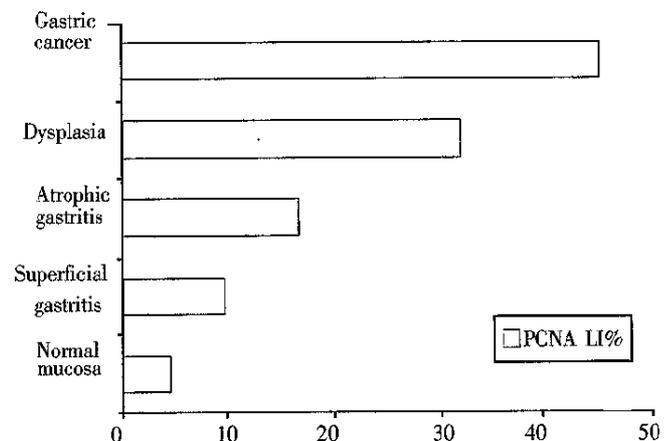
## RESULTS

Table 1 shows the demographic profile and *H. pylori* status in 161 subjects. Figure 1 shows PCNA LI% in the study groups regardless of *H. pylori* status. PCNA LI% in normal gastric mucosa, superficial gastritis, atrophic gastritis, dysplasia and gastric carcinoma group was  $6.31 \pm 1.67$  ( $\bar{x} \pm s$ ),  $10.04 \pm 1.32$ ,  $17.11 \pm 2.55$ ,  $32.46 \pm 4.16$  and  $46.05 \pm 4.63$ , respectively. PCNA LI% was progressively increased from normal mucosa to gastric carcinoma, and there was significant difference between the groups ( $P < 0.01$ ).

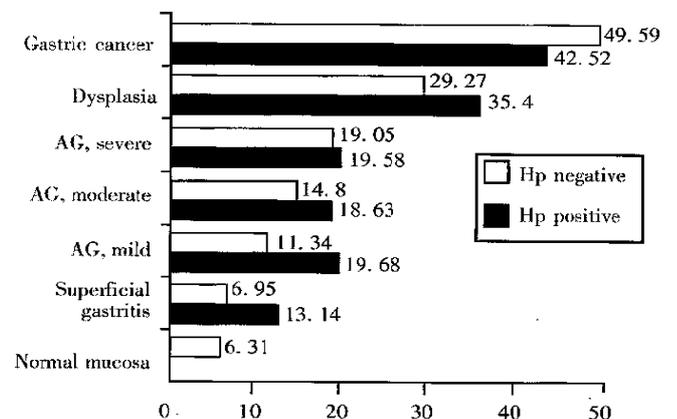
**Table 1** The demographic profile and *H. pylori* status in 161 subjects

Study groups	No. of patients	Femal/ male	Average ages(yrs)	No. of Hp positive	No. of Hp negative
Normal gastric mucosa	11	2/9	44.5	0	11
Superficial gastritis	32	10/22	43.6	16	16
Atrophic gastritis					
Mild	32	11/21	42.1	16	16
Moderate	26	11/15	51.0	15	11
Severe	25	8/17	56.1	10	15
Dysplasia	25	7/18	53.2	13	12
Gastric Carcinoma	10	3/7	66.2	5	5

Figure 2 shows the PCNA LI% in the groups associated with *H. pylori* status. Atrophic gastritis group was further classified into mild, moderate and severe subgroups based on the scores of atrophy and intestinal metaplasia. In superficial gastritis and mild atrophic gastritis groups, PCNA LI% in *H. pylori* positive patients was  $13.14 \pm 1.6$  and  $19.68 \pm 2.22$  respectively, significantly higher than  $6.95 \pm 0.78$  and  $11.34 \pm 1.89$  in *H. pylori* negative patients ( $P < 0.01$ ); but there was no such difference in other groups ( $P > 0.05$ ).



**Figure 1** PCNA LI% in the study groups from normal gastric mucosa to gastric carcinoma regardless of *H. pylori* status.



**Figure 2** PCNA LI% in study groups associated with *H. pylori* status from normal mucosa to gastric carcinoma. AG=atrophic gastritis.

## DISCUSSION

The etiology of gastric carcinoma has not been fully elucidated. In 1988, Correa<sup>[10]</sup> proposed a human model of gastric carcinogenesis based on the epidemiological, pathological and clinical findings. He postulated that gastric cancer develops through a complex sequence of events from normal to superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally to intestinal type gastric carcinoma, and that the chronic gastritis in the first step in the progression to malignancy. This postulation was proved correct later by several studies<sup>[11,12]</sup>.

*H. pylori* is the primary etiological cause of chronic gastritis<sup>[13,14]</sup>, and is associated with a sixfold increased risk of gastric carcinoma<sup>[2]</sup>. Long-term studies of *H. pylori* infection have provided evidence of a progression from *H. pylori* gastritis to atrophic gastritis, intestinal metaplasia, and dysplasia. *H. pylori* has been listed by the WHO as class 1 carcinogen for gastric cancer. But the exact mechanisms that *H. pylori* participates in gastric carcinogenesis are not clear.

Excessive cell proliferation increases the chance of spontaneous error of DNA replication, and potentiates the action of any carcinogen targeting DNA, and thereby enhances the risk of neoplastic transformation of cells<sup>[15]</sup>. The assessment of epithelial cell proliferation as an indicator of risk has been validated in gastric carcinoma, even before *H. pylori* was found to be associated with chronic gastritis<sup>[6]</sup>. Several recent studies have shown that *H. pylori* infection can promote gastric epithelial proliferation<sup>[16,17]</sup> so that *H. pylori* infection links to increased risks of gastric carcinogenesis. But few studies have pursued the effect of *H. pylori* infection on gastric epithelial proliferation in the progression from normal mucosa to gastric carcinoma<sup>[18]</sup>.

Our results showed that gastric epithelial proliferative activity expressed as PCNA LI% increased progressively from normal mucosa to superficial gastritis, atrophic gastritis, dysplasia and gastric carcinoma, suggesting that increased gastric epithelial proliferation is associated with gastric precancerous changes and gastric cancer, and can be used as an indicator for evaluating the risk of gastric carcinogenesis.

The analysis of the effect of *H. pylori* infection on gastric epithelial proliferation in progression from normal mucosa to gastric carcinoma indicated that the increase in gastric epithelial proliferation associated with *H. pylori* infection was only seen in superficial gastritis and mild atrophic gastritis, and in other groups, there

was no significant difference in PCNA LI% between *H. pylori* positive and negative patients. The results suggested that *H. pylori* infection causes increased gastric epithelial proliferation primarily in the stages of superficial and mild atrophic gastritis, and it may not have so strong influence in the late stages of gastric carcinogenesis. There, *H. pylori* infection may be the precipitating factor in triggering gastric carcinogenesis.

Once *H. pylori* gastritis develops to the stage of gastric atrophy, the gastric acid secretion will decrease markedly, leading to changes in gastric bacterial flora, and subsequently promoted the endogenous formation of carcinogenic N-nitrosocompounds<sup>[19,20]</sup>, and gastric epithelial proliferation. This may explain why *H. pylori* infection has less effect on gastric epithelial proliferation in late stages of gastric carcinogenesis.

## REFERENCES

- Forman D, Newell DG, Fullerton F, Yarnell JWG, Stacey AR, Wald N *et al*. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ*, 1991;302(6788):1302-1305
- Eurogast Study Group. An international association between *Helicobacter pylori* infection and gastric cancer. *Lancet*, 1993;341(8857):1359-1362
- Parsonnett J, Friedman GD, Vandersteen DP, Chang Y, Vogelmann JH, Orentreich N *et al*. *Helicobacter pylori* infection and risk of gastric cancer. *N Engl J Med*, 1991;325(16):1127-1131
- Craanen ME, Blok P, Dekker W, Tytgat GNJ. *Helicobacter pylori* and early gastric cancer. *Gut*, 1994;35(10):1372-1374
- Aska M, Kimura T, Kato M, Kudo M, Miki K, Ogoshi K *et al*. Possible role of *Helicobacter pylori* infection in early gastric cancer development. *Cancer*, 1994;73(11):2691-2694
- Xu YL, Zheng ZT. Cell proliferation kinetics in chronic gastritis and gastric cancer. *Chin Med J*, 1984; 97(8): 526-532
- Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res*, 1988; 48(2):235-245
- Hall PA, Ivison DA, Woods AL, Yu CC, Kellock DB, Watkins JA *et al*. Proliferating cell nuclear antigen (PCNA) immunolocalization in some neoplasia. *J Pathol*, 1990;162(4):285-294
- Yamada K, Yoshitaka K, Sato M and Ahnen DJ *et al*. Proliferating cell nuclear antigen in normal, and colonic epithelium in the rat. *Gastroenterology*, 1992; 103(1):160-167
- Correa P. A human model of gastric carcinogenesis. *Cancer Res*, 1988;48(13): 3554-3560
- Correa P. A human model of gastric carcinogenesis. *Cancer Res*, 1988;48(13): 3554-3560
- Correa P, Haenszel W, Cuello C, Zavala D, Fonthanm E, Zarama G *et al*. Gastric precancerous process in a high risk population: Cohort follow-up. *Cancer Res*, 1990;50(15):4737-4740
- Jiang SJ, Liu WZ, Zhang DZ, Shi Y, Xiao SD, Zhang ZH. Campylobacter-like organisms in chronic gastritis, peptic ulcer and gastric carcinoma. *Scand J Gastroenterol*, 1987;22(6):553-555
- Wyatt JL. Histopathology of gastroduodenal inflammation: The impact of *Helicobacter pylori*. *Histopathology*, 1995;26(1):1-15
- Ames BN, Gold LS. Too many rodent carcinogens: Mitogenesis increases mutagenesis. *Science*, 1990;249(4970):970-971
- Fan XG, Kelleher D, Fan XI, Xia HX, Keeling PWN. *Helicobacter pylori* increases proliferation of gastric epithelial cells. *Gut*, 1996;38(1):19-22
- Lynch DAF, Mapstone NP, Clarke AMT, Sobala GM, Jackson P, Morrison L *et al*. Cell proliferation in *Helicobacter pylori* associated gastritis and the effect of eradication therapy. *Gut*, 1995;36(3):346-350
- Cahill RJ, Kilgallen C, Beattie S, Hamilton H, O'Morain C *et al*. Gastric epithelial cell kinetics in the progression from normal mucosa to gastric carcinoma. *Gut*, 1996;(2)28:177-181
- Correa P. *Helicobacter pylori* and gastric carcinogenesis. *Am J Surg Pathol*, 1995;19(suppl 1):S37-43
- Sobala GM, Pignatelli B, Schorah CJ, Bartsch H, Sanderson M, Dixon MF *et al*. Levels of nitrite, nitrate, nitroso compound, ascorbic and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach. *Carcinogenesis*, 1991;12(2):193-198

# Serum IgG response to differentiated antigens of *Helicobacter pylori*

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**Subject headings** *Helicobacter pylori*; spiral form; coccoid form; ELISA; *Helicobacter pylori* infection; *Helicobacter pylori* antigens, bacterial; IgG; antigens, differentiation; antigen-antibody reactions; stomach ulcer; stomach neoplasms; gastritis

## Abstract

**AIM** To detect antibodies against *Helicobacter pylori* spiral and coccoid antigens in human sera.

**METHODS** Blood samples were collected from 278 patients with gastric diseases. A 3-day-old culture of *H. pylori* on chocolate blood agar was used to provide spiral form. 'Synchronous' coccoids were cultured in (BHY) (brain heart infusion supplemented with 10% horse serum and 0.4% yeast extract) medium in a chemostat. Antigens from spiral and coccoid form were prepared using acid glycine extraction. Enzyme-linked immunosorbent assay (ELISA) was performed to detect serum IgG antibodies against spiral and coccoid forms of *H. pylori*.

**RESULTS** Seroprevalence of *H. pylori* infection was higher in patients with gastric ulcer (79%) and gastric cancer (83%) than those with non-ulcer dyspepsia (NUD) (44%) and other diseases (45%) ( $P < 0.05$ ). IgG antibodies against spiral and coccoid antigens were detected in 50.7% (141/278) and 49.6% (138/278), respectively.

**CONCLUSION** The spiral and coccoid forms of *H. pylori* coexist in patients infected with the bacterium.

## INTRODUCTION

It was reported that *Helicobacter pylori* can convert to coccoid form from spiral form *in vitro* after prolonged incubation<sup>[1]</sup> or under antibiotic stress<sup>[2]</sup>. By histological examination, Chan *et al*<sup>[3]</sup> and Janas *et al*<sup>[4]</sup> observed two morphological forms of the bacteria in *H. pylori*-positive gastric specimens. The dimorphism of *H. pylori* has stimulated researchers to investigate whether coccoid form of *H. pylori* is viable and pathogenic<sup>[1,5,6]</sup>. Coccoid form like its spiral form has similar proteins except that a high-molecular-mass antigenic fraction (>94kDa), absent in spiral forms, was detected during coccoid conversion<sup>[6]</sup> and that a smaller number of immunoreactive bands were recognized in the coccoid antigens compared with the spiral antigens<sup>[5]</sup>. In this study, we used ELISA technique to detect immune response of IgG to spiral and coccoid antigens.

## MATERIALS AND METHODS

### Serum specimens

Blood samples were collected from 278 patients with gastric disorders at the National University Hospital, Singapore. Informed consent was obtained from all the patients. After collection, blood specimens were allowed to clot at room temperature for 36mins-60mins. The sera were removed from the clot and any remaining insoluble material removed by centrifugation at 2 000×g for 10min at 4°C. Sera were stored at -20°C until use.

### Antigen preparation

A local *H. pylori* strain V2 isolated from a patient with non-ulcer dyspepsia was used. A 3-day-old culture of *H. pylori* on chocolate blood agar was used to provide spiral form. 'Synchronous' coccoids were cultured in BHY (brain heart infusion supplemented with 10% horse serum and 0.4% yeast extract) medium in a chemostat. Antigens from spiral and coccoid form were prepared according to a modified method of Goodwin *et al*<sup>[7]</sup> as described by Vijayakumari *et al*<sup>[5]</sup>.

### Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed according to the method described previously<sup>[8]</sup>. Briefly, flat-bottomed microtitre plates (Nunc) were coated with acid

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Received 1998-04-25

glycine extract antigens of *H. pylori*. The sera tested were diluted at 1:100. Each diluted serum was examined in triplicate. Positive control serum was diluted at 1:100, 1:200, 1:400, 1:800, 1:1 600 and 1:3 200 and negative control serum at 1:100. Horse radish peroxidase-labelled rabbit anti-human IgG (Dako) was used as conjugates. The substrate used was ophenylenediamine dihydrochloride (OPD, Sigma). The optical density at wavelength 490 and 620nm reference filter was read immediately using an ELISA reader (Ceres 900 Bio-Tek Instruments, Inc). The cut-off value for the ELISA was derived according to Khin and Ho<sup>[8]</sup>.

## RESULTS

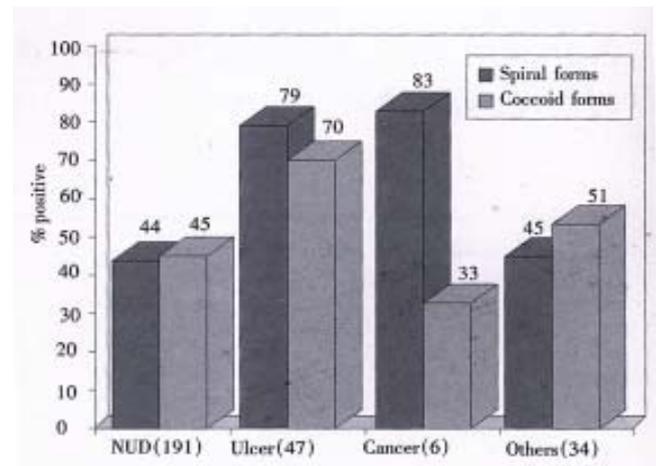
Seroprevalence of *H. pylori* infection was higher in patients with gastric ulcer (79%) and gastric cancer (83%) than those with non-ulcer dyspepsia (NUD) (44%) and other diseases (45%) ( $P < 0.05$ ). Furthermore, of the 278 sera, IgG antibodies against NCTC 11637 spiral and coccoid antigens were detected in 141 (50.7%) cases and 138 (49.6%) cases.

In 191 NUD patients, 84 (43.9%) and 86 (45.0%) were detected with IgG antibodies to spiral and coccoid antigens, respectively. There was no significant difference in seroreactivity to spiral and coccoid antigens in patients with NUD ( $P > 0.05$ ) (Figure 1).

Of the 47 peptic ulcer patients, 37 (78.7%) and 33 (70.2%) showed antibodies IgG against spiral and coccoid antigens ( $P > 0.05$ ). Among the peptic ulcer patients, 69.2% (9/13) and 76.9% (10/13) of the gastric ulcer patients while 82.1% (23/28) and 67.9% (19/28) were positive for spiral and coccoid antigens, respectively. Serum IgG antibodies against spiral antigens were detected in 83.3% (5/6) patients with both gastric and duodenal ulcer, while 66.7% (4/6) of these were seropositive for IgG antibodies against coccoid antigens (Figure 1).

Of the 6 cases of gastric cancer antibodies IgG 5 (83.3%) cases were positive with IgG antibodies to spiral antigens and 2 (33%) cases were positive with IgG antibodies to coccoid antigens. No statistical difference was found in IgG antibodies against spiral and coccoid for antigens in this group ( $P > 0.05$ ) (Figure 1).

Of the 34 cases of other diseases, there was 45.4% (15/33) and 51.5% (17/33) had positive seroreactivity against *H. pylori* spiral and coccoid antigens respectively. There was no significant difference ( $P > 0.05$ ) (Figure 1).



**Figure 1** Prevalence of antibodies against *H. pylori* spiral and coccoid antigens in patients with different gastric diseases.

## DISCUSSION

Most adult patients colonized with *H. pylori* elicit a measurable systemic antibody response which comprises predominantly IgG. In this study, the systemic immune response of 278 sera was examined by ELISA. IgG to *H. pylori* spiral and coccoid antigens was found to be 50.7% and 49.6%, respectively. The serological response to both spiral and coccoid antigens indicates the possibility of coexistence of two forms of *H. pylori* in these patients which leads to the production of similar IgG responses in patients. It has been shown earlier that there were differences in protein profiles of spiral and coccoid antigens<sup>[6]</sup>. *in vitro*, coccoid form could be detected 6 hours after exposure to 10ng/L of amoxicillin<sup>[2]</sup>. Janas *et al*<sup>[4]</sup> reported the presence of two different morphological forms of *H. pylori* in gastric antrum specimens. Could these two morphological forms of spiral and coccoid of *H. pylori* be the complete cell cycle *in vivo*? The morphological changes could have been triggered under physical or chemical stress which is unfavourable to *H. pylori*. Spiral form may then convert into coccoid form. When the environment becomes favourable, the bacteria may revert from coccoid form to spiral form *in vivo*. However, what could have led to the revision is still unknown. Some *in vitro* studies indicated that coccoid form of *H. pylori* might be viable<sup>[1,9-11]</sup>. Furthermore, Vijayakumari *et al*<sup>[5]</sup> showed that the adherence patterns of coccoid form of *H. pylori* on kato III *in vitro* were similar to those observed with spiral form in gastric biopsy specimens *in vivo*. It was reported that the vital cytotoxic proteins of spiral forms were also conserved in the coccoids<sup>[6]</sup>. Therefore, like its counterpart, coccoid form could be an infective, transmissible, immunogenic and pathogenic form of *H. pylori* which participates in

the pathogenesis of gastroduodenal diseases.

The coccoid form was present above damaged epithelial cells<sup>[4]</sup> and is not easy to be detected by the techniques now being used. Patients with morphological conversion of *H. pylori* from spiral to coccoid form after treatment may be neglected and considered as eradication of *H. pylori*. The possible cell cycle of *H. pylori* may have clinical relevance. Xia *et al*<sup>[12]</sup> reported that recurrence of *H. pylori* infection was probably caused by recrudescence in the patients studied. One possible reason of relapse could be morphological reversion of *H. pylori* form coccoid form to spiral form after treatment.

This study demonstrated the presence of antibodies to different antigens of *H. pylori* in patients. Coccoid form like its counterpart spiral form may have clinical relevance in the outcome of gastric diseases.

## REFERENCES

- 1 Hua J, Ho B. Is the coccoid form of *Helicobacter pylori* viable *Microbioscience*, 1996;87:103-112
- 2 Berry V, Jennings K, Woodnutt G. Bactericidal and morphological effects of Amoxicillin on *Helicobacter pylori*. *Antimicrob Agents Chemother*, 1995;39:1859-1861
- 3 Chan W. Y., Hui P. K., Leung K. M. and Thomas T. M. M. Modes of *Helicobacter* colonization and gastric epithelial damage. *Histopathology*, 1992;21:521-528
- 4 Jans B, Czkwianianc E, Bak Romaniszyn L, Bartel H, Tosik D, Ptaneta-Malecka I. Electro microscopic study of association between coccoid forms of *Helicobacter pylori* and gastric epithelial cells. *Am J Gastroenterol*, 1995;90:1829-1833
- 5 Vijayakumari S, Khin MM, Jiang B, Ho B. The pathogenic role of the coccoid form of *Helicobacter pylori*. *Cytobioscience*, 1995;82:251-260
- 6 Benaissa M, Babin P, Quillard N, Pezennec L, Cenatiempo Y, Fauchere JL. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect Immun*, 1996;64:2331-2335
- 7 Goodwin CS, Blincow E, Peterson G, Sanderson C, Cheng W, Marshall BJ, Warren JR, McCulloch R. Enzyme-linked immunosorbant assay for *Campylobacter pyloridis*: correlation with presence of *C. pyloridis* in the gastric mucosa. *J Infect Dis*, 1987;155:488-494
- 8 Khin MM, Ho B. Immunological detection of *Helicobacter pylori* pregnant woman. *Biomedical Letters*, 1994;52:71-78
- 9 Mai U, Geis G, Lying H, Ruhl G, Opferkuch S. Dimorphism of *Campylobacter pylori*. In: Megraud F, Lamouliate H, eds. *Gastroduodenal pathology and Campylobacter pylori*. Amsterdam: Elsevier Science, 1989:29-33
- 10 Bode G, Mauch F, Malfertheiner P. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiol Infect*, 1993;111:483-490
- 11 Sorber M, Nilsson M, Hanberger H, Nilsson LE. Morphologic conversion of *Helicobacter pylori* from bacillary to coccoid form. *Eur J Clin Microbiol Infect Dis*, 1996;15:216-221
- 12 Xia HX, Windle HJ, Marshall DG, Smyth CJ, Keane CT, O'Morain CA. Recrudescence of *Helicobacter pylori* after apparently successful eradication: novel application of randomly amplified polymorphic DNA fingerprinting. *Gut*, 1995;37:30-34

# In vitro production of TNF $\alpha$ , IL-6 and sIL-2R in Chinese patients with ulcerative colitis \*

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**Subject headings** tumor necrosis factor alpha; interleukin 6; interleukin 2, receptor; colitis, ulcerative

## Abstract

**AIM** To determine the tumor necrosis factor alpha (TNF $\alpha$ ), interleukin 6 (IL-6) and soluble interleukin 2 receptor (sIL-2r) from peripheral blood mononuclear cells (PBMC) in 25 Chinese patients with ulcerative colitis and 20 healthy controls.

**METHODS** PBMC were isolated by density gradient centrifugation of heparinized blood and cultures for 24 or 48 hours by stimulation with LPS or PHA. TNF $\alpha$  and sIL-2r were measured by ELISA method and IL-6 measured by bioassay.

**RESULTS** TNF $\alpha$  production stimulated by LPS and sIL-2r production by PHA in ulcerative colitis were significantly lower than in healthy controls (TNF $\alpha$  509(46-7244)ng/L vs 1995(117-18 950)ng/L,  $P < 0.05$ ; sIL-2r 320U/ml $\pm$ 165U/ml vs 451U/ml $\pm$ 247U/ml,  $P < 0.05$ ). Spontaneous TNF $\alpha$  and sIL-2r production were not significantly different between ulcerative colitis and controls (TNF $\alpha$  304(46-7044)ng/L vs 215(46-4009)ng/L,  $P > 0.05$ ; sIL-2r 264U/ml $\pm$ 115U/ml vs 236U/ml $\pm$ 139U/ml,  $P > 0.05$ ). IL-6 production by spontaneous release from PBMC in ulcerative colitis group was 109U/ml $\pm$ 94U/ml vs 44U/ml $\pm$ 39U/ml for those in healthy controls,  $P < 0.01$ . IL-6 stimulated by LPS in ulcerative colitis group was (261U/ml $\pm$ 80U/ml) higher than in healthy controls (102U/ml $\pm$ 54U/ml,  $P < 0.01$ ). No correlation of TNF $\alpha$ , IL-6, sIL-2r production was found to disease activity, disease location and medication.

**CONCLUSION** Cytokine production from PBMC was also disturbed in Chinese patients with ulcerative colitis.

## INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD). Its etiology is still unknown so far. Immunological abnormality demonstrated in IBD patients and imbalance between proinflammatory cytokines and anti-inflammatory cytokines play an important role in the initiation and regulation of the immune responses<sup>[1,2]</sup>. Tumor necrosis factor alpha (TNF $\alpha$ ) produced by activated mononuclear cells is a potent proinflammatory and immunoregulatory cytokine<sup>[3]</sup>. It was named TNF at beginning because of its cytotoxic and anti-tumor activities<sup>[4]</sup>. Now it is known to have a wide spectrum of importance in IBD, e.g., TNF antibodies have been successfully used for treatment of Crohn's disease<sup>[5]</sup>.

IL-6 is produced by a variety of cells, such as monocytes, macrophages, T lymphocytes, B lymphocytes, fibroblasts, endothelial cells, etc. It may affect the proliferation of epithelial cells and act as an autocrine growth factor for enterocytes<sup>[6,7]</sup>. It has been shown to stimulate T cell and B cell activation and proliferation<sup>[8-10]</sup>, and to increase immunoglobulin synthesis from epithelium of human intestine<sup>[11,12]</sup>. It is interesting to know that IL-6 has been proposed as a marker of inflammation in IBD and its concentration was elevated in serum, peripheral blood mononuclear cells (PBMC), mucosa biopsy and lamina propria mononuclear cells (LPMC) in IBD patients<sup>[13-16]</sup>. Some papers also show that IL-6 is increased in the systemic circulation in Crohn's disease and is not elevated in UC patients<sup>[17]</sup>.

Soluble interleukin-2 receptor (sIL-2r) is one form of IL-2 receptor secreted by activated T cells and other monocytes<sup>[18]</sup>. It corresponds to the "Tac" antigen (alpha chain) and could bind IL-2, and then participate in the regulation of IL-2 mediated lymphocyte activation<sup>[19,20]</sup>. Recent studies reported that sIL-2r was increased in vivo and in vitro in active IBD patients and may be an index for IBD activity<sup>[21-23]</sup>. The present study was to determine in vitro production of TNF $\alpha$ , IL-6 and sIL-2r in PBMC in Chinese UC patients and analyze the relation of these three cytokine production from PBMC to disease activity, location and medication.

## MATERIALS AND METHODS

### Patients

Twenty-five patients with UC were studied (13 male,

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\*Project supported by IBD cooperative study between the Second Affiliated Hospital of Hubei Medical University and Free University Hospital of Amsterdam.

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Received 1997-04-18 Revised 1997-05-05

12 females; mean age 43 and range 23-67 years). Diagnosis of UC was based on the conventional clinical, radiological, endoscopic and pathological criteria designed by Lennard-Jones<sup>[24]</sup>. Assessment of its activity followed Sutherland's score criteria<sup>[25]</sup>. Of 25 patients, 7 patients had Sutherland's scores 1-4, 12 had scores 5-8, and 6 patients 9-12; 3 patients had proctitis, 14 left-sided colitis, 8 total colitis; 16 patients were on oral sulphasalazine (SASP) treatment (2 patients were being treated with corticosteroid), 8 patients were on Chinese medicine and one patient was treated with metronidazole.

Twenty healthy volunteers served as controls (9 male, 11 females; mean age 37 and range 21-66 years). There is no statistical difference in age and sex between these two groups.

#### Laboratory methods

From each patient, five ml of heparinized blood were collected. Vials were coated and PBMC isolated within two hours after blood collection.

**Stimulation of PBMC** PBMC were isolated by density gradient centrifugation of heparinized blood and washed three times with Hank's balanced salt solution (without Ca<sup>++</sup> and Mg<sup>++</sup>). After washing, cells were suspended in RPMI 1640(GIBCO) with 15% fetal calf serum (GIBCO), 2mM L-glutamine, 100U/ml penicillin and 100  $\mu$ g/ml streptomycin. The isolated cells were cultured for 24 hours (for TNF $\alpha$  and IL-6 determination) or 48 hours (for sIL-2r determination) at 37°C under 5% CO<sub>2</sub> and 100% humidified air in small sterile culture flasks in 2ml culture medium at a concentration of 1 $\times$ 10<sup>6</sup>/ml. The cells were cultured in the presence of 100mg/L of LPS (SIGMA) for TNF $\alpha$  and IL-6 stimulation production, and in the presence of 200mg/L PHA (SIGMA) for sIL-2r stimulation production. For spontaneous production of these cytokines, no stimulators were added in cell culture medians. After culturing, cell culture supernatant was harvested, added 1mmol/ml PMSF, aliquoted and stored at -30°C until assay. Viability was determined using 3% trypan blue. Only more than 95% viable cells were used in this study.

**TNF $\alpha$  measurement** TNF $\alpha$  was determined using specific TNF $\alpha$  ELISA kit (Beijing Biotin Biomedicine Co.). In brief, 100 $\mu$ l/well TNF $\alpha$  standard markers and supernatant samples (in duplicate) were added into immunoplates and incubated for 2 hours at 20°C and washed three times with 0.01M PBS (pH 7.4) and 0.05% Tween 20. The plates were subsequently incubated with 100  $\mu$ l/well horseradish labelled polyclonal rabbit-anti-TNF $\alpha$  (1:1 000) for 1 hour and washed three times

with 0.01M PBS (pH 7.4) and 0.05% Tween 20. Then 100  $\mu$ l/well substrate Ophenylenediamine added. Incubation was allowed for 15 minutes in the dark at room temperature. The reaction was stopped by 50  $\mu$ l/well 2 M H<sub>2</sub>SO<sub>4</sub> solution. Absorption was read at 492nm on ELISA reader. A standard curve was drawn according to OD values of TNF $\alpha$  markers. TNF $\alpha$  concentration of each sample was read to its OD value within standard curve and expressed as ng/L.

**IL-6 bioassay** The IL-6 bioassay was performed using the IL-6 dependent cell line B9 according to Hou<sup>[26]</sup>. Briefly, each 100  $\mu$ l of different samples and standard recombinant human IL-6 was added into 96 well microtitration plate (CORNING). Meanwhile, the IL-6 dependent cell line B9 cells were suspended in RPMI 1640 containing 10% fetal calf serum (GIBCO) and regulated at a concentration of 5 $\times$ 10<sup>4</sup> cells/ml. Each 100  $\mu$ l/ml of the cells were added into the plate and cultured at 37°C in 5% CO<sub>2</sub> for 68 hours. Ten  $\mu$ l/well of 5g/L methyl thiazolyl tetrazolium blue (MTT, FLUKA) was added. The culture was continued for another 4 hours. Absorption value was read at 570nm on an automatic ELISA Reader and represented B9 cell proliferation. The concentration of IL-6 in a supernatant sample was calculated as follows: IL-6(U/ml)=the diluted concentration of supernatant of giving rise to half maximal proliferation of B9 cells  $\times$  standard IL-6 activity (100U/ml)  $\div$  the diluted concentration of standard IL-6 giving rise to half maximal proliferation of B9 cells.

**sIL-2r measurement** sIL-2r concentration in supernatants from 48-hour culture of PBMC with or without PHA stimulation was determined using specific sIL-2r ELISA kit (Beijing Biotin Biomedicine Co.). In brief, 48-well plate was coated with monoclonal anti-IL-2r alpha antibody and blocked with 1% BSA in PBS. IL-2r markers and samples (in duplicate) were added, and incubated for 2 hours at 37°C. After washing three times with 0.01M PBS (pH 7.4) and Tween 20, 100  $\mu$ l/well horseradish labelled polyclonal rabbit-anti-IL-2r alpha antibody (1:40) was added and incubated at 37°C for one hour and a half, then washed three times, and added substrate Ophenylenediamine for 30 minutes at room temperature. The reaction stopped with 50  $\mu$ l of 2M H<sub>2</sub>SO<sub>4</sub>. The absorption was read at 490nm on a ELISA reader. sIL-2r concentration was expressed as U/ml.

#### Statistical analysis

All values were transformed by log transformation.

Then *t* test was used for comparison of TNF $\alpha$ , IL-6 and sIL-2r between UC and healthy control groups. Relations of TNF $\alpha$ , IL-6 and sIL-2r production to disease activity, disease location and medication were analyzed by linear correlation. *P* value less than 0.05 was considered statistically significant.

## RESULTS

**TNF $\alpha$ , IL-6 and sIL-2r production** The spontaneous and stimulated TNF $\alpha$ , IL-6 and sIL-2r production are shown in Table 1. TNF $\alpha$  production stimulated with LPS and sIL-2r production by PHA in UC group was significantly lower than that in healthy control group. Spontaneous TNF $\alpha$  and spontaneous sIL-2r production were not different significantly between UC and control groups. Large inter individual differences were observed in TNF $\alpha$  production. However, both spontaneous and stimulated IL-6 production in UC group were significantly higher than those in healthy controls.

**Linear correlation of TNF $\alpha$ , IL-6 and sIL-2r production from PBMC to disease activity, location and medication** No significant correlation was found between TNF $\alpha$ , IL-6 and sIL-2r production and disease activity, disease location and medication, but a tendency of correlation was shown between spontaneous IL-6 production and disease activity ( $r = 0.37$ ) and medication ( $r = 0.38$ ).

**Table 1 Spontaneous and stimulated production of TNF $\alpha$ , IL-6 and sIL-2r from PBMC**

	UC (n = 25)	HC (n = 20)
TNF $\alpha$ (spontaneous) ng/L	304(46-7044)	215(46-4009)
TNF $\alpha$ (stimulated) ng/L	509(46-7244)*	1995(117-18950)
IL-6 (spontaneous) U/ml	109 $\pm$ 94*	44 $\pm$ 39
IL-6 (stimulated) U/ml	261 $\pm$ 80*	102 $\pm$ 54s
IL-2r (spontaneous) U/ml	264 $\pm$ 115	236 $\pm$ 139
sIL-2r (stimulated) U/ml	320 $\pm$ 165*	451 $\pm$ 247*

*P*<0.05. Median values shown in TNF $\alpha$ , range in brackets; Mean values with standard deviation shown in IL-6 and sIL-2r; HC: healthy controls.

## DISCUSSION

In this study there was a marked decrease in LPS stimulated release of TNF $\alpha$  and a significant decrease in PHA stimulated release of sIL-2r by PBMC from patients with UC as compared with healthy controls. No significant difference was found in spontaneous release of TNF $\alpha$  or sIL-2r by PBMC between these two groups. Our study also showed that both spontaneous and stimulated IL-6 production was increased from PBMC as compared to healthy controls. These implied that the release of TNF $\alpha$ , IL-6 and sIL-2r by activated PBMC may not always be paralleled.

IL-1 $\beta$ , TNF $\alpha$  and IL-6 are three important

proinflammatory cytokines which respond to the initial stimulation. Many studies on TNF $\alpha$  production in UC have been reported with a different results<sup>[27-31]</sup>. Our previous study in Dutch population showed a tendency towards lower TNF $\alpha$  production in PBMC from UC patients<sup>[32]</sup>. The present study in Chinese UC patients confirmed this result. A large inter-difference was observed in TNF $\alpha$  production in Chinese UC patients.

Several studies have shown that IL-6 concentration is increased in active IBD and may be an index of disease activity<sup>[13-16]</sup>. Our study confirmed these observations, but only a tendency of correlation between spontaneous IL-6 concentration and disease activity was found in UC patients. The explanation may be that, the patients with mild inflammation often take maintenance dose of SASP or no medicine, only the patients with severe diseases were administered with high dose of SASP and corticosteroid. The latter medicine may have an inhibiting effect on immune reaction of the body. Our data also showed that IL-6 had no relation to the disease location. These results suggested that IL-6 from PBMC may reflect the active stage of the disease.

sIL-2r concentrations were increased in serum, tissue homogenates and PBMC from IBD patients, especially in Crohn's disease<sup>[20-22]</sup>. However, Schreiber *et al* reported a moderately increased spontaneous release of sIL-2r and significantly less sIL-2r secretion stimulated by pokeweed mitogen in 14 days culture of colonic LPMC from UC patients<sup>[33]</sup>. Our study had a similar result that sIL-2r concentration was slightly higher in spontaneous release from PBMC in UC patients than in healthy controls. When PBMC were cultured with PHA only for 48 hours, less release of sIL-2r was observed in UC group than in healthy controls.

PBMC is a very heterogeneous cell population. Macrophages and T cells may be mostly responsible for release of these three cytokines. The circulatory changes of proinflammatory cytokines may reflect the original status for cytokine secretion. For this reason, studies on local tissue production of cytokines are more accurate and more exact than studies on circulation. The changes of the proinflammatory cytokines may also reflect different genetic background. Our previous study showed in Dutch population TNF gene polymorphisms are present in five combinations<sup>[34]</sup> and TNF $\alpha$  production is associated with TNF haplotypes. These data strongly supported the concept that a different immunogenetic background may determine the degree of the immune response in IBD.

Our findings showed that TNF $\alpha$ , sIL-2r in vitro production were reduced and IL-6 was increased

from PBMC activation in Chinese UC patients. Further study is necessary to confirm these *in vitro* findings to *in vivo* conditions. Studies at local intestinal level should be undertaken in order to assess the significance of the cytokine dysregulation in the inflammatory response.

## REFERENCES

- McCabe RP, Dean P, Elson CO. Immunology of inflammatory bowel disease. *Curr Opin Gastroenterol*, 1996;12(4):340-344
- Sartor RB. pathogenesis and immune mechanism of chronic inflammatory bowel disease. *Am J Gastroenterol*, 1997;92(Suppl 12):5S-11S<sup>a</sup>
- Ruddle NH. Tumor necrosis factor (TNF-A) and lymphotoxin (TNF-A). *Curr Opin Immunol*, 1992;4(3):327-332
- Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA*, 1975;72(9):3666-3670
- Derckx B, Taminiau J, Radema S, Stronkhorst A, Wortel C, Tytgat G *et al.* Tumour necrosis-factor antibody treatment in Crohn's disease. *Lancet*, 1993; 342(8864):173-174
- Krueger J, Ray A, Tamm I, Sehgal PB. Expression and function of interleukin-6 in epithelial cells. *J Cell Biochem*, 1991;45(4):327-334
- Jones SC, Trejdosiewicz LK, Banks RE, Howdle PD, Axon AT, Dixon MF *et al.* Expression of interleukin-6 by intestinal enterocytes. *J Clin Pathol*, 1993; 46(12):1097-1100
- Van Seventer GA, Shimizu Y, Horgan KJ, Luce GE, Webb D, Shaw S. Remote T cell co-stimulation via LFA-1/ICAM-1 and CD2/LFA-3: demonstration with immobilized ligand/mAb and implication in monocyte mediated co-stimulation. *Eur J Immunol*, 1991;21(7):1711-1718
- Hilbert DM, Cancro MP, Scherle PA, Nordan RP, Van Snick J, Gerhard W *et al.* Tcell derived IL-6 is differentially required for antigen-specific antibody secretion by primary and secondary B cells. *J Immunol*, 1989;143(12):4019-4024
- Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6/BSF-2 function as a killer helper factor in the *in vitro* induction of cytotoxic T cells. *J Immunol*, 1988;141(5):1543-1549
- Roldan E, Brieve JA. Terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion: role of bone marrow stromal cells and interleukin 6. *Eur J Immunol*, 1991;21(11):2671-2677
- McGhee JR, Fujihashi K, Beagley KW, Kiyono H. Role of interleukin 6 in human and mouse mucosal IgA plasma cell responses. *Immunol Res*, 1991;10 (3-4):418-422
- Holtkamp W, Stollberg T, Reis HE. Serum interleukin-6 is related to disease activity but not disease specificity in inflammatory bowel disease. *J Clin Gastroenterol*, 1995;20(2):123-126
- Susiki Y, Saito H, Kasanuki J, Kishimoto T, Tamura Y, Yoshida S. Significant increase of interleukin 6 production in blood mononuclear leukocytes obtained from patients with active inflammatory bowel disease. *Life Sci*, 1990; 47(24):2193-2197
- Reinecker HC, Steffen M, Witthoef T, Pflueger I, Schreiber S, MacDermott RP. Enhanced secretion of tumour necrosis factor- $\alpha$ , IL-6, and IL-1 $\beta$  by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol*, 1993;94(1):174-181
- Hyams JS, Fitzgerald JE, Treem WR, Wyzga N, Kreutzer DL. Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. *Gastroenterology*, 1993;104(5):1285-1292
- Gross V, Andus T, Caesar I, Roth M, Sch-Imerich J. Evidence for continuous stimulation of interleukin 6 production in Crohn's disease. *Gastroenterology*, 1992;102(2):514-519
- Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R *et al.* Soluble interleukin 2 receptors are released from activated human lymphoid cells *in vitro*. *J Immunol*, 1985;135(5):3172-3177
- Rubin LA, Jay G, Nelson DL. The released interleukin 2 receptor binds interleukin 2 efficiently. *J Immunol*, 1986;137(12):3841-3844
- Jacques Y, Le-Mauff B, Boeffard F, Godard A, Soullilou JP. A soluble interleukin 2 receptor produced by a normal alloreactive human T cell clone binds interleukin 2 with low affinity. *J Immunol*, 1987;139(7):2308-2316
- Brynskov J, Tvede N, Plasma interleukin-2 and a soluble shed interleukin-2 receptor in serum of patients with Crohn's disease. Effect of cyclosporin. *Gut*, 1990;31(7):795-799
- Mahida YR, Gallagher A, Kurlak L, Nawkey CJ. Plasma and tissue interleukin 2 receptor levels in inflammatory bowel disease. *Clin Exp Immunol*, 1990;82 (1):75-80
- Mueller C, Knoflach P, Zielinski CC. T-cell activation in Crohn's disease. Increased levels of soluble interleukin 2 receptor in serum and in supernatants of stimulated peripheral blood mononuclear cells. *Gastroenterology*, 1990;98(3):639-646
- Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol*, 1989;24(Suppl 170):2-6
- Sutherland LR, Martin F, Greer S. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and proctitis. *Gastroenterology*, 1987;92(6):1894-1898
- Hou J, Kong XT. Interleukin 6 bioassay by MTT colorimetry. *Chin J Med Lab Technol*, 1993;16:208-210
- Maeda M, Watanabe N, Neda H, Yamauchi N, Okamoto T, Sasaki H *et al.* Serum tumor necrosis factor activity in inflammatory bowel disease. *Immunopharmacol Immunotoxicol*, 1992;14(3):451-461
- Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*, 1991;32(8):913-917
- Mahmud N, O'Connell MA, Stinson J, Goggins MG, Weir DG, Kelleher D. Tumor necrosis factor- $\alpha$  and microalbuminuria in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol*, 1995;7(3):215-219
- Mazlam MZ, Hodgson H. Peripheral blood monocyte cytokine production and acute phase response in inflammatory bowel disease. *Gut*, 1992;36(6): 773-778
- Nielsen OH, Brynskov J, Bendtzen K. Circulating and mucosal concentrations of tumour necrosis factor and inhibitor(s) in chronic inflammatory bowel disease. *Dan Med Bull*, 1993;40(2):247-249
- Bouma G, Oudkerk Pool M, Scharenberg JGM, Kolkman JJ, von Blomberg BME, Scheper RJ *et al.* Differences in the intrinsic capacity of T-cells to produce the cytokines tumor necrosis factor alpha and beta in patients with inflammatory bowel disease and healthy controls. *Scand J Gastroenterol*, 1995;30(11):1095-1100
- Schreiber S, Raedler A, Conn AR, Rombeau JL, MacDermott RP. Increased *in vitro* release of soluble interleukin 2 receptor by colonic lamina propria mononuclear cells in inflammatory bowel disease. *Gut*, 1992;33(2):236-241
- Bouma G, Xia B, Crusius JBA, Bioque G, Koutroubakis I, von Blomberg BME *et al.* Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol*, 1996;103(3):391-396

# Protective effect of YHI and HHI-I against experimental acute pancreatitis in rabbits

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**Subject headings** pancreatitis/pathology; pancreatitis/therapy; Yuanhu; Huoxuehuayu; amylase/blood; interleukin-6/blood; microcirculation; rabbits

## Abstract

**AIM** To observe the protective effect of combined i.v. administration of Yuanhu injection (YHI) and Huoxuehuayu injection-I (HHI-I) against acute pancreatitis (AP) in rabbits.

**METHODS** Severe acute pancreatitis (SAP) was induced by retrograde infusion of artificial bile juice into biliary-pancreatic duct, and treated with YHI and HHI-I intravenously. The protective effect was judged by the survival time and rate, serum amylase, serum interleukin-6, pancreatic microcirculation and pathological alteration.

**RESULTS** Combined use of YHI and HHI-I could markedly increase the rabbits' 5-day survival rate after AP (83.3% in the treatment group and 33.3% in control). The serum amylase value ( $\bar{x}\pm s$ ) decreased to 1596.6U/L $\pm$ 760.50U/L in the 5th day from the high level (6320.83U/L $\pm$ 2614.12U/L) in the 1st day after AP in the treatment group, while in the control group the amylase activity in the 5th day was 2095.0U/L $\pm$ 1081.87U/L, being significantly different from that before AP (837.17U/L $\pm$ 189.12U/L). YHI and HHI-I also obviously improved the pancreatic microcirculation and lowered the serum interleukin-6 level, one of the indices of severe pancreatitis. Pathological examination indicated all the changes typical for AP in YHI and HHI-I treatment group were milder than those in the control.

**CONCLUSION** YHI and HHI-I used in combination might have protective effect against acute pancreatitis in rabbits.

## INTRODUCTION

Autodigestion of pancreatic enzymes in pancreatic tissues and pancreatic ischemia are two most important pathogenic factors in acute pancreatitis (AP). In order to lower the morbidity and mortality of AP, inhibition of pancreatic digestive enzymes and prevention and treatment of pancreatic ischemia are the two necessary therapies. Recently, many experimental researches and clinical trials are being addressed to these fields. Yuanhu (*Rhizoma Corydalis*) injection (YHI) is the most effective inhibitory Chinese medicine screened from the decoction used in our institute for AP. Huoxue Huayu Injection I (HHI-I) is one of best Huoxue Huayu (promoting blood circulation to remove blood stasis) decoctions. It has been demonstrated that YHI can inhibit the activity of trypsin and elastase and HHI-I can improve the pancreatic blood flow and oxygen supply<sup>[1]</sup>. In this experiment, we have observed the protective effect of YHI and HHI-I against AP in rabbits.

## MATERIAL AND METHOD

### *Animal model of AP*

The AP model in rabbit was produced according to Klar's method with slight modification<sup>[2]</sup>. Male white healthy rabbits weighing 2.5kg $\pm$ 0.4kg were used. After overnight fasting, the animals were anesthetized with intravenous 6% pentobarbital (20mg/kg) and intramuscular ketamine (25mg/kg). Under sterile condition, the abdomen was opened and biliary pancreatic duct at duodenum wall was found and inserted with a polyethylene catheter. A mixture of 5% sodium taurocholate, trypsin (24U/ml) and homologous blood (30  $\mu$ l/ml), incubated at 37  $^{\circ}$ C for 60min was infused at 0.8ml/kg body weight into the duct under 5.3kPa, at an interval of about 10min.

### *Reagent*

Sodium taurocholate (Sigma Co, USA); Trypsin (Boehringer Mannheim CO), Activity was determined by the Biological Department, Nankai University; and Kit of amylase (Zhongsheng Co, Beijing).

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Received 1997-12-30

### Instrument and assay method

Dynamic analyzer of Doppler laser microcirculation (JM-200, Jinke Co, Tianjin). Serum amylase activity was determined with UNIFAST analyzer. Serum interleukin-6 was assayed by the MTT method.

### Experimental design

Twelve rabbits were divided into treatment and control groups 6 each. During experiment, the abdomen was opened, the state of pancreatic microcirculation was determined and AP was then induced. Immediately, 30, 60, 90 and 120min after the AP was induced, microcirculation was evaluated once more. The abdomen was then closed and the survival time and rate were observed. Before AP and 1 and 2 days after the operation blood samples were obtained from auricle vein to measure the serum amylase and interleukin-6 levels. Normal saline was infused intravenously in control animals immediately after AP and 1 and 2 days after that. The treatment group was administered with YHI and HHI-I simultaneously at 1g/kg desolved in 10ml normal saline. When the animals died, sample of pancreas was harvested for pathological examination.

## RESULTS

### Survival time and survival rate

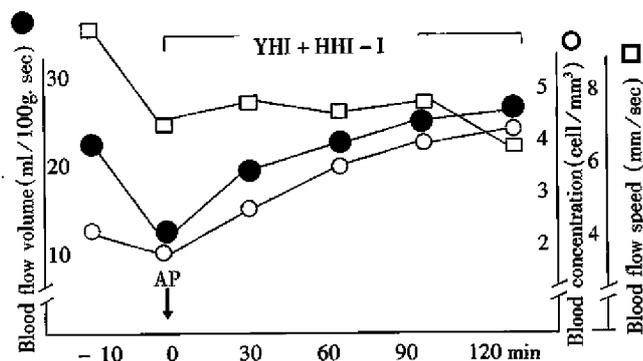
Two of 6 animals in the control group died 12 and 24 hours after AP was produced respectively. Another two died at 96 hours and the remaining survived till the end of experiment, with a 5-day survival rate of 33.3%. In treatment group, one rabbit died at 72 hours after AP and all the others survived, with a 5-day survival rate of 83.3%. An increased trend of survival rate was observed in treatment group but was not different significantly from the control group ( $P < 0.05$ ).

### Serum amylase

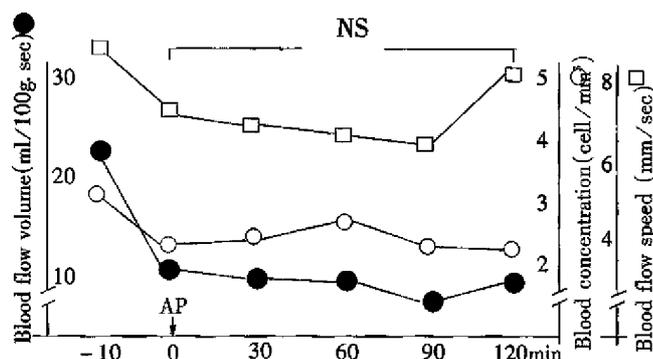
Serum amylase activity increased obviously in the control group at the 1st and 3rd day. At the 5th day it began to decrease, but still remained markedly higher than before AP. In treatment group, higher levels of amylase activity could be seen at first day after AP. At the 3rd day, however, it decreased significantly and almost reached to the normal level at the end of experiment (Table 1).

### Changes of pancreatic microcirculation

Immediately after AP, the microcirculation of pancreatic tissue became obviously deteriorated. In treatment group pancreatic blood flow was improved somewhat at 30, 60, 90 and 120min after AP was induced, but in the control group it was getting worse and worse (Figures 1 and 2).



**Figure 1** Effect of YHI and HHI-I on pancreatic blood flow, blood concentration, flow speed in rabbits with SAP.



**Figure 2** Effect of normal saline on pancreatic blood flow, blood concentration and flow speed in rabbits with SAP.

### Serum interleukin-6

All animals after AP had increased level of serum interleukin, which remained high in the control group at the 3rd and 5th day while in treatment group it decreased more significantly than the control group (Table 2).

### Pathologic examination

Most parts of pancreatic and fat tissue in control group had necrosis and large areas of hemorrhage. In the necrotic tissues there were many foci of PMN infiltrations and abscesses. The necrosis dimension and the number or size of hemorrhage in the treatment group were obviously lower than in the control. The extent of PMN leukocyte infiltration and the size or number of abscesses were also milder, with some more intact pancreatic lobules. Even though some lobules were destroyed severely, the was still intact (Table 3).

**Table 1 The effect of YHI and HHI-I on serum amylase of rabbit with SAP ( $\bar{x}\pm s$ )**

Group	Before AP	After AP (d)		
		1	3	5
Treatment(U/L)	867.67±374.62	6320.83±2614.12(6) <sup>b</sup>	4774.0±1859.36(5) <sup>b</sup>	1596.6±760.50(5)
Control(U/L)	838.17±189.12	7586.67±3745.03(5) <sup>b</sup>	7380.0±3687.41(4) <sup>b</sup>	2095.0±1081.87(2) <sup>a</sup>

( ) number of survived, <sup>a</sup> $P<0.05$  and <sup>b</sup> $P<0.01$  compared with before AP.

**Table 2 The effect of YHI and HHI-I on serum interleukin-6 in AP rabbits ( $\bar{x}\pm s$ )**

Group	Before AP	After AP (d)		
		1	3	5
Control(U/L)	32.37±9.11(6)	69.75±19.18(5)	72.35±10.14(4)	70.25±10.34(2)
Treatment(U/L)	30.0±16.40(6)	59.50±17.59(6)	50.33±16.57(5) <sup>a</sup>	46.50±14.94(5) <sup>a</sup>

( ): animal number, <sup>a</sup> $P<0.05$  compared with control.

**Table 3 Effect of YHI and HHI-I on pathologic changes of pancreas in rabbits with SAP**

	Control group	Treatment group
Lobule structure	Destroyed totally	Relatively intact
Acinus	Destroyed	Atrophied
Intralobular duct	Destroyed seriously	Existed, expanded
Interlobular duct	Residually existed	Existed
Fatty tissue	Destroyed totally	Mild-moderately destroyed
Inflammatory cell	Abscess formed	A few abscesses and WBC
Focal hemorrhage	Large size	Small size and a few number
Interstitial hyperplasia of fibrous tissue	A few around abscess and necrosis	Much more around abscess and necrosis
Island	No	Existed

## DISCUSSION

Aprotinin was an antiprotease used firstly in clinical practice. However, the therapeutic effect for SAP was not satisfactory possibly because its molecular weight was too heavy to enter into acinar cells, and the half-life was short in blood circulation. Subsequently, some other antiproteases with low molecular weight were found such as Gabexate Mesilate (GM,  $Mr$  417.5) and Camostat (FOY-305,  $Mr$  494.5). Several experiment studies showed that GM was a very valuable medicine for prevention and treatment of AP, especially when it was before the disease was induced. The results of the first multi-center clinical research was also encouraging. A report of a case-control clinical trial indicated that GM (900mg/d) could decrease the number of operations associated with AP. Owing to the relative low dosage used, Büchler and his co-workers increased the dose of GM to 4000mg/d in 223 patients with moderate to severe pancreatitis (average Ranson score at 3.7) for 7 days, and found there was no difference in the mortality, pancreatitis-associated operation, hospitalized days, complication score and some parameters of biochemical assays as compared with those in the control group. Their conclusion was that GM was not effective in prevention of complications and

death<sup>[3]</sup>. According to the result of the recent retrospective reviews GM could obviously decrease the incidence of complications in operations. Being expensive in price could only be used in those patients at high risk to create a better cost-effect ratio<sup>[4]</sup>.

Possessing wider enzyme-inhibitory spectrum, FOY-305 could markedly increase the survival rate in experimental SAP animals<sup>[4]</sup>. The beneficial effect of FOY-305 could be obtained by FOY-305 and its metabolites which could be absorbed from intestine into blood circulation; by stimulating the endogenous secretion of secretin and CCK to increase the exocrine pancreas; and decreasing the intra-acinar vacuolization. Recently, it was demonstrated that oral administration of FOY-305 could also decrease the extent of pancreatic edema and vacuolization of acinar cells in AP rats induced by cerulein. FOY-305 could decrease the level of trypsin in pancreatic tissues as well. By means of above actions, FOY-305 might be beneficial to cerulein-induced AP rats<sup>[6]</sup>. To date no information was found on clinical application of FOY-305.

Antiprotease was not satisfactory for treating AP clinically made the researchers suspect the correctness of the concept of enzyme-inhibition in treatment AP. It was important to recognize that

the patients could not be admitted to hospital 12 to 24 hours after the occurrence of inflammation, during which the patients' kinin and complement system were already activated, capillary endothelium cells injured, some enzymes released from activated leukocytes. All these were related to the poor therapeutic effect of trypsin antagonist used after that period.

It was demonstrated that Chinese medicines, such as Banxia (*Penellia ternata*), Dahuang (*Rheum palmatum*), Huangqin (*Scutellaria baicalensis*), Hulan (*Rhizoma picrorhizae*) and Baishao (*Radix paeoniae alba*), that had enzyme inhibitory actions. The results from our institute indicated that among the 9 medicines investigated, Yuanhu (*Rhizoma corydalis*) was the most potent inhibitor for trypsin activity.

Pancreatic ischemia was the etiologic and deteriorating factor for AP, and played an important role in necrosis of pancreas in the early period of AP<sup>[7,8]</sup>. Pancreatic ischemia imposed on pancreatic edema could induce necrotic AP. After AP was induced by ligation of pancreatic duct and overstimulation to exocrine pancreas, blockade of pancreatic artery for only 5 minutes could induce parenchymal necrosis of pancreas. Temporary ischemia of pancreas made the acinar cells more sensitive to the degradation effect of enzyme and more easy to form necrosis. Mithofer used bleeding method to induce blood hypotension (30mmHg, 30min) in rats, resulting in pancreatic ischemia, and found the serum amylase increased in 1 hour after ischemia and rose at the 4th hour. Trypsinogen activative peptide (TAP) of pancreatic tissue was also increased at the first hour, and further increase occurred at 24 hour. Pancreatic edema and necrosis were observed as well. In our opinion, besides deteriorated action, pancreatic ischemia itself, if serious, could also initiate AP<sup>[9]</sup>. On the other hand, changes of pancreatic blood circulation could be found in the course of AP. That reported that 5min after AP was induced, segmental spasm of arteriole and venule of acinus could be observed, and there was blood stasis at 15min. Using vital microscopy technique, Klar observed that perfusion of pancreatic capillaries of rabbit diminished gradually 30min after AP and stopped completely at 3 hours<sup>[2]</sup>. Klar also found that isovolemic hemodilution with dextran 60 could maintain pancreatic capillary perfusion, and the relative number of vacuolization in acinar cells and parenchymal edema were both obviously lower than in the control group. Clinically, Klar carried out a

trial treatment in 13 patients with SAP (mean Ranson score 4.6). The time interval between appearance of symptoms of AP to the beginning of hemodilution was 38 (19-90) hours. The hematocrit (HCT) decreased to 34%±6% at the first hour and 31%±4% at the second hour. The mean exchanging time was 45.70 minutes and the exchanging volume was 750ml-170ml. The ultimate results showed a mortality of only 7.7%. Therefore, we think that the isovolemic hemodilution had no harmful side-effect and could be used in further clinical trial<sup>[11]</sup>.

*In vitro* screening test in our research indicated that YHI was the most potent inhibitor for pancreatic digestive enzymes among the nine screened Chinese herbs. Optimized selection experiment showed that HHI-I was the most beneficial herb for both intestinal and pancreatic blood flow and oxygen consumption. In this experiment satisfactory results were obtained in treatment of AP in rabbits with YHI and HHI-I in combination. These were related with their enzyme-inhibitory action and improvement of pancreatic blood circulation.

However, this is a preliminary experiment only, further investigation on toxicity, side-effect, effective dosage, and pharmacodynamics etc. are needed.

## REFERENCES

- 1 Zhao LG, Wu XX, Zhu ZM, Chen YL, Liu FS, Chen JT. Screening and pharmacologic study of new Chinese medicine treating severe acute pancreatitis. *China Natl J New Gastroenterol*, 1996;2(3):136-138
- 2 Klar E, Herfarth C, Messemer K. Therapeutic effect of isovolemic hemodilution with dextran 60 on the impairment of pancreatic microcirculation in acute biliary pancreatitis. *Ann Surg*, 1990;211(7):346-353
- 3 Büchler M, Malfertheiner P, Waldemar UHL, Jürgen S, Fritz S, Guido A *et al.* Gabexate mesilate in human acute pancreatitis. *Gastroenterology*, 1993;104(4):1165-1170
- 4 Messiru A, Rampazzo R, Scroccaro G, Olivato R, Bassi C, Falconi M *et al.* Effectiveness of gabexate mesilate in acute pancreatitis. *Dig Dis Sci*, 1995;40(4):734-738
- 5 Leonhared V, Seidensticker F, Fussek M, Stockmann F, Creutzfeldt W. Camostat (FOY-305) improves the therapeutic effect of peritoneal lavage on taurocholate induced pancreatitis. *Gut*, 1990;31(8):934-937
- 6 Kisfalvi K, Papp M, Friess H, Büchler M, Goracz UG. Beneficial effects of preventive oral administration of camostat on cerulein-induced pancreatitis in rats. *Dig Dis Sci*, 1995;40(3):546-547
- 7 Klar E, Messmer K, Warshaw AL, Herfarth C. Pancreatic ischemia in experimental acute pancreatitis: mechanism, significance and therapy. *Br J Surg*, 1990;77(11):1205-1210
- 8 Kyogoku T, Manabe T, Tobe T. Role of ischemia in acute pancreatitis. Hemorrhagic shock converts edematous pancreatitis to hemorrhagic pancreatitis in rats. *Dig Dis Sci*, 1992;37(9):1409-1417
- 9 Mithofer K, Castillo CF, Frick TW, Foitzik T, Bassi DG, Lewandrowski KB *et al.* Increased intrapancreatic trypsinogen activation in ischemia-induced experimental pancreatitis. *Ann Surg*, 1995;221(4):364-371
- 10 Klar E, Mall G, Messemer K, Herfarth C, Rattner DW, Warshaw AL. Improvement of impaired pancreatic microcirculation by isovolemic hemodilution protects pancreatic morphology in acute biliary pancreatitis. *SGO*, 1993;176(2):144-150
- 11 Klar E, Foitzik T, Buhr H, Messmer K, Herfarth C. Isovolemic hemodilution with dextran 60 as treatment of pancreatic ischemia in acute pancreatitis. *Ann Surg*, 1993;217(4):369-374

# Therapeutic effect of Zijin capsule in liver fibrosis in rats

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**Subject headings** liver cirrhosis/therapy; Zijin capsule; integrated TCM-WM; diseases models, animal; rats

## Abstract

**AIM** To confirm the therapeutic effect of Zijin capsule on liver fibrosis in rat model.

**METHODS** Model group: Bovine serum albumin (BSA) Freund's incomplete adjuvant 0.5ml was injected subdermally at d1 d15 d22 d29 and d36 for primary sensitization. Seven days after the fifth injection, BSA antibody in the serum was detected by double agar diffusion method. Normal saline of 0.4ml was injected through cauda vein to BSA antibody-positive rat twice a week for fifteen times. Traditional Chinese medicine (TCM) decoction group and Zijin capsule group: In the attack injection period, Chinese medicinal decoction or Zijin capsule was given ig, the others were the same as in the model group. NS was used in the control group. The collagen content of rat liver was determined by Bergman's method and expressed as  $\bar{x} \pm s$ . The liver pathological changes were divided into four grades and expressed as the average of the total rank sum.

**RESULTS** The collagen content (mg/g) of the liver in the control group ( $7.2 \pm 1.9$ ) was significantly lower than that in the other groups; it was higher in the model group ( $31.7 \pm 16.6$ ) than that in the two therapeutic groups; and lower in Zijin capsule group ( $9.7 \pm 2.8$ ) than that in the TCM decoction group ( $11.5 \pm 5.3$ ). The pathological changes were more aggravated in the model group (37.4) than those in the two therapeutic groups; and more severe in the TCM decoction group (30.2) than in the Zijin capsule group (22.9).

**CONCLUSION** The therapeutic effect of Zijin capsule on the model was confirmed.

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\*Supported by the key Project Fund of Scientific Committee of Hubei Province.

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Received 1998.02.25

## INTRODUCTION

The pathological changes of the rat liver fibrosis induced by bovine serum albumin (BSA) injections are similar to those in human portal cirrhosis. Compared with the TCM decoction proved effective in clinic the therapeutic effect of Zijin capsule in rat liver fibrosis was observed.

## MATERIALS AND METHODS

### Materials

Wistar rats (female 30, male 30, 150g-210g) were purchased from Hubei Medical Institute. BSA was product of Shanghai Medical Testing Agent Plant, prepared as 18g/L in normal saline, clean from bacteria through filtration, and stored at 4°C. Freund's incomplete adjuvant: One gm lipid from sheep hair (CP Third Lipid Company in Shanghai) was mixed with 2 gm liquid paraffin (Fushan Chemical Industry), sterilized in a steam autoclave and stored at 4°C. L-hydroxyproline, standard sample, was from Biochemistry Institute of the Chinese Academy of Sciences. Zijin capsule, prepared mainly from *herba swertiae puniceae* and *endothelium corneum gigeriae galli* in Hubei College of Traditional Chinese Medicine. The concentration of crude drugs was

5g/1ml. The TCM decoction provided by the Department of Infectious Diseases, the Affiliated Hospital of Hubei TCM College, containing mainly *Radix astragali*, *Radix condonopsis pilosulae*, *Rhizoma atractylodis macrocephalae*, *Rhizoma polygoniti*, *fructus lycii*, *fructus corni*, *radix rehmanniae* and *Radix rehmanniae preparata*.

### Method

Wistar rats with free access to water were randomly divided into four groups.

Control group ( $n = 12$ ). Normal saline was used for immunological primary (sensitization) and second (attack) injection instead of BSA, the others were the same as those in the model group.

Model group ( $n=12$ ). BSA Freund' incomplete adjuvant 0.5ml was injected subdermally at d<sub>1</sub> d<sub>15</sub> d<sub>22</sub> d<sub>29</sub> and d<sub>36</sub> for primary sensitization. Seven days after the fifth injection, BSA antibody in rats' serum was detected by double agar diffusion method. BSA of 0.4ml in normal saline was administered once through cauda vein for attack injection in BSA antibody-positive rats, twice a week for fifteen times, the concentration of BSA

was 5.00, 5.25, 5.50, 5.75, 6.00, 6.25, 6.50, 6.75, 7.00, 7.25, 7.50, 8.50, 9.00, 9.50 and 10.00g/L. The rats were given 10ml · kg · d N.S. ig at the same period. All animals were killed with decollation nine days after the last injection. The livers were taken for biochemistry detection and morphological observation. The whole period was 95 days.

**TCM decoction group ( $n = 18$ ).** In the attack injection period, the Chinese medicinal decoction equivalent to 36g crude drugs/kg · d was given ig, the others were the same as those in the model group.

**Zijin capsule group ( $n = 18$ ).** In the attack injection period, Zijin capsule, equivalent to 12.8g crude drugs/kg · d, was given ig, the others were the same as those in the model group.

The collagen content in rat liver was determined by Bergman's method<sup>[1]</sup>, and expressed as  $\bar{x} \pm s$  and analyzed by *t* test. Value of  $P < 0.05$  was considered at a significant level. The rat livers were embedded with paraffin and stained with HE. The pathological changes were classified into four grades according to the quality and quantity of the liver histological features, expressed as the average of the total rank sum in each group and analyzed by *H*-test. Value of  $P < 0.05$  was considered at a significant level.

## RESULTS

### *Collagen content in rat liver*

The indexes are shown in Table 1. The collagen content was significantly lower in the control group than that in the model group ( $P < 0.01$ ), the TCM decoction group ( $P < 0.05$ ) and Zijin capsule group ( $P < 0.05$ ); it was higher in the model group than that in the TCM decoction group ( $P < 0.01$ ) or Zijin capsule group ( $P < 0.01$ ); and not significantly lower in Zijin capsule group than that in the TCM decoction group ( $P > 0.05$ ).

**Table 1** The collagen content in rat liver ( $\bar{x} \pm s$ )

Groups	Dosage	<i>n</i>	Content (mg/g)
Control	10.0ml/(kg · d)	10	7.2±1.9
Model	10.0ml/(kg · d)	6	31.7±16.6 <sup>b</sup>
TCM decoction	36.0g/(kg · d)	12	11.5±5.3 <sup>ad</sup>
Zijin capsule	12.8g/(kg · d)	9	9.7±2.8 <sup>ad</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs control group; <sup>d</sup> $P < 0.01$ , vs model group.

### *The pathologic change grade in rat livers*

After a comprehensive observation on the pathologic changes, a distinct grading standard was defined as follows:

**Grade 0 (normal rat liver)** Liver capsula was a thin connective tissue. The parenchyma consisted of hepatic lobules and portal areas. The hepatic lobules were similar to round balls, their central veins, laminae of hepatocytes (radiated arrange) and sinusoids were normal and clear. The hepatic lobule boards were hepatic cell layers as limiting laminae. There was hepatic sinusoid between the hepatic laminae, their endothelia were distributed regularly, Kupffer's cells can be seen obviously. The ratio of the hepatic laminae to the hepatic sinusoids was 3 to 2. The hepatocytes are polygonal cells, cytoplasm was normal acidophil, the hepatic nuclears in round shape, the chromatin distributed rarefactionally along the nuclear membrane with nucleole. The portal area was mainly connective tissues, including the interlobular hepatic artery, the portal vein branches and interlobular bile ducts. Some lymphocytes and plasma cells had infiltrated into the portal areas. The necrosis within a few hepatocytes and their lymphocyte or plasma cell infiltration (spotty necrosis) were occasionally observed in one hepatic lobule or two. There were many red-dyed microgranules (slight cloudy swelling) in some hepatocytes.

**Grade 1 (liver injury change).** Serious and extensive changes were present as the degeneration and necrosis of hepatocytes and the congestion or bleeding in the hepatic sinusoid. Cloudy swelling of hepatocytes: enlarged volume, round shape, many small red-dyed granules in hepatic cytoplasm were observed; hepatic laminae became wider, and hepatic sinusoid were pressed to ischemia. Thin hepatic cytoplasm: the hepatocyte volume became larger, red-dyed cytoplasm thinner and less homogeneous; the nuclears stained dim; and the ratio of hepatic laminae to the hepatic sinusoids became higher. Ballooning degeneration of hepatocytes: the volume of hepatocytes became extremely large, round shaped; the cytoplasm appeared empty (light transparency) like a balloon; nuclear not located at the cell centre. Lytic necrosis of hepatocytes: the ballooning degeneration of hepatocytes further developed to karyopyknosis, karyorrhexis and karyolysis; the whole structure of the hepatocyte body even disappeared, only the network of the reticular fibers remained; however, the lymphocytes infiltration in necrotic focus was not obvious; and the necrosis of hepatocytes did not occur at the limiting laminae in the hepatic lobules. The congestion and bleeding in the hepatic sinusoid: the hepatic sinusoids were expanded and filled with blood. The bleeding in Disse's cavity was extensive. Two kinds of congestion and bleeding in hepatic lobules were observed in these cases, one

distribution was limited around central veins, which was seldomly observed, the other mainly appeared near the limiting laminae of hepatic lobules, which were predominant. At the same time, there were light hyperemia, edema and fibrous proliferation in the portal areas (Figure 1).

Grade 2 (liver prefibrosis). The necrosis within a few hepatocytes occurred at the limiting laminae (piecemeal necrosis); and the necrosis range may be expanded to a large region of hepatic necrosis which connected the central veins or portal areas (bridging necrosis). Serious congestion and bleeding near the limiting laminae in the hepatic lobules were common. The hepatic laminae became thinner in the same region accompanied by hepatocyte atrophy, cloudy swelling and fatty degeneration and the hepatocytes even disappear in the same region. The congestion may be connected with the central veins or portal areas (bridging congestion). In those cases, the Kupffer's cells in the hepatic sinusoid enlarged and proliferated obviously with more processes. In the portal areas, fibroblasts proliferated obviously and collagen increased, which made the boundary in the normal hepatic lobules more clear, but they were not stretching into the hepatic lobules through the limiting laminae.

Grade 3 (liver fibrosis). The hepatocyte injury (degeneration and necrosis) at the limiting laminae has advanced obviously, while the proliferated collagen at portal areas has invaded into the hepatic lobules along with the injured limiting laminae. It was called as liver fibrosis while the proliferated collagen has not been completely contacted each other and absolutely separated the hepatic lobules. It was the cirrhosis when the proliferated collagen has been contacted each other completely, and absolutely separated the hepatic lobules, leading to the formation of pseudolobules (many round islands of hepatocytes). Their hepatic laminae have not radiated regularly, the central vein dystopy (asymmetry, disappear or several veins), the structure of portal areas was located in the pseudolobules, there were some atrophy, fatty degeneration and necrosis of the hepatocytes in the pseudolobules; they were separated continuously with the proliferated collagen, the cholestatic bile capillaries appeared and their bile thrombus formed. Among the pseudolobules, the fibrous bands were continuous, homogeneous and delicate; and there are more infiltrated lymphocytes. The collagenous fibers were stained bright-red (Figure 2).

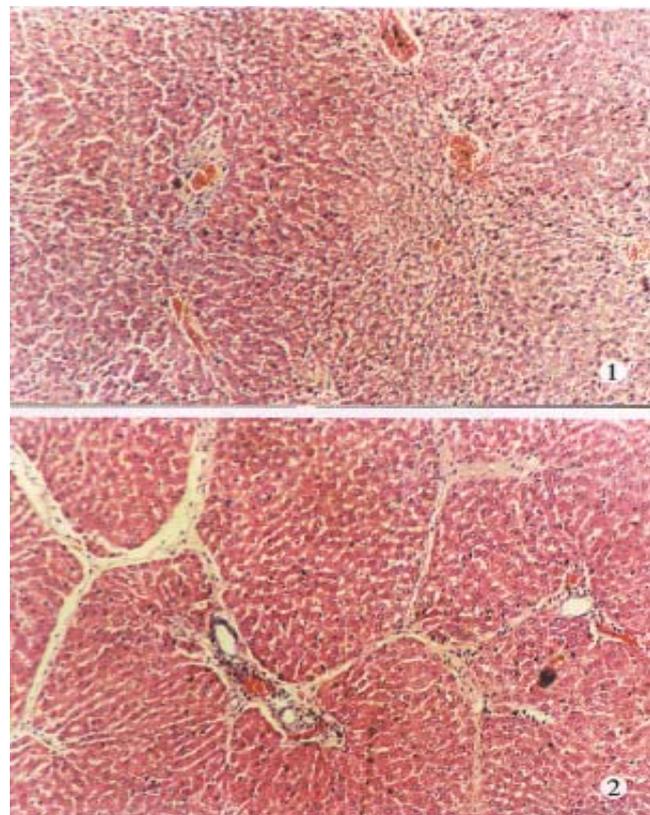
According to the grading, *H* test was used to analyse statistically the liver pathological changes among those groups. The pathological changes are more moderate in the control group than those in the other three groups ( $P < 0.01$ , Table 2). They were more severe in the model group than those in the two therapeutic groups; and more aggravated in

the TCM decoction group than those in the Zijin capsule group.

**Table 2** The pathological change grades in rat liver

Groups	Dosage	n	Pathologic grade				Rang sum	$\bar{x}$ -Rang sum
			1	2	3	4		
Control	10.0ml/kg · d	10	0	0	0	10	55.0	5.50
Model	10.0ml/kg · d	0	1	3	5	9	337.0	37.44 <sup>b</sup>
TCM decoction	36.0g/kg · d	0	5	5	3	13	393.0	30.23 <sup>bc</sup>
Zijin capsule	12.8g/kg · d	0	11	4	0	15	343.0	22.87 <sup>bde</sup>

<sup>b</sup> $P < 0.01$  vs control group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs model group; <sup>e</sup> $P < 0.05$  vs TCM decoction group (*H* test).



**Figure 1** Grade 1 pathologic change (liver injury).HE×250

**Figure 2** Grade 2 pathologic change (liver fibrosis).HE×250

## DISCUSSION

Rat liver fibrosis induced by BSA injections After BSA sensitization injection (sc), the rats were intravenously injected with BSA as attack injection, the CIC (the circulating immune complex formed with BSA antigens and their antibodies) or the remained antigens (BSA) has deposited in some tissues of the rats, leading to classical type III or/and type II of the allergic reactions, subsequently local tissue injuries. The administration of antigen, including the route, time and dose of the injected antigen decides the location and features of injury lesions. Based on the references about the experimental methods, BSA injected with various

dosages, at different times and dates produced serious injuries (37/37) of livers and even obvious fibrosis or cirrhosis (8/37). The locations and features of the pathologic changes have suggested the pathogenesis of cirrhosis, i.e., the pathological changes were advanced near the limiting laminae in the hepatic lobules, because the CIC deposited earlier and more seriously at the location according to the circulative dynamics in the hepatic lobules. The features of the pseudolobules (such as shape, size, laminae arrange, cellular injuries) and the fibrous bands (delicate, homogeneous) were similar to the pathological changes in human portal cirrhosis. The hepatocytes did not regenerate to form pseudobobules obviously. However, the liver structures were normal ( $P < 0.01$ ) and the collagen content was low ( $P < 0.05$ ) in the control group. The results indicate that the rat cirrhosis models induced by BSA injections according to the immunological principles are successful<sup>[1,2]</sup>.

#### ***The therapeutic effect of Zijin capsule on rat liver fibrosis***

In contrast to the model group, the collagen was low ( $P < 0.01$ ), and the pathologic changes were slight ( $P < 0.05$  or  $P < 0.01$ ) in Zijin capsule group

as well as the TCM decoction group. It confirms the effect of Zijin capsule to treat the rat liver fibrosis. Compared with that in the TCM decoction group with confirmed therapeutic effect in human liver fibrosis<sup>[3]</sup>, the collagen content remained the same level ( $P > 0.05$ ) in Zijin capsule group. The main active components<sup>[4]</sup> of Zijin capsule can increase the permeability of capillary, lead to the CIC depositing in different locations, enhance the ability of the mononuclear phagocyte system (it may promote the elimination of the CIC from the circulating blood), activate the hepatocyte metabolism to normalize their biology, and protect the hepatocytes from injuries. All these may be associated with the therapeutic effect of Zijin capsule in the rat liver fibrosis induced by BSA injections.

#### **REFERENCES**

- 1 Zhu QG, Fang BW, Wu HS, Lan SL, Fu QL. The study of the immunity liver fibrosis animal model induced by bovine serum albumin. *Chin J Pathol*, 1993; 22(2):121-122
- 2 Wang BE. The study of the experimental model in immunity liver fibrosis. *Chin Med J*, 1989;69(9):503-506
- 3 Fang BW, Zhu QG, Zhu JE, Wu HS, Lan SL, Fu QL. The experimental study of the drugs for supplementing Qi and activating blood circulation in the liver fibrosis animal model by bovine serum albumin. *J Chin Traditio West Med*, 1992;12(12):738-739
- 4 Chen JC, Huang XS. The chemical component analysis of Swertia Punicea Plant in Hubei Province. *Chin Materia Med*, 1990;13(2):29-31

# A new method of extensive resection for gastric carcinoma: selective type III operation

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**Subject headings** stomach neoplasms/surgery; lymphatic metastasis; lymph node excision/method; life quality; survival rate; prognosis

## INTRODUCTION

One of the most important factors related to the prognosis of progressive gastric carcinoma is the metastasis to lymph nodes. Since 1968, we have made researches on the lymphatic metastasis and the proper scope of resection of lymph node in progressive gastric carcinoma. On the basis of this research, we designed a new method of extensive radical operation with special reference to the resection of lymph nodes.

## MATERIALS AND METHODS

### *Theoretical basis of selective D3 operation*

Since 1968, we have studied comprehensively the lymphatic metastasis in gastric carcinoma in 181 patients and reviewed 1317 cases reported in literature<sup>[1,2]</sup>. There were 784 cases of carcinoma in gastric antrum, and the overall rate of lymphatic metastasis in stations 1 and 2 were 16.5%-58.1%. The rate of lymphatic metastasis in station 3 was group 12, 20.7%; group 14, 13.4%; group 10, 13.2%, group 15, 12.8%; group 13, 11.6%; group 11, 9.0%, group 2, 4.1%; and in station 4: group 16, 3.7%. There were 481 cases of carcinoma in the gastric body, the overall rate of lymphatic metastasis were 13.5%-60.4% in stations 1 and 2. The rate of lymphatic metastasis in station 3 was: group 12, 12%; group 13, 4.9%; group 14, 4.3%; group 15, 2.4%; and in station 4: group 16, 3.8%. There were 162 cases of carcinoma in gastric cardia,

the overall rate of lymphatic metastasis in stations 1 and 2 were 8.1%-60.2%. The rate of lymphatic metastasis in station 3 was group 12, 7%; group 11, 5%; group 13, 2.5%; group 14, 15 and in station 4: group 16, 1%. There were 71 cases of carcinoma in the whole stomach, and the overall rate of lymphatic metastasis in stations 1, 2, 3 were 8.6%-80.3%.

According to the above results, in cases of progressive gastric carcinoma, the resection of only station 2 lymph nodes will result in incomplete resection of lymph node metastases, and if routine resection of station 3 lymph nodes is performed, the operational injury and postoperative complications will be increased, leading to unnecessary total gastrectomy. All these may influence the quality of post-operational life. On the basis of our study and with reference to the Japanese Clinical Pathological Standard for Gastric Carcinoma, we designed selective D<sub>3</sub> operation.

### *The scope of lymph node resection in selective D3 operation*

This operation is characterized by the routine resection of lymph nodes in stations 1 and 2, and high lymph nodes metastasis rate in station 3.

In the cases of carcinoma of gastric antrum, the lymph nodes in groups 3, 4, 5 and 6 in station 1 and groups 1, 7, 8 and 9 in station 2, and the groups 11, 12, 13, 14, 15 in station 3 should be resected. The groups 2 and 10 lymph nodes in station 3 and group 16 in station 4 should not be routinely resected, unless they are suspected of having metastasis. In the cases of carcinoma of gastric body, the lymph nodes in groups 3, 4, 5 and 6 of station 1 and groups 2, 7, 8, 9, 10 and 11 of station 2 and groups 12 and 13 of station 3 should be resected. The lymph nodes in groups 14 and 15 in station 3 and group 16 in station 4 are not routinely resected, unless they are suspected to have metastasis. In the cases of carcinoma of gastric cardia, the lymph nodes in groups 1, 2, 3 and 4 of station 1 and groups 5, 6, 7, 8, 9, 10, 11 and 110 of station 2, and groups 12 of station 3 should be resected. The lymph nodes in groups 13, 14, 15 and 111 of station 3 and group 16 of station 4 are not routinely resected, unless they are suspected to have metastasis.

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**Presented at the 8th Sino-Japanese Conference of Gastroenterological Surgery, 1996**

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Received 1997-06-10 Revised 1997-07-26

## RESULTS

### *Complications and mortality*

In the 834 cases of gastric carcinoma treated from 1960 to 1982 in our hospital, the rates of complication treated with different operational modalities were D<sub>1</sub> 2.4% and D<sub>2</sub> 4.7%; and selective D<sub>3</sub> 6.7% and D<sub>3</sub> 6.7%, with no significant statistical difference ( $P>0.05$ ). The mortality rates were D<sub>1</sub> 0.7% and D<sub>2</sub> 2.3%; and selective D<sub>3</sub> 2.1% and D<sub>3</sub> 6.7%, with no significant statistical difference ( $P>0.05$ )<sup>[3]</sup>. No complications and death occurred. In the 216 cases of stage III carcinoma of gastric antrum and body treated from 1972 to 1989 with D<sub>2</sub> or selective D<sub>3</sub> in our hospital.

### *Survival time*

In the 834 cases of gastric carcinoma from 1960 to 1982, no significant statistical difference was observed in the 243 stage I and II cases, whether treated with D<sub>1</sub>, D<sub>2</sub> or selective D<sub>3</sub>. The 5-year survival rate were 2.8%, 49.2%, 68.3% and 83.8%, respectively in the stage III gastric carcinoma treated with D<sub>1</sub> in 139 cases, D<sub>2</sub> in 181 cases, selective D<sub>3</sub> in 88 cases and D<sub>3</sub> in 6 cases. The 5-year survival rate in cases treated with D<sub>3</sub> and selective D<sub>3</sub> were significantly higher than that of cases treated with D<sub>1</sub> and D<sub>2</sub> ( $P<0.01$ ), but no significant difference was noted between cases treated with D<sub>3</sub> and selective D<sub>3</sub> ( $P>0.05$ ). Among 216 cases of stage III gastric antrum and body carcinoma managed from 1975 to 1989 in our hospital. The 5-year survival rate was 35.7% in 114 cases treated with D<sub>2</sub>, and 56.3% in 102 cases treated with selective D<sub>3</sub>, difference was significant statistically ( $P<0.01$ ).

## DISCUSSION

Surgical resection is the treatment of choice for gastric carcinoma and the resection of lymph nodes is a very important part of the operation. To reduce the residual lymphatic metastasis as much as possible, we conducted a research into the lymph node metastasis of gastric carcinoma, and found that there was a close relationship between the site of tumor, the depth of invasion, the size of tumor and the biological behavior of the tumor. And there was a definite role of lymph node metastasis, except

for the carcinoma involving the whole stomach.

Based on the research, we designed the selective D<sub>3</sub> operation. This operation includes mainly: a. The complete resection of lymph nodes in stations 1 and 2, and those of higher rate of metastasis in station 3. b. As to the low lymph node metastasis rate of stations 3 and 4, whether they should be resected or not may be judged by if any metastasis was found during the operation in combination with the clinical pathological factors. No statistical difference of complication and mortality rate was noted between selective D<sub>3</sub> and D<sub>1</sub> and D<sub>2</sub> modality. Long-term clinical practice showed that the selective D<sub>3</sub> operation can significantly prolong the survival of stage III cases and part of the stage IV cases, with significant statistical difference from D<sub>1</sub> and D<sub>2</sub>. No significant statistical difference in survival time was noted in comparison with D<sub>3</sub>, but the operational injury was less severe and unnecessary resection of whole stomach can be avoided in part of the selective D<sub>3</sub> cases with better quality of post-operational life.

Through more than 20 years of practice, we consider the indications for selective D<sub>3</sub> operation are: a. carcinoma with invasion to the serosa without involvement of liver and peritoneum; b. direct invasion to neighboring tissues and organs, which can be radically resected by combined resection; and c. minor metastasis to peritoneum close to the primary lesion and isolated metastasis to liver, which can be completely resected. Contraindications: a. carcinoma with invasion to mucosa and submucosa only. In case except there was metastasis to lymph nodes in station 3, D<sub>2</sub> was used routinely; b. carcinoma involving whole stomach (>2 regions). If these lesions can be completely resected, D<sub>3</sub> was used; and c. extensive metastases to liver and peritoneal cavity.

## REFERENCES

- 1 Lin CH, Wang RS, Ma XZ, Qian YQ, Chen QS, Zhang YT *et al*. Gastric carcinoma study of lymphatic metastasis. *Chin Med J*, 1984;97(10):741-746
- 2 Lin CH. Gastric carcinoma study of lymphatic metastasis. *J Abdom Surg*, 1992;5(1):43-44
- 3 Lin CH. Clinical experience in the surgical treatment of 1399 cases of gastric carcinoma. *Chin Med J*, 1987;100(4):273-280

# Expression of nm23 gene in hepatocellular carcinoma tissue and its relation with metastasis

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; nm23 gene; gene expression; neoplasm metastasis; immunohistochemistry

## INTRODUCTION

Among the mostly expressed 23 genes in nonmetastatic tumors, *nm23* had the highest frequency. Steeg *et al*<sup>[1]</sup> first identified and cloned its complementary DNA and confirmed that its lower expression was related to the high metastatic activity of melanoma cell lines. Many studies found afterwards that the expression of *nm23* at the RNA or protein level was inversely correlated with the development of metastasis or poor clinical course in cohorts of several human tumor types, including breast, colorectal and gastric carcinomas. But the effects of *nm23* on metastasis of hepatocellular carcinoma (HCC) is still unclear. In this study we have investigated *nm23* expression in HCC with immunohistochemical techniques and the correlation between its expression level and metastatic progression.

## MATERIALS AND METHODS

### Subjects

Specimens of 24 cases of human HCC were obtained from surgical resections in Tongji Hospital. Observations were carried out on tissues from tumor areas, nonneoplastic areas and their boundary areas when available. Ten of them showed cancer cell emboli in portal vein or metastasis in portal lymph nodes or in distant organs, e.g. in the lung. Fourteen cases without metastasis were characterized by no findings of tumor invasion into the surrounding tissues at operation or no metastasis outside the liver by X-ray and sonography. The samples were fixed with 4% paraformaldehyde and embedded with paraffin. Successive sections were

stained with HE, as well as immunohistochemically with the SP method. The staining was considered negative (-) when no cells were stained on the section, and weakly (+), moderately (++) and strong (+++) positive, when a few, more and a lot of cancer cells were darkly stained, respectively.

## RESULTS

The positive signal revealed brown grains in cytoplasm of tumor cells. *nm23* protein expressed highly in HCC, but was not obviously related to the degree of malignancy histologically. The positive rate was 67% (16/24). The expression of *nm23* was heterogeneous in different cancer cell nodules and in the same nodule. The positive cells presented focal distribution or scattered through the cancer nodules. *nm23* protein also expressed in the normal liver tissues around the carcinoma. The positive rate of *nm23* was 86% in the group without metastasis, and 40% in the group with metastasis. The *nm23* expression level in metastatic HCC was significantly lower than that in nonmetastatic HCC ( $P < 0.05$ , Table 1).

**Table 1 Relationship between <sup>a</sup>«nm23<sup>a</sup>» expression and metastasis of HCC**

Groups	n	nm23 expression				Positive rate(%)
		-	+	++	+++	
Nonmetastatic	14	2	3	3	6	85
Metastatic	10	6	2	1	1	40 <sup>a</sup>

<sup>a</sup> $P < 0.05$  compared with metastatic group.

## DISCUSSION

*nm23* is a suppressor gene for tumor metastasis that encodes nucleoside diphosphokinase (NDPK). NDPK causes activation of a G protein pathway involved in the signal transduction of many growth factors and hormones. Expression of *nm23* at the RNA or protein level was shown to be inversely correlated with the staging and differentiation of human breast cancer. In later period of poorly differentiated tumors, *nm23* showed in general a lower expression and their recidive rate was higher, and survival rate was low<sup>[2]</sup>. Similar results were obtained by prostate and thyroid carcinoma<sup>[3]</sup>. Our data showed that the expression level of *nm23* was

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\*Project supported by the National Natural Science Foundation of China, No. 39070376

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Received 1997-09-10

significantly lower in cases of HCC with metastasis than that without metastasis, suggesting that *nm23* had some effects of inhibiting metastasis of HCC. However, no relation between expression of *nm23* and lymph node metastasis was reported by Haut *et al.*<sup>[4]</sup>. However, Cohn *et al.*<sup>[5]</sup> found that *nm23* was associated with distant metastasis after operation in colorectal carcinoma. Moreover, *nm23* was reported to be related with lymph node metastasis in pulmonary squamous cell carcinoma, but not in pulmonary adenocarcinoma<sup>[6]</sup>. Our preliminary study also showed that there was no *nm23* expression in 2 nonmetastatic HCC tissues, but stronger expression in 1 metastatic HCC. These suggested that some

other regulatory factors may exist evidently in the process of metastasis of HCC.

## REFERENCES

- 1 Steeg PS, Bevilacqua G, Kopper L, Thorgeirsson UP, Talmadge JE, Liotta LA *et al.* Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst*, 1988;80(3):200-204
- 2 Hennessy C, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ. Expression of the antimetastatic gene *nm23* in human breast cancer and associated with good prognosis. *J Natl Cancer Inst*, 1991;83(4):281-285
- 3 Konishi N, Nakaoda S, Tsuzuki T, Matsumoto K, Kitahori Y, Hiasa Y *et al.* Expression of *nm23-H1* and *nm23-H2* proteins in prostate carcinoma. *Jpn J Cancer Res*, 1993;84(10):1050-1054
- 4 Haut M, Steeg PS, Willson JKV, Markowitz SD. Induction of *nm23* gene expression in human colonic neoplasms and equal expression in colon tumors of high and low metastatic potential. *J Natl Cancer Inst*, 1991;83(10):712-716
- 5 Cohn KH, Wang F, Desoto-Lapaix F, Solomon WB, Patterson LG, Arnold MR *et al.* Association of *nm23-H*, allelic deletions with distant metastasis in colorectal carcinoma. *Lancet*, 1991;21.Sep.338(8769):722-724

# Detection of blood AFPmRNA in nude mice bearing human HCC using nested RT-PCR and its significance

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**Subject headings** carcinoma,hepatocellular/pathology; lung neoplasms/secondary;kidney neoplasms/secondary; alpha-fetoproteins/analysis; RNA, messenger; polymerase chain reaction

Prognosis of patients with HCC is estimated by several factors, such as histological differentiation of tumor cells, tumor size, and extent of lymphatic or hemotogenous spread. In this study, we detected the AFPmRNA in the blood of nude mice bearing human hepatocellular carcinoma (HCC) using nested reverse transcriptase polymerase chain reaction (nested RT-PCR) and study its significance and role in blood spread and distant metastasis.

## MATERIALS AND METHODS

**Animal** SMMC-LINM cell lines which can secrete AFP (provided by the pathology department of our university<sup>[1]</sup>) were inoculated into the neck and back of nude mice (BALB/C/NU, 4 weeks after birth), (107 HCC cells to each mouse). After 6 weeks, 20 nude mice (15g-20g in weight) with tumors growing to 2cm-4cm were used in the experiment.

**Collection of samples** Both eyes of the nude mice bearing HCC were scooped out and blood (about 1ml) was collected and placed into a 12ml centrifuge tube. The subcutaneous tumors were resected, and their integrity and relationship with the surrounding tissues were observed. The liver, lung, kidneys and other organs of the mice were cut and examined pathologically.

**Methods** One ml whole blood was collected from the peripheral vein of each subject into a centrifuge tube. AFPmRNA was detected with nested RT-PCR. The detailed procedures were as follows.

**Detection of AFPmRNA** Heparinized whole blood was centrifuged and the plasma fraction was removed. The cellular fraction was enriched for mononuclear cells or possible tumor cells according to the method by Komeda<sup>[2]</sup>. Total cellular RNA was extracted by a single-step method of RNA isolation<sup>[3]</sup>. The reverse transcription reaction was carried out in 20 µl reaction mixture using a first-strand cDNA synthesis kit (Promega USA) according to the manufacturer's instructions. Nested PCR was conducted by addition of 5 µl solution of cDNA to 100 µl reaction mixture containing 10mM Tris HCl (pH 9.0), 50mM potassium chloride, 4.5mM magnesium chloride, 250nM dNTP 15pmol of each outer primer (EX-sense and EX-antisense) and 2.5 units of Taq DNA polymerase (Promega, USA). The reaction mixtures were subjected to 35 cycles of amplification in a programmable thermal cycler (Perking-Elmer Cetus, USA) using the following sequence: 94 °C for 1.5min, 57 °C for 1.5min and 72 °C for 2.5min, and a final extension step at 72 °C for 10min. A sample of 10 µl of the first amplification product was further amplified using an inner pair of primers (IN-sense and IN-antisense). To verify the amplified AFP DNA fragment, the samples were digested with the restriction enzyme Pst I and analysed by electrophoresis on a 2% agarose gel and stained with ethidium bromide for the specific bands of 174 base pairs (first amplification product) and 101 base pairs (second amplification product). Nested PCR was performed two or three times for samples with conflicting results. The external and inner pair of primers were designed as follows:

EX-sense 5'-ACTGAATCCACAACACTGCATAG-3'

EX-antisense 5'-TGCAGTCAATGCATCTTCACCA-3'

IN-sense 5'-TGGAAATAGCTTCCATATTGGATTTC-3'

IN-antisense 5'-AAGTGGCTTCTTGAACAAACTGG-3'

According to this design, the PCR products of 176 and 101 base pairs were amplified from AFPcDNA by external (EX-sense and EX-antisense) and internal (IN-sense and IN-antisense) primer pairs, respectively. EX-sense was located in exon 1 (AFPmRNA nucleotides 90-112), EX-antisense in exon 2 (AFPmRNA nucleotides 241-263), IN-sense over exon 1 and exon 2 (AFPmRNA nucleotides 122-145) and IN-antisense in exon 3

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Received 1998-04-01

(AFPmRNA nucleotides 200-222). cDNA sequences followed the method reported previously<sup>[4]</sup>.

**Statistical analysis** The relationship between the AFPmRNA in peripheral blood and various clinical parameters was examined by Chi-square test.

## RESULTS

AFP mRNA was detected in the blood of 6 (30.0%) mice bearing HCC, 4 (66.67%) of 6 nude mice had distant metastasis in lungs, liver or kidneys (Figures 1-3).

**Table 1** The relationship between detectable rate of AFPmRNA and distant metastasis

Types	Cases	Cases of metastasis	Metastasis rate
AFPmRNA (+)	6	4	66.67 <sup>b</sup>
AFPmRNA (-)	14	0	0.00

<sup>b</sup> $P < 0.01$ , vs AFPmRNA (-).

The diameters of HCC in 6 mice with positive AFP mRNA in blood were more than 3cm, no distant metastasis occurred in tumors below 3cm in diameter (Table 2).

**Table 2** The relationship between tumor diameters and distant metastasis

Diameter	Cases	Cases of AFPmRNA (+)	Positive rate
>3cm	9	6	66.67 <sup>b</sup>
<3cm	11	0	0.00

<sup>b</sup> $P < 0.01$ , vs tumors below 3cm in diameter.

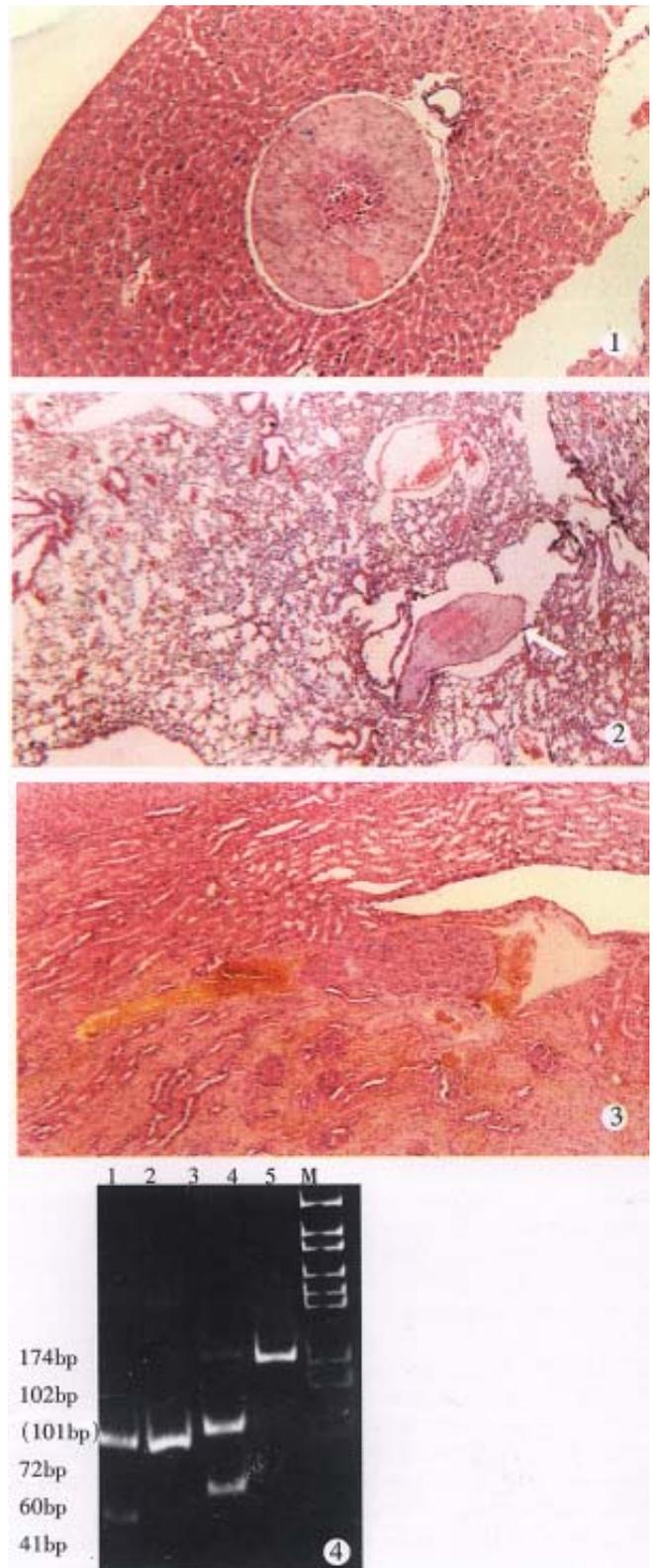
AFPmRNA was detected among 12 (50%) nude mice bearing HCC with serum AFP levels beyond 4 000  $\mu\text{g/L}$  while no AFPmRNA was found in 8 nude mice with serum AFP levels below 4 000  $\mu\text{g/L}$  (Table 3).

**Table 3** The relationship between AFP levels in serum and detectable AFPmRNA

AFP levels( $\mu\text{g/L}$ )	Cases	AFPmRNA (+)	Positive rate
>4000	12	6	50.0 <sup>b</sup>
<4000	8	0	0.0

<sup>b</sup> $P < 0.01$ , vs nude mice with serum AFP levels below 4 000  $\mu\text{g/L}$ .

The first-cycle PCR products, 174 base pairs, can be cut into two pieces of 102 and 72 base pairs by restriction enzyme Pst I. The second-cycle PCR product, 101 base pairs, can be cut into two pieces of 60 and 41 base pairs (Figure 4).



**Figure 1** Metastasis in liver. (×30)

**Figure 2** Metastasis in lungs. (×30)

**Figure 3** Metastasis in kidney. (×30)

**Figure 4** The first-cycle PCR products was cut into two pieces of 102 bp and 72 bp by restriction enzyme Pst I; and the final PCR products was cut into two pieces of 60 bp and 41 bp.

## DISCUSSION

Recently, there have been some reports about the employment of the reverse transcriptase-polymerase chain reaction (RT-PCR) technique to detect tumor cells spreading into the peripheral blood, bone marrow, and lymph nodes<sup>[5-7]</sup>. These trials have aimed to amplify tumor-specific gene transcripts which can not be detected in these tissues under normal conditions. Free mRNA is so fragile under conditions of abundant RNase activity that the specific mRNA in blood can indicate the presence of intact cells producing such proteins just before the extraction of RNA.

This study showed that AFPmRNA was detected in 6 (30.0%) of 20 nude mice bearing human HCC 4 (66.67%) of them had distant metastasis. None of 14 nude mice with negative AFPmRNA in blood had distant metastasis ( $P < 0.01$ ), suggesting that the distant metastasis occurred via blood circulation. The detectable rates of AFPmRNA was significantly related with AFP

levels, tumor size and distant metastasis ( $P < 0.01$ ). In other words, AFPmRNA in blood may be a prerequisite for distant metastasis of HCC.

In conclusion, AFPmRNA may be an effective and sensitive marker for HCC metastasis in blood and distant metastasis as well.

## REFERENCES

- 1 Ji YY, Liu YF, Chen ZN. Radioimmuno-detection and autoradiographic localization of monoclonal antibody against human hepatocellular carcinoma in xenografts. *Cancer*, 1992;69:2055-2059
- 2 Komeda T, Fukuda Y, Sando T, Kita R, Furukawa M, Nishida N *et al*. Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. *Cancer*, 1995;75(9):2214-2219
- 3 Chomzynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 1987;162:156-159
- 4 Morinaga T, Sakai M, Wegmann TG, Tamaoki T. Primary structures of human  $\alpha$ -fetoprotein and its mRNA. *Proc Natl Acad Sci USA*, 1980;80:4604-4608
- 5 Schoenfeld A, Luqmani Y, Smith D, Reilly SO, Shousha S, Sinnett HD *et al*. Detection of breast cancer micrometastasis in axillary lymph nodes by using polymerase chain reaction. *Cancer Res*, 1994;54:2986-2990
- 6 Ghossein RA, Scher HI, Gerald WL, Kelly WK, Curley T, Amsterdam A *et al*. Detection of circulating tumor cells in patients with localized and metastatic prostatic carcinoma: clinical implications. *J Clin Oncol*, 1995;13(5):1195-1200
- 7 Pfliederer C, Zoubek A, Gruber B, Kronberger M, Ambros PF, Lion T *et al*. Detection of tumor cells in peripheral blood and bone marrow from ewing tumor patients by RT-PCR. *Int J Cancer*, 1995;64:135-139

# Biological effects of hepatoma cells irradiated by $25\text{MeV/u}^{40}\text{Ar}^{14+}$

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**Subject headings** carcinoma,hepatocellular/radiotherapy; liver neoplasms/radiotherapy;argon/therapeutic use

## INTRODUCTION

Radiotherapy was initiated when Grubbe treated tumor with X-rays in 1896<sup>[1]</sup>. Afterwards, radioisotopes such as Ra and Rn, were used for clinical diagnosis and treatment. Basic researches on the biological effects of X-rays,  $\gamma$ -rays, fast neutron, and so on discovered that damages to mammalian cells induced by high-LET irradiation were more serious than that by low-LET<sup>[2]</sup>.

Because of their low oxygen enhancement ratio (OER) and high relative biological effectiveness (RBE), heavy ions can kill carcinoma cells efficiently<sup>[3]</sup>, among which about 5%-20% was hypoxia. There was a Bragg peak along the energy deposition of heavy ions, so that more dose could reach to the tumor while less to the normal tissues<sup>[4]</sup>. Therefore, heavy ion beam is believed to play an important role in the future in the radiotherapy for tumors.

The treatment with heavy ion beam has been studied and put into clinical practice since the 1970s in America and since 1994 in Japan. But in our country, this research is still in its initial stage. This is our preliminary report on the dose--response and fractionated irradiation with  $25\text{MeV/u}^{40}\text{Ar}^{14+}$  in human hepatoma SMMC-7721 cells.

## MATERIALS AND METHODS

### Cell culture

The human hepatoma SMMC-7721 cell line was obtained from the Second Military Medical University<sup>[5]</sup>. The cells were cultured in RPMI-1640

medium (Gibco Inc.) supplemented with 10% calf, 100u penicillin and 100  $\mu\text{g/ml}$  streptomycin. The medium was placed at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>. The cells were inoculated in the glass flasks with a diameter of 35mm and density of  $5 \times 10^4$  cells/ml 2 days before irradiation.

### Irradiation

$25\text{MeV/u}^{40}\text{Ar}^{14+}$  was accelerated by HIRFL. The intensity of the beam was  $2.1 \times 10^6$  ions/s.

Before irradiation, the medium was removed and the cells were washed twice with D-Hank's buffer. The flasks were enveloped with 4  $\mu\text{m}$  mylar membrane. A part of cells were irradiated at the dose of 0.68 Gy, 6.8 Gy, 68 Gy, 680 Gy and 6800 Gy, respectively. The others were irradiated with fractionated dose of 68 Gy for 1, 2, 3 and 4 times, respectively, at an interval of 2 hours. After irradiation, each flask was added 2ml medium and kept in 37 °C incubator for 24 hours.

### Analysis of samples

After washed with D-Hank's buffer, the cells were fixed for 4 hours and then stained with acridine orange (0.01mg/L, pH 6.8) for 10min and differentiated with stiller water for about 5min. Cells with micronuclei were counted under the fluorescence microscope to obtain frequency of micronuclei (FM). Afterwards, cells were washed once with PBS (pH 6.8) and stained with Giemsa (1:20, pH 6.8) for 8 min to gain number of cells per  $\text{mm}^2$  (NC).

## RESULTS

### Observation of cellular configuration

Under the fluorescence microscope, the null were bright yellow while cytoplasm and nucleolus were red. After irradiation, many types of aberration were observed, such as micronuclei, small nuclei, free chromosomes, chromosome bridge, and so on (Figure 1).

Cells treated with 680 Gy and 6 800 Gy were dead, but the remnants appeared different. The remnants induce by 680 Gy were far smaller than controls and the nuclei were bright yellow and the cytoplasm was light yellow, while those induced by 6 800 Gy change little in size, but the nucleus and the cytoplasm could not be distinguished. The death of the former may be caused by the change of the permeance ability of cellular membrane caused by

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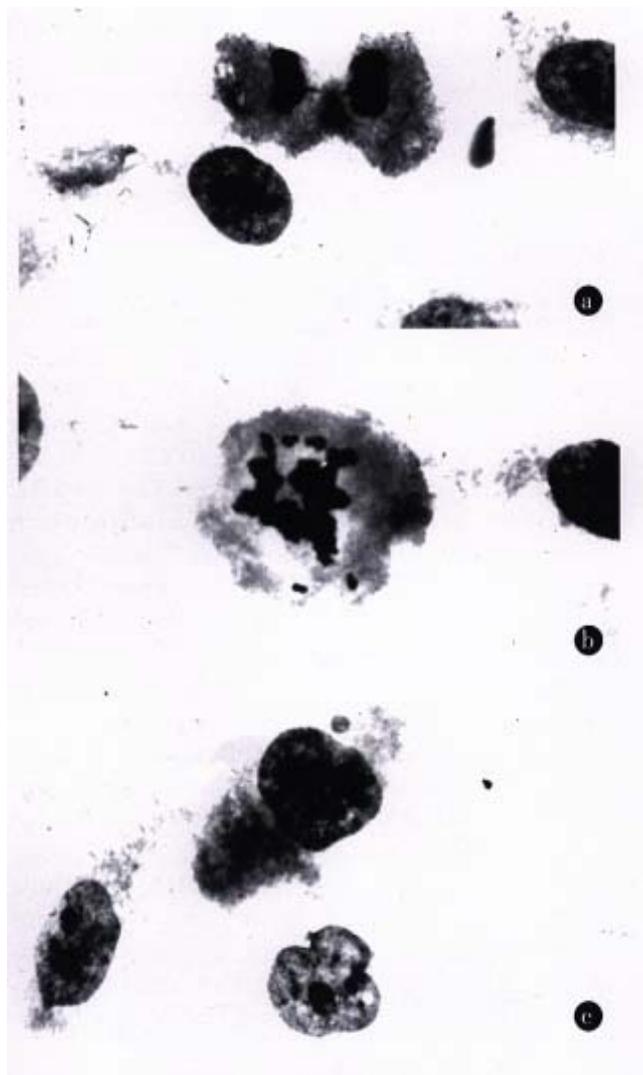
Presented at the 4th Symposium on Radiation Research and Radiation Technology, Changchun, 24-27 June, 1996.

\*Supported by the Top Project of National Fundamental Research, No. 01-3.

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Received 1997-11-26 Revised 1998-01-04

the high dose, and shrinking of the cells. When fixed, the nuclei were smaller than controls but not disperse. The dose for the latter one was even higher, and cells died quickly after irradiation. So, the nuclear membrane was broken, and the nuclei entered the cytoplasm when fixed.



**Figure 1** Abnormal nuclei and chromosome aberration induced by  $25\text{MeV}/u^{40}\text{Ar}^{14+}$   
a. micronuclei, b. free chromosome, c. chromosome bridge.

### Dose-response

As shown in Table 1, FM of the samples was higher than the control, which was correlated positively with the dosage ( $r = 0.9952$ ) while NC was negatively correlated with dosage ( $r = -0.9279$ ). This is consistent with other approaches<sup>[6]</sup>.

### Response of fractionated irradiation

As shown in Table 2, with the increase of irradiation times FM decreased and NC increased. The correlation coefficient was  $r = -0.9590$  and  $r = 0.9681$ , respectively.

**Table 1** Dose-response of single irradiation with heavy ions

Dose (Gy)	Number of cells	FM (%)	NC
0	861	2.56	380
0.68	1952	4.27	391
6.8	2008	4.83	235
68	1960	5.62	207

**Table 2** Biological effects of fractionation irradiation

Times of irradiation	Number of cells	FM (%)	NC
1	1960	5.62	207
2	971	5.13	213
3	974	2.47	250
4	1000	1.80	281

## DISCUSSION

The damages induced by heavy ions were intensified with the increasing dose, but NC induced by 0.68 Gy was higher than that of the control, possibly because very low dose can stimulate cell propagation.

When the times of irradiation were increased, the repair mechanism is activated and the efficiency of repair was enhanced. FM induced with four divided doses was less than that of control. This may be due to the high repair efficiency.

MF peaked when cells underwent one division cycle after irradiation<sup>[6]</sup>. The time of a division cycle was about 24 hours because of the delayed division induced by irradiation. According to our approach, FM was not the highest one (the data are not shown), but there was significant correlation between MF and dosage, MF and the times of irradiation. Micronucleus was one kind of nuclear structure, less than 1/5 of the normal nucleus, forming fragments induced by irradiation during the cell division. Therefore, FM after one division cycle represented the direct effects of irradiation with heavy ion beam.

NC was closely correlated with FM, but NC represented by the lethal effect more directly than FM. The correlation coefficient was  $-0.8870$  for single one. Both as the endpoints of radiosensitivity, they reflected the same results.

## REFERENCES

- Manuel Lederman MB, FRCR. The early history of radiotherapy: 1895-1939. *Int J Radiat Oncol Biol Phys*, 1981;7(5):639-648
- Ritter MA, Cleaver JE, Tobias CA. High-LET radiations induce a large proportion of non-rejoining DNA breaks. *Nature*, 1977;266(5603):653-655
- Zhu RB, Liu Y, Luo ZY. *Radiobiology*. 1st ed, Beijing: Science Press, 1987: 572-592
- Lu ZL. Application of accelerator in medicine. *Trends Nucl Phys*, 1989;6(1): 38-42
- Dong RC, Zhou RH, Lu FD. The establishment and biological study of human hepatoma SMMC-7721 cells. *Second Military Med Univ*, 1980;1(1):5-9
- Midander J, Revesz L. The frequency of micronuclei as a measure of cell survival in irradiated cell populations. *Int J Radiat Biol*, 1980;38(2):237-242

# Effects of Yibei multi-active elements on mesenteric microcirculation in rats

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**Subject headings** microcirculation; mesentery; Yibei multi-active elements (YBMAE); blood flow velocity

*Mytilus edulis linnaeus* (Yibei) belongs to mytilide, gill lamella, and mollusc. There are rich resources in Bohai and Huanghai of China. Yibei multi-active elements (YBMAE)<sup>[1]</sup> come from *mytilus edulis linnaeus* containing taurine, EPA, Zn, Ci, Ferris, etc. Its preparation and composition were described previously<sup>[2]</sup>. This paper aims at studying the effect of YBMAE on the mesenteric microcirculation in rats.

## MATERIALS AND METHODS

### Materials

YBMAE was provided by the Department of Pharmacology, Medical College of Qingdao University. Sterile amniotic fluid and Panax Notoginsenosidum (PNS) were obtained from Taishan Medical College. Wistar rats were purchased from the Animal Center of Shandong Medical University. The microcirculation monitoring system was product of Xuzhou Optic Instrument Factory, China.

### Methods

Thirty Wistar rats (male or female, weighing 300g±50g) were divided into 5 groups: YBMAE group I (1.2g/kg), group II (3g/kg), group III (6g/kg), PNS group (40mg/kg) and control group (with saline). Each group consisted of 6 rats. The drugs were given qd for 28 days<sup>[3-5]</sup>. Thirty minutes after the last administration of drugs, the rats were anaesthetized with vinbarbitol 50mg/kg

ip. A 2-cm incision was made on the abdominal wall. The blood color, flow velocity and vessel wall clarity of tertiary blood vessels were observed, meanwhile blood flow and flow velocity were monitored with microcirculation monitoring system. Sterile amniotic fluid (1ml/kg) was given intravenously. The above-mentioned indexes were monitored and video recorded immediately 10 and 30min after amniotic fluid injection.

**Statistical analysis** Student's *t* test was used for the statistical study.

## RESULTS

### Effect of YBMAE on blood flow of Wistar rat mesenteric microcirculation

Immediately, 10min and 30min after amniotic fluid injection, blood flow of the control group was decreased significantly ( $P<0.05$ ,  $P<0.01$ ,  $P<0.01$ ), while the blood flow of YBMAE groups I and II showed no obvious changes compared with that before amniotic fluid injection, but more significant changes than that of the control groups ( $P<0.05$ ,  $P<0.01$ ). In YBMAE group III, the blood flow at 10min was lower than that before amniotic fluid injection ( $P<0.05$ ), significantly higher at 30min than the control group ( $P<0.01$ ). In PNS group, the blood flow had no obvious changes immediately and 10min after amniotic fluid injection, and increased at 30min ( $P<0.01$ ), being significantly different compared with the control group ( $P<0.05$ ,  $P<0.01$ ) (Table 1).

### Effect of YBMAE on blood flow velocity of rat mesenteric microcirculation

The blood flow velocity was greatly decreased immediately, 10min and 30min after amniotic fluid injection in the control group ( $P<0.05$ ,  $P<0.01$ ), while in the YBMAE group I, there was little change in the blood flow velocity, but greater than that of the control group ( $P<0.05$ ,  $P<0.01$ ). In YBMAE group II, right after amniotic injection, the velocity was significantly decreased ( $P<0.05$ ), but still faster than that of the control group. At 10min, 30min, the velocity was increased, but not faster than that before amniotic injection. In YBMAE group III, the velocity was decreased

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\*One of the National "8.5" Key Projects of Scientific and Technical Researches, No.85-08-07-09.

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Received 1997-11-26 Revised 1998-01-04

immediately and 10min after injection ( $P<0.05$ ), and increased at 30min, which was significantly faster than that of the control group at any time ( $P<0.05, 0.01$ ). In PNS group, the velocity had no obvious changes after injection, but significantly faster than the control group ( $P<0.05$ ) (Table 2).

#### **Effect of YBMAE on microcirculation status, blood color and vessel wall clarity of rat mesentery**

The microcirculation condition of the control group changed from linear to linear granular flow after

amniotic fluid injection. At 30min, 90% of the vessels turned to linear granular flow, the blood color was dark red, the vessel wall was not clear, and there was stasis in the venous blood. At 12 h, 50% of the animals died. In the three YBMAE groups and PNS group, 40%, 10%, 0% and 0% vessels had linear granular flow after amniotic fluid injection. The blood color changed from bright red to dark red in about 30%, 20%, 20%, 10% vessels respectively. Thirty minutes later, it returned to normal, and the vessel walls became clear. No animals died within 12 h.

**Table 1** Effect of the drugs on blood flow ( $\mu\text{m}^3 \cdot \text{s}^{-1}$ ) of mesenteric microcirculation in rats ( $\bar{x} \pm s$ )

Groups	Dosage (g/kg)	n	Before amniotic fluid injection	After amniotic fluid injection		
				Immediately	10min	30min
NS			6616±91.79	432±132.68 <sup>a</sup>	358±64.32 <sup>b</sup>	348±84.30 <sup>b</sup>
YBMAE	1.2	6	576±67.41	545±105.47	593±70.63 <sup>d</sup>	560±57.12 <sup>d</sup>
	3.0	6	560±57.88	450±86.49	561±77.96 <sup>c</sup>	588±50.75 <sup>d</sup>
	6.0	6	733±100.76	602±99.26	578±93.03 <sup>ac</sup>	665±73.12 <sup>d</sup>
PNS	0.04	6	586±60.58	616±67.34 <sup>c</sup>	638±55.11 <sup>d</sup>	766±60.40 <sup>bd</sup>

Compared with before amniotic fluid injection, <sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ ; compared with NS, <sup>c</sup> $P<0.05$ , <sup>d</sup> $P<0.01$ .

**Table 2** Effect of the drugs on the blood flow velocity of rat mesenteric microcirculation ( $\bar{x} \pm s$ )

Groups	Dosage (g/kg)	n	Before amniotic fluid injection	After amniotic fluid injection		
				Immediately	10min	30min
NS			60.51±0.10	0.35±0.05 <sup>a</sup>	0.32±0.10 <sup>a</sup>	0.32±0.08 <sup>a</sup>
YBMAE	1.2	6	0.56±0.05	0.52±0.07 <sup>c</sup>	0.49±0.07 <sup>c</sup>	0.55±0.05 <sup>d</sup>
	3.0	6	0.54±0.05	0.45±0.07 <sup>a</sup>	0.53±0.07 <sup>d</sup>	0.55±0.04 <sup>d</sup>
	6.0	6	0.64±0.06	0.48±0.08 <sup>a</sup>	0.49±0.08 <sup>ac</sup>	0.59±0.06 <sup>d</sup>
PNS	0.0	6	0.50±0.07	0.45±0.06	0.49±0.06 <sup>c</sup>	0.52±0.07 <sup>c</sup>

Compared with before amniotic fluid injection, <sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ ; compared with NS, <sup>c</sup> $P<0.05$ , <sup>d</sup> $P<0.01$ .

## DISCUSSION

Recent studies showed that the flow velocity, blood flow, flow status, the agglutination ability of platelet and red blood cell, the amount of opening capillary were important factors determining the functional status of the flow velocity, improve the blood flow status, the blood color, the vessel wall clarity, increased the amount of opening capillaries<sup>[6]</sup>. The results of this study demonstrated that YBMAE could increase the blood flow, and could improve the microcirculation status. The increment of flow velocity and blood flow were proportional to the amount of YBMAE before and 30min after amniotic fluid injection. According to the literature, taurine could regulate  $\text{Ca}^{++}$  metabolism and prevent arterial atherosclerosis<sup>[7]</sup>. EPA could inhibit the blood vessel constriction induced by norepinephrine, and vasoconstrictin A2 and increase elasticity of RBC, decrease the blood

viscosity and synthesis of  $\text{TXA}_2$ <sup>[8]</sup>. YBMAE contains plenty of taurine, EPA, amino acid and unsaturated lipid acid, therefore the effect of YBMAE in improving microcirculation may be related to its components.

## REFERENCES

- 1 Health Department of Logistic Ministry of Navy, PLA, China. Pharmacological marine creature. 1st ed. Shanghai: Shanghai People's Publication, 1977: 68-69
- 2 Ming L, Shao BQ, Zhang Y, Li WP, Xu SY. The influence of YBMAE on quail experimental atherosclerosis. *Chin Pharmacol Bull*, 1996;12(6):554-556
- 3 Xu SY (editor). Pharmacological experimental methodology. 2nd ed. Beijing People's Health Publishing House, 1994:992-1003
- 4 Li YK (editor). Pharmacological experimental methodology. 1st ed. Shanghai: Shanghai Science and Technology Publishing House, 1991:141-147
- 5 Xu SK (editor). Antiaging material media. 1st ed. Beijing: China Medical and Pharmaceutical Science and Technology Press, 1994:185-193
- 6 Pang DW, Liu MY, Tan JM, Li ZY, Shi SH. The influence of aspirin and rephedrine on the microcirculation of white mouse mesentery. *Chin Pharmacol Bull*, 1996;12(4):356
- 7 Wang SN, Zhang HL, Wu DC, Wang H. The pharmacological research advancement and clinical usage of taurine. *China Pharmacy*, 1993;4(4):33-34
- 8 Wu BJ (editor). Pharmacology of animal biochemical medicine. 1st ed. Beijing: China Commercial Press, 1993:54-63

# Effects of erythromycin on pressure in pyloric antrum and plasma motilin and somatostatin content in dogs

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**Subject headings** erythromycin/pharmacology; somatostatin/blood; motilin/blood; pyloric antrum/drug effects

## INTRODUCTION

Erythromycin (EM) is a potent agonist of motilin (MTL) receptors<sup>[1]</sup>. EM may enhance the gastroenteric motion by binding with MTL receptors<sup>[2]</sup>. However, effects of EM pyloric antrum and its mechanism are not clear. The purpose of this study was to investigate the relation between EM, plasma MTL and somatostatin in regulation of pyloric sphincter muscle function in dogs.

## MATERIALS AND METHODS

A randomized study was performed using male or female dogs weighing between 11kg-19kg. Before operation, dogs was prohibited from eating food for 24h.

Animals were anaesthetised with i.v. injection of pentobarbital (2.5%, 1mg/kg). The upper medial incision of abdomen was performed. The anterior wall of gastric antrum was cut about 0.5cm, the tube of the gastric pressure meter (WYY-1 type, Sapceflight Medicine Engineer Research Institute) was inserted and fixed. The pressure graph was recorded by the pressure transducer.

Erythromycin lactate was dissolved in 5% glucose liquid, and transfused i.v. (5mg/kg per hour). Isoptin (1mg/kg) was injected i.v. at an interval of 60min. At 90min i.v. atropine sulfoacid (0.1mg/kg) was given. During 120min of pre- and post-infusion, pressure measure was done for 2min and

1ml blood sample was collected from dog femoral vein every 15min. Nine blood samples (1ml each) were collected from each dog. 3ml EDTA-Na<sub>2</sub> and 200KIu aprotinin was added into the samples. Blood samples were immediately centrifuged at 3 500r/min for 15min at 4°C. The plasma was stored at -70°C. MTL and SS was assayed by radioimmune method. (MTL and SS radioimmune reagent box, East-Asia Immune Technique Research Institute). The concentration of MTL and SS in blood was counted with a FJ-2003-50G counter.

## Statistical analysis

Statistical analysis was carried out using the Statistical analysis System. When a significant analysis of variance was found, Student's *t* test between two samples was performed.

## RESULTS

The changes of pyloric pressure and the concentration of MTL and SS in plasma before and after i.v. transfusion (Table 1).

Our results showed that the dog pyloric antrum basic pressure, total pressure and wave amplitude significantly increased after administration of EM. The interval time of high pressure wave amplitude was reduced and the frequency increased. After i.v. injection of antagonists isoptin and atropine, the pyloric pressure was inhibited rapidly. The level of MTL in plasma of dogs and the change of the pyloric pressure induced by EM was related significantly, and were also influenced by atropine and isoptin. The concentration of SS in plasma of dogs was increased after EM administration and not inhibited by atropine and isoptin.

## DISCUSSION

EM is one of most common antibiotics. To investigate the gastroenteric side effects of EM, Pilot and Itol, *et al*<sup>[3]</sup>, have found that the i.v. infusion of EM might mimic the migrating synthetical electric current of muscles (or contraction) during dog digestion induced by MTL. The effect of EM was similar with MTL in vivo or vitro, EM competitively inhibited the combination of receptors and MTL, therefore EM is considered one of the agonists on MTL receptors. Sarna, *et al*<sup>[4]</sup> have found that i.v. EM 1mg • kg<sup>-1</sup>/h-3mg • kg<sup>-1</sup>/h

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\*Supported by the National Natural Science Foundation of China, No. 39570885

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Received 1997-12-18

(far below the dose of antibiotics) might induce migrating synthetical electric current of III phase muscles, beginning at stomach and migrating downward, which was related to MTL release. The main physiological action of MTL is to enhance the gastroenteric motion and increase the gastric and pyloric pressure. By i.v. EM 5mg • kg<sup>-1</sup>/h, the pyloric pressure increased immediately, suggesting that EM could increase the pressure in pyloric antrum and it was highly sensitive; and the effect of EM was related with MTL. We believe that EM might be used to treat the gastroduodenal reflux diseases in future.

It has been found that the effect of EM on dog stomach, duodenal and gall doct might be inhibited by atropine<sup>[5]</sup>, indicating that EM act on prejunctional receptors of cholinergic. Some studies have suggested that EM and MTL have a similar gastroenteral action and race specificity. EM could induce the contraction of rabbit gastric smooth muscles, and was not inhibited by atropine,

but blocked by antagonists of calcium passway Nifedipine. This indicated EM effect was related with calcium passway. Our result is similar with other investigators'.

There have been a lot of investigations on the effect of gastroenteric hormone. Increase in pyloric pressure was increased by human or dog duodenal infusion of HCl or florence oil, suggesting that gastroenteric hormone could regulate pyloric motion. We observed for the first time the plasma SS changes, and found that the plasma SS level was not blocked by isoptin and atropine after i.v. EM, and the plasma SS was higher in late stage. This phenomenon may be related to be autoregulation in vivo in order to maintain the balance among gastroenteral hormones like MTL and normal pyloric pressure.

In summary, the study suggested EM may increase the pressure in pyloric antrum. The effect may be related to the plasma motilin and somatostatin level.

**Table 1 Pyloric pressure and plasma MTL and SS content before and after infusion of erythromycin in dogs. (n = 10,  $\bar{x} \pm s$ )**

Parameters	Before drug administration	Erythromycin	Verapamil	Atropine
Total pressure (kPa)	20.1±2.2	34.5±3.1 <sup>a</sup>	10.3±0.4 <sup>a</sup>	8.2±0.2 <sup>a</sup>
Basic pressure (kPa)	4.1±2.5	6.9±0.9 <sup>a</sup>	4.4±0.8	5.2±0.2
Wave amplitude pressure (kPa)	16.0±14.4	27.6±9.6 <sup>a</sup>	5.9±0.4 <sup>a</sup>	3.0±0.1 <sup>b</sup>
Wave frequency (time/min)	9.8±4.5	5.4±0.5 <sup>a</sup>	2.9±0.4 <sup>a</sup>	2.0±1.0 <sup>b</sup>
Wave interval (s)	3.0±1.1	6.7±0.6 <sup>a</sup>	3.8±0.4	3.1±0.1
Plasma MTL (ng/L)	426.9±53.4	553.9±87.2 <sup>a</sup>	447.9±67.6	378.3±8.2 <sup>a</sup>
Plasma SS (ng/L)	64.6±13.7	75.2±4.7 <sup>a</sup>	85.6±2.9 <sup>b</sup>	105.6±0.2 <sup>b</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01 vs before used medicine.

## REFERENCES

- 1 Peeters T, Itch Z. Erythromycin is a motilin receptor agonist. *Am J Physiol*, 1989;257(3):470-477
- 2 Weber FH, Richards RD, McCallam RM. Erythromycin: a motilin agonist and gastrointestinal prokinetic agent. *Gastroenterology*, 1993;104(3):485-493
- 3 Itoh Z, Peeters T. Erythromycin mimics exogenous motilin in gastrointestinal contractile acting in the dog. *Am J Physiol*, 1984;247(6):688-694
- 4 Vantrappen G, Janssens J, Tack J. Erythromycin is a potent gastrokinetic in diabetic gastroparesis. *Gastroenterology*, 1989;96(5):525-529
- 5 Sarna SK, Ryan RP, Brandon A. Erythromycin acts on presynaptic neurons to stimulate gastrointestinal motor activity. *Gastroenterology*, 1991;100(5):490-498

# Molecular biology of Barrett's esophagus and esophageal cancer: role of p<sup>53</sup>

Alan G. Casson, MB ChB MSc FRCS FACS

**Subject headings** esophageal neoplasms/epidemiology; adenocarcinoma; Barrett's esophagus; p53 gene

Despite recent advances in multimodality therapy, the prognosis for invasive esophageal cancer is poor, with a five-year survival generally below 10%. While improvements in survival may be anticipated with early detection, improved staging, and rational use of adjuvant therapies, it is likely that significant progress in the treatment of this disease will only occur with an improved understanding of its tumor biology, and careful evaluation of clinically relevant molecular markers.

Over the past decade, various molecular alterations have been reported in human esophageal cancers. As the p53 tumor suppressor gene appears to have a central role in human neoplasia, the aim of this review is to discuss the potential clinical applications of this biomarker in esophageal cancer.

## ESOPHAGEAL CANCER EPIDEMIOLOGY<sup>[1]</sup>

Squamous cell carcinoma of the esophagus is one of the most frequent malignancies worldwide. The epidemiology of this disease is characterized by a striking geographic variation in incidence, not only between countries, but also within distinct geographic regions and among ethnic groups. Epidemiologic and experimental studies from high-incidence areas have implicated several environmental factors with the development of esophageal cancer, including nutritional deficiencies, dietary nitrosamine precursors, alcohol intake and tobacco smoking. However, the relative influence of each factor appears unique to the region studied.

Recent reports from North America and Europe confirmed clinical suspicions that adenocarcinomas of the lower esophagus and cardia were being seen more frequently. The 10% annual rate of increase

in white males exceeded that for any other solid tumors. Furthermore, a proximal shift in gastric carcinomas towards the upper third has also been described in recent years. Although the factors for this changing pattern of disease are unknown, molecular epidemiologic studies may provide further insight into the etiology and biology of esophagogastric carcinomas.

## ESOPHAGEAL ADENOCARCINOMA

Primary esophageal adenocarcinomas are frequently confused with proximal gastric (or cardia) cancers. Despite apparently different clinical and biologic behaviour, there currently appears to be no clear way to accurately distinguish between these tumors. This is of particular importance in view of the changing epidemiology of this disease, and in planning treatment strategies. One recent classification<sup>[2]</sup> proposed a definition based on tumor measurements related to the anatomic esophagogastric junction (EGJ). Primary esophageal adenocarcinomas were defined as tumors centred 1 to 5cm above the EGJ; cardiac carcinomas, between 1cm above and 2cm below the EGJ; and subcardial gastric carcinomas, with a tumor centre from 2cm to 5cm below the EGJ.

In 1991, we proposed guidelines in an attempt to establish the primary esophageal origin of adenocarcinomas<sup>[3]</sup>. These criteria (summarized below), incorporating clinical and pathologic characteristics of these tumors, were determined by preoperative endoscopy, radiology, at surgery, and on pathologic examination of the resected foregut.

a. An associated Barrett's epithelium. When present, this is virtually a diagnosis of a primary esophageal adenocarcinoma. However, approximately 50% of tumors will not have a demonstrable Barrett's mucosa, presumably because this has been incorporated into the tumor mass. In this situation, the following criteria assume increasing importance.

b. Greater than 75% of the tumor mass involving the tubular body of the esophagus.

c. Direct histologic invasion of periesophageal tissues.

d. Minimal gastric involvement.

e. Clinical symptoms of esophageal obstruction (i.e. dysphagia).

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**Received** 1998-05-29

These criteria have been crucial to the design and conduct of our laboratory/translational studies, and are increasingly used in current clinical practice.

### BARRETT'S ESOPHAGUS<sup>[4]</sup>

Barrett's esophagus is characterized by replacement of normal squamous epithelium, by intestinalized columnar epithelium. Previous definitions of a columnar epithelium lined esophagus required variable lengths of replacement proximal to an arbitrary 2cm-3cm of "normal columnar lining" of the lower esophagus. However, the histologic finding of intestinal type goblet cells is now accepted to be a prerequisite for the diagnosis of Barrett's mucosa, which incorporates "short segment" Barrett's esophagus (i.e. less than 3cm).

Barrett's esophagus is thought to be an acquired condition resulting from chronic gastroesophageal reflux disease. In symptomatic patients (i.e. dyspepsia, heartburn), the prevalence of Barrett's epithelium is estimated at 10%, whereas less than 1% of asymptomatic patients will have this diagnosis. The importance of this finding is that it is premalignant. The risk that patients with preexisting Barrett's mucosa will develop invasive esophageal carcinoma was estimated by two prospective studies to be at least fifty times greater than the general population. Dysplasia is widely regarded as the precursor of invasive cancer, and high-grade dysplasia in Barrett's epithelium is frequently associated with primary esophageal adenocarcinoma. Dysplastic change may be characterized histologically by experienced pathologists in biopsy specimens obtained at esophagoscopy. Recent reports suggest that endoscopic surveillance can detect early adenocarcinoma in Barrett's epithelium, and that early detection and surgical resection may decrease the mortality rate for esophageal carcinoma<sup>[5]</sup>.

### THE p53 TUMOR SUPPRESSOR GENE<sup>[6,7]</sup>

Certain genes appear necessary to regulate cell growth and prevent oncogene-driven clonal expansion and uncontrolled cellular proliferation. Loss of tumor suppressor function (thereby providing a selective growth advantage for clonal expansion) is thought to require inactivation of both alleles, and this may occur through several mechanisms including chromosomal deletion, point mutation, or by neutralization of the protein product by interaction with other cellular proteins.

The p53 protein was first discovered in 1979 as a cellular 53-kD nuclear phosphoprotein bound to the large transforming antigen (T antigen) of the Simian virus 40 (SV40), and subsequently to oncoproteins from other tumor viruses. The normal

function of p53 protein is not known with certainty, but it is believed to be a transcription factor which plays a role in cell cycle progression. DNA damage induces a rapid accumulation of cell nuclear p53 protein, resulting in transactivation of target genes including the cyclin kinase inhibitor p21waf-1, bax-1, a regulator of apoptosis, and other key proteins regulating DNA replication, transcription and repair.

The p53 gene is localized to the short arm of chromosome 17 (17p13), and comprises 11 exons with five evolutionarily conserved domains within the coding region. 95% of known p53 mutations fall within these DNA-binding regions, the majority of which are missense, which lead to stabilization and cell nuclear accumulation of p53 protein. Various p53 mutations may result in different biological properties, and this varies depending on the cell system. The molecular basis of p53 mutations / function is not fully understood. Exons encoding the N and C terminal domains contain a higher proportion of nonsense mutations.

The diversity of p53 mutations in human solid tumors suggests that this may be a potentially useful biomarker for molecular epidemiologic studies. For example, mutations arising from endogenous events, such as methylation and deamination of cytosine to thymine, result in C to T transitions at CpG dinucleotides. Mutations do not form at an equal rate at all base positions, and a strand bias in distribution suggests exogenous mechanisms. Mutation patterns may also suggest DNA damage induced by exogenous carcinogens. For example, A:T to T:A transversions in patients with hepatic angiosarcomas following exposure to vinyl chloride; and G to T transversions in patients with lung cancers associated with tobacco (benzo-a-pyrene) exposure. The most frequently mutated p53 codons in lung cancer are 157, 248, and 273, which may be preferential sites for DNA adduct formation. Codon 157 is rarely mutated in other tumors, and may be seen as a "hot spot" for lung carcinoma. By contrast, while codons 248 and 273 are frequently mutated in several other cancers, patterns of mutation may differ at these sites. For example, in breast and colorectal cancers, C to T transitions at CpG dinucleotides suggest an endogenous mechanism.

### p53 ALTERATIONS IN ESOPHAGEAL CANCER AND BARRETT'S MUCOSA

p53 gene mutations were first reported in esophageal squamous cell carcinomas by Hollstein *et al* in 1990<sup>[8]</sup>. Using strict criteria to define adenocarcinomas of esophageal (vs. gastric) origin, we reported p53 mutations in primary esophageal adenocarcinomas and associated Barrett's

epithelium<sup>[3]</sup>

These original observations have now been confirmed by other investigators, documenting additional p53 mutations, altered expression of p53 mRNA, p53 protein accumulation and loss of heterozygosity of chromosome 17p<sup>[9]</sup>.

Patterns of p53 mutations differ widely for squamous cell carcinomas and adenocarcinomas of the esophagus. To date, most esophageal tumors studied have been squamous cell carcinomas, where 46% of tumors were found to have p53 mutations, predominantly at codons 175, 193, 194, 195 and 270. The high frequency of G:C to A:T transversions and A to T transitions at CpG dinucleotides, suggesting that p53 mutations in these tumors may occur spontaneously by deamination or mismatch repair.

### POTENTIAL CLINICAL APPLICATIONS<sup>[10]</sup>

It has been proposed that p53 alterations in esophageal tissues may have potential clinical applications: for early diagnosis of invasive esophageal cancer in patients with Barrett's esophagus undergoing endoscopic surveillance, for staging, as a stratification factor in future clinical trials, and as a molecular target for novel anticancer therapies.

#### Early detection

Although the natural history of Barrett's epithelium is not known, it is considered a premalignant tissue. Endoscopic surveillance is currently used to identify patients at increased risk for developing malignancy. However, conventional histologic markers such as dysplasia do not reliably predict the development of invasive carcinoma.

The timing of p53 alterations in esophageal tumorigenesis is not known with certainty. However, the finding of p53 mutations in metaplastic (non dysplastic) Barrett's mucosa, suggests this may be an early event in esophageal tumorigenesis. This is in keeping with other tumors of the upper aerodigestive tract (i.e. oropharynx, lung), where p53 mutations are believed to occur as relatively early molecular events. The finding of a p53 alteration in Barrett's mucosa, may, in the future, be used to identify subsets of patients at increased risk for esophageal malignancy, prompting further intervention (increased surveillance or resection). Prospective clinical studies are warranted to test this hypothesis.

#### Prognosis

Fundamental differences in esophageal tumor biology may explain different clinical outcomes for patients with histologically similar tumors within each well defined stage. Incorporation of molecular

markers, such as p53, into conventional clinicopathologic staging schema may improve reliability of predicting tumor recurrence, metastasis or survival.

To date, published studies evaluating the prognostic value of p53 protein in esophageal cancer have reported conflicting results. We recently reported a prospective study, evaluating p53 mutations and protein accumulation in esophageal adenocarcinomas, in an attempt to clarify the role of p53 as a predictor of survival following surgical resection. We found that p53 alterations were associated with significantly reduced disease-free and overall survival, by both univariate and multivariate analysis, suggesting p53 to be a potentially useful prognostic marker in this disease<sup>[11]</sup>.

#### Therapy

There has been increased interest in the use of chemotherapy in the multimodality therapy of esophageal cancer. It has been suggested that p53 may be used to stratify patients in future clinical trials evaluating induction chemotherapy. This approach was recently supported by experimental studies of esophageal cancer cell lines, where mutational status of p53 protein was predictive of chemosensitivity<sup>[12]</sup>.

The correction of underlying molecular alterations for preventing or treating cancer is a goal of gene therapy. Current approaches use modified or attenuated viral vectors to deliver genetic material into the tumor cell nucleus. Preliminary results with lung cancer have been encouraging, and it is believed that this approach will be successful for other upper aerodigestive tract tumors.

### REFERENCES

- 1 Casson AG, McKneally MF. Esophageal cancer epidemiology. In: Pearson FG, Deslauriers J, Ginsberg RJ, Hiebert CA, McKneally MF, Urschel HC, eds. Esophageal surgery. New York: Churchill Livingstone, 1995:551-559
- 2 Holscher AH, Siewert JR. Classification of adenocarcinomas of the esophagogastric junction. In: Peracchia A, Rosati R, Bonavina L, Fumagalli U, Bona S, Chella B, eds. Recent advances in diseases of the esophagus. Bologna: Mondizzi Editore, 1996:549-555
- 3 Casson AG, Mukhopadhyay T, Cleary KR *et al*. p53 gene mutations in Barrett's epithelium and esophageal cancer. *Cancer Res*, 1991;51:4495-4499
- 4 Dent J, Bremner CG, Collen MJ *et al*. Barrett's esophagus. *J Gastroenterol Hepatol*, 1991;6:1-22
- 5 Streitz JM, Andrews CW, Ellis FH. Endoscopic surveillance of Barrett's esophagus. Does it help. *J Thorac Cardiovasc Surg*, 1993;105:383-388
- 6 Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature*, 1991;351:453-456
- 7 Hollstein M, Sidranski D, Vogelstein B *et al*. p53 mutations in human cancers. *Science*, 1991;253:49-53
- 8 Hollstein MC, Metcalf RA, Welsh JA *et al*. Frequent mutation of the p53 gene in human esophageal cancer. *Proc Natl Acad Sci USA*, 1990;99:958-9961
- 9 Montesano R, Hollstein M, Hainaut P. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. *Int J Cancer*, 1996; 225-235
- 10 Harris CC, Hollstein M. Clinical implications of the p53 tumor suppressor gene. *N Engl J Med*, 1993;329:1318-1327
- 11 Casson AG, Tammemage M, Eskandarian S *et al*. p53 alterations in esophageal cancer: association with clinicopathological features, risk factors, and survival. *J Clin Pathol: Mol Pathol*, 1998;51:71-79
- 12 Nabeya Y, Loganzo F, Maslak P *et al*. The mutational status of p53 protein in gastric and esophageal adenocarcinoma cell lines predicts sensitivity to chemotherapeutic agents. *Int J Cancer*, 1995;64:37-46

# A review of reflux esophagitis around the world

Edwin J. Zarling, M.D.

**Subject headings** gastroesophageal reflux/epidemiology; gastroesophageal reflux/ pathophysiology; gastroesophageal reflux/complications;gastroesophageal reflux/diagnosis; gastroesophageal reflux/therapy

In the Western world, esophageal reflux is a common condition. In other parts of the world, the prevalence is less well defined, but appears to be swiftly approaching the levels seen in the West. As complications of this condition such as Barrett's epithelium and esophageal adenocarcinoma have seen huge increases over the past several decades in the West, there seems a likelihood that the rest of the world is at risk for a similar explosion in esophageal complications. This article will review the epidemiologic information about esophageal reflux, and pathophysiologic components of acid migration and altered peristaltic motility, and examine the advantages of treating this growing medical illness with acid suppression and prokinetic medication, as well as other nonpharmacologic therapies.

## EPIDEMIOLOGY

The prevalence and incidence of esophageal reflux are difficult to establish. These values are dependent on the population under study. Reported frequencies tend to be lower if the studied population is based on the general population as opposed to a study of patients from a medical facility. Frequencies based upon endoscopic observation tend to be substantially lower than symptom surveys, which is consistent with the observation that endoscopic evidence of damage is present in only 10% of symptomatic patients.

Despite the differences in methods for collecting prevalence and incidence data, there appears to be variability in frequencies of reflux around the world (Table 1). In a 1997 U.S. report, the prevalence of weekly heartburn symptoms in the general population was 19.8% (95% confidence interval 17.7-21.9)<sup>[1]</sup>. This appears to be higher than the often quoted weekly heartburn rate of 14% from 1976<sup>[2]</sup>, suggesting an increase in reflux over the past two decades. Reflux was reported to be

rare in Nigeria fifteen years ago<sup>[3]</sup>, and the low frequency was postulated to be due to protective differences in the structure of the lower esophageal sphincter. The frequency of symptoms in Taiwan recently was thought to be similar to the U.S. frequency reported two decades ago<sup>[4]</sup>, and the authors expressed concern that westernization of the diet, aging of the population, and increasing obesity would put the Taiwanese at higher risk of reflux and its sequelae.

**Table 1** Prevalence of reflux in various geographic sites(%)

	Weekly symptoms	Monthly symptoms	Endoscopic damage
U.S.A. <sup>[1,2,10]</sup>	19.8	36	7
Europe <sup>[8,37,39,40]</sup>	12-23	21-34	2
East Asia <sup>[4,7,8,9]</sup>	14		1.5-5

In the U.S., heartburn occurring at least once per month is reported in 36%<sup>[2]</sup> of patients. This seems similar to the 40% prevalence of dyspepsia in the British population<sup>[5]</sup>, and heartburn prevalence of 38% in Danish males and 30% in Danish females<sup>[6]</sup>.

The prevalence of endoscopic esophagitis in the U.S. is approximately 7%. In non-Western countries, the prevalence of esophagitis seems to be lower. It is seen during endoscopic observation in 1.5% to 5% of cases in China<sup>[7,8]</sup>, 2.7% in Korea<sup>[9]</sup>, and 2% in Germany<sup>[10]</sup>.

The pattern of higher prevalence of reflux in the Western countries, lower prevalence in Asia, and the lowest prevalence in Africa is similar to the frequencies of hiatal hernia around the world. As reviewed by Wienbeck and Barnert<sup>[10]</sup>, hiatal hernia are found most frequent in the industrialized countries.

## CLINICAL PRESENTATION

Reflux can present at any age, but seems most common in the 20 to 50 year age group. Symptoms occur equally often in males and females<sup>[10]</sup>, and the like lihood of hospitalization is similar for the two genders<sup>[11]</sup>. However, occurrence of esophageal damage is two to three times more often in males both in the West<sup>[6-10]</sup> and in China<sup>[7]</sup>. As individual patients age, they become less likely to experience symptoms of reflux but their risk for esophageal damage remains constant<sup>[4]</sup>. Patients with Barrett's epithelium are substantially less likely to feel

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Received 1998-05-25

discomfort during acid regurgitation. Mild and moderate obesity is strongly associated with the development of reflux.

Typical symptoms of esophageal reflux are epigastric or lower retrosternal pain. The pain lasts one or two hours and occurs one hour after a meal, or sooner if aggravating food was consumed such as spicy food, tomatoes, citrus fruit, onions or alcohol. The pain usually is described as burning, and may be accompanied by nausea or regurgitation. If the reflux occurs during sleep, the patient often notices a bile taste and hyper-salivation. Regurgitation without vomiting is also frequently reported. Many patients report a history of attempted therapy with over the counter antacids which usually provide relief that is effective but of too short duration.

In addition to the typical presentation, some patients reports symptoms which should serve as a warning that the reflux may be more severe or complicated than the classical case. Such warning symptoms may include dysphagia, weight loss, and anemia. If present, dysphagia may be suggestive of a motor dysmotility if it occurs to both solids and liquids, occurs intermittently, and is not associated with the need for vomiting in order to obtain relief. The presentation is more suggestive of an esophageal stricture if it occurs regularly and in response to solid food, or of regurgitation is the only method for relief.

Atypical presentations of reflux are being recognized in a widening arena of medical conditions. Certainly, many patients with atypical chest pain derive their symptoms from gastrointestinal reflux<sup>[12]</sup>. Pulmonary symptoms such as chronic bronchiectasis or repeat aspiration pneumonia may be due to esophageal reflux. More commonly, small volumes of reflux such as micro-aspiration or acid regurgitation without aspiration are thought to produce chronic cough, hoarseness, or asthma. The asthmatic events usually occur in non-allergic young adults who have nocturnal reflux. Over 80% of adults with asthma have esophageal reflux. This is mediated by a vagal reflex and may require anti-reflux surgery for long term treatment. Recently, chronic nasal sinusitis is being investigated for possible association with GERD.

Regardless which form of presentation occurs, reflux is most often seen in patients with mild to moderate obesity. Interestingly, 50 massively obese patients with a mean BMI of 42 had a prevalence of symptoms that was similar to that of normal controls, and they were not found to have macroscopic evidence of reflux<sup>[13]</sup>. It is unclear why this occurs. Aside from mild obesity, reflux occurs more often in cigarette smokers. Patients with *H. Pylori* infections are less likely to have reflux<sup>[14]</sup>. The mechanism of this phenomenon may be related to decreased acid production in patients with chronic *H. Pylori* infections.

## PATHOPHYSIOLOGY

Esophageal reflux occurs when gastric contents move in a retrograde direction into the esophagus. This regularly occurs in everyone on a daily basis. Problems develop when the gastric contents have prolonged exposure time to esophageal mucosa. This happens when the lower esophageal sphincter fails to provide an adequate mechanical barrier, when the esophageal peristaltic contractions fail to provide adequate clearing of the gastric contents, or when gastric contents are available for a prolonged time due to gastroparesis. The various physiologic events which contribute to the development of reflux are listed in Table 2.

**Table 2 Pathophysiologic factors contributing to the development of reflux**

Incompetent lower esophageal sphincter
Low pressure sphincter
Short sphincter length
Poor esophageal peristalsis
Decreased amplitude of contractions
Absence of propagated peristalsis
Delayed gastric emptying
Inadequate gastric contractions
Partial gastric outlet obstruction
Mucosal susceptibility to refluxate
Acid
Pepsin
Bile
Duodenal fluid

The lower esophageal sphincter pressure is low in one third of reflux patients, and normal in the remainder. Reflux may occur in the setting of normal sphincter pressure if the functional length of the sphincter is short, as shown by DeMeester *et al*<sup>[15]</sup>. As there is some inherited predisposition towards development of reflux,<sup>[16]</sup> it seems possible that worldwide variations in reflux prevalence may in part be due to genetically determined differences in sphincter length.

The normal action of peristaltic clearing of esophageal contents may fail in reflux disease. The magnitude of the failure of esophageal clearance is directly proportional to the severity of esophageal mucosal injury<sup>[17]</sup>. The most common peristaltic malfunction is a decreased amplitude of contractions in the distal esophagus, and occasionally a total failure of peristaltic propagation through this region. With these failures, gastric contents which have gained access to the esophageal mucosa will have extended exposure to this tissue. While it is unclear whether the peristaltic failure or the mucosal damage occurs first, it is commonly seen in clinical practice that pharmacologic resolution of the tissue injury is followed by return of the contractile activity.

A delay in gastric emptying can contribute to esophageal reflux, because it provides a substantial pool of acidic gastric contents for an extended period of time. Approximately half of patients with esophageal reflux have abnormal gastric emptying<sup>[18]</sup> which can be measured by radio nucleotide scintiscan or by electrogastrogram. Patients who fail to improve on acid suppression therapy should be evaluated for this comorbid condition.

A large sliding hiatal hernia traps gastric contents in its pouch above the diaphragm. This leads to free retrograde flow of acid into the esophagus<sup>[19]</sup> and is associated with poor esophageal emptying. Large hernias typically are at least four or five centimeters in length. More common smaller hernias are associated with comparatively normal esophageal emptying, and usually do not contribute to symptoms of reflux.

The injurious agent in reflux disease principally is gastric acid, however pepsin and perhaps other compounds also contribute. Because pepsin requires acid for activation, it is felt to be a less important caustic agent. In some surgical conditions such as partial gastrectomy, symptoms of reflux and the presence of esophageal damage may be due to regurgitation of duodenal fluid including bile into the esophagus. This may be directly measured and assessed with specialized research equipment<sup>[20]</sup>.

## COMPLICATIONS

Chronic reflux can lead to esophageal stricture formation, hemorrhage, and development of Barrett's mucosa. Strictures usually occur in patients with chronic esophageal ulceration. Hemorrhage is an uncommon complication, and most often is an acute problem seen in patients confined to an intensive care unit.

Barrett's mucosal transformation is found in Caucasians more often than in Africans or Orientals<sup>[4]</sup>. This may be a reflection of the lower prevalence of reflux in those populations. However, reflux is becoming more common in Asia, and there is concern that the risk of Barrett's and adenocarcinoma may rise in the coming decades<sup>[4]</sup>. Barrett's mucosa is eventually found in 15% of patients with reflux disease, however the condition is probably under diagnosed. In a study of Minnesota residents, the clinically diagnosed prevalence of Barrett's was 18 per 100 000 adults but the autopsy diagnosed prevalence was 376 per 100 000 cases<sup>[21]</sup>. This under diagnosis may occur because Barrett's patients are less likely to feel symptoms of reflux, and thus are less likely to present for medical evaluation. In addition, the extent of Barrett's mucosal changes may be very limited. These cases of short segment Barrett's are detected by extensive biopsy, or more recently by biopsy directed through the endoscopic use of stains such as methylene blue or an iodine solution.

According to a study which was age and gender matched, Barrett's patients develop reflux symptoms at an earlier age (onset at the age of 35 vs 44 for non Barrett's GERD patients), have a longer duration of symptoms (16 years vs 12 years), and were more likely to have smoking histories<sup>[22]</sup>. Efforts to reverse Barrett's changes with long term, high dose acid suppression by proton pump inhibitors have been unsuccessful. Recent reports suggest preliminary success with mucosal ablation by laser, cautery, and photo therapy<sup>[23]</sup>.

## DIAGNOSTIC APPROACH

The American College of Gastroenterology suggests that empirical therapy is appropriate in patients with typical symptoms of uncomplicated reflux disease<sup>[24]</sup>. All others should be considered for a diagnostic evaluation to confirm the diagnosis and define the extent of disease. The evaluation options include endoscopy, barium esophagram, 24 hour pH monitoring, and esophageal motility testing.

Endoscopy is a very valuable tool in the evaluation of esophageal reflux. It permits assessment of the mucosa, both visually and by biopsy. Over 85% of reflux patients have a normal visual findings at endoscopy, which can leave the origin of their symptoms in doubt. The use of biopsy in the distal esophagus is invaluable for confirming the presence of reflux which fortunately is too mild to produce visible damage.

Barium esophagram seems to have lost popularity after the advent of endoscopy. Currently, the best use of radiography is to establish the presence of minor strictures which can produce dysphagia. This uncommon condition may be hard to detect by endoscopy. One study reported that patients primarily evaluated by radiography rather than gastrointestinal consultation or endoscopy had higher total costs for their care<sup>[25]</sup>.

The 24 hour pH is monitored to confirm and quantify the existence of esophageal reflux, and to correlate the occurrence of symptoms with the presence of acid in the esophagus. This tool is most often used in patients with atypical presentations. It is also useful in patients with typical symptoms who do not improve on maximal medical therapy. In these patients, the 24 hour test should be done while the patient consumes the full pharmacologic regimen prescribed.

Esophageal motility testing is most useful prior to the decision for surgery. Patients found to have poor esophageal peristalsis are at risk for dysphagia after an antireflux procedure.

## TREATMENT

### *Lifestyle changes*

The prevalence of smoking is higher in reflux patients than in healthy controls. This suggests that smoking may promote the development of esophageal reflux damage, perhaps due to its

relaxation of the lower esophageal sphincter. Symptoms in patients who smoke are more difficult to control than those in non smokers. Thus it is beneficial in the treatment of reflux for the patient to stop the use of tobacco products.

With the observation that moderate obesity aggravates or initiates reflux symptoms, patients are advised to achieve a modest weight loss. Symptoms are improved once there is a weight loss of 5% to 10%.

Elevation of the head of the bed aids the gravitational drainage of the esophagus. Since a substantial portion of reflux patients have impaired esophageal emptying, the lifting of the head of the bed by six inches should be beneficial. This has been demonstrated in a study which noted equivalent rates of healing of esophageal ulcers when bed elevation was compared to standard dose of a histamine receptor antagonist<sup>[26]</sup>.

### Pharmacologic options

As listed in Table 3, the currently available options for reflux treatment include acid suppression drugs such as histamine receptor antagonists (H2RA) or proton pump inhibitors (PPI), and promotility agents. While some studies have reported a beneficial effect of isolated promotility drugs in cases of mild esophagitis, most others suggest a lesser role. Several authors recommend that mild to moderate reflux patients be treated initially with H2RA, and that severe cases use PPI drugs<sup>[27,28]</sup>. This practice suggestion is consistent with observations of actual practice patterns by primary care physicians and gastroenterologists<sup>[29]</sup>. Prokinetic agents may be added to acid suppression therapy for additional symptomatic and healing effects.

Several review papers have reported that all four H2RA agents are similar in efficacy<sup>[24]</sup>. However, there are very few direct studies which attempt to compare two or more of these agents using equivalent doses in appropriate clinical populations. Thus it is unclear whether all branded H2RA agents, or their generic equivalents now beginning to appear on the markets, are truly similar.

**Table 3 Pharmacologic agents used in the treatment of reflux**

	Dosage*	Possible mechanism
H2 receptor antagonist		
Cimetidine	800mg bid or 400mg qid	Reduce acid
Famotidine	20mg bid or 40mg bid	Reduce acid
Nizatidine	150mg bid	Reduce acid, prokinetic
Ranitidine	150mg qid	Reduce acid
Proton pump inhibitor		
Lansoprazole	30mg qd	Reduce acid
Omeprazole	20mg qd	Reduce acid
Prokinetic		
Cisapride	10mg qid or 20mg qid	Prokinetic
Metoclopramide	15mg qid	Prokinetic

\*Dosages for erosive esophagitis, except for cisapride which is suggested for symptomatic treatment only<sup>[41]</sup>.

A review of the medical literature suggests several potential differences among the H2RA drugs. In order to clinically measure whether these differences are important in clinical practice, carefully designed studies will be needed which compare H2RA drugs in the mild and moderate severity groups of reflux esophagitis. A potential point of differentiation among the H2RA drugs in the observation that one agent, nizatidine, has prokinetic activity. In a dog model, the intensity of this activity may exceed that of cisapride<sup>[30]</sup>. Prokinetic activity was not significantly present in the other H2RA. Nizatidine has been shown in human, dog and rat models to improve gastric emptying<sup>[30-32]</sup>. The mechanism of the prokinetic activity may be due to nizatidine's antiacetylcholinesterase activity<sup>[30]</sup>. Another point of differentiation is that two agents, famotidine and nizatidine, have no interaction with the hepatic P-450 enzyme system. As this system is capable of metabolizing many medications, interference with its function by one of the other H2RA drugs has the potential to alter the clearance of other medications taken by a patient. Whether such a hypothetical interaction has clinical significance is not known.

The proton pump inhibitor drugs available in the U.S. are both capable of healing esophageal ulcers and relieving symptoms of reflux. At least one study suggests that lansoprazole (30mg per day) was better at symptom relief than omeprazole (20mg per day)<sup>[33]</sup>. The two drugs seem to differ in that lansoprazole has fewer interferences with blood levels of other medications.

### Surgical therapy

While the vast majority of patients with esophageal reflux can be successfully treated with lifestyle and pharmacologic therapies, a small number need surgical help. Patients who fail to respond to maximal medical therapy are candidates for surgical correction. Another group of candidates are the patients who are unable to stop their medication and yet unwilling to take the medicine over a long time frame. Both groups of medically resistant patients are often younger than the usual reflux patient, and tend to have a near normal body weight. A third group of surgical candidates are those with atypical symptoms such as asthma. Often they do not resolve their atypical symptoms on maximal medical therapy.

Of the patients undergoing a Nissen fundoplication, over 90% can anticipate cures of their symptoms. This figure appears to hold true for both the open procedure as well as the laparoscopic approach. The rate of conversion from laparoscopic to open Nissen procedure is less than 2%<sup>[34]</sup>. The reoperation rate for this laparoscopic approach is 3.9%. Unfortunately, the laparoscopic Toupet procedure may not promote such good long term results, as one study reported the Toupet procedure

was associated with a high incidence of recurrent postoperative esophageal reflux disease<sup>[35]</sup>.

### Maintenance therapy

After a course of anti-reflux medication has been completed, those patients who succeeded in their lifestyle changes are the ones most likely to be symptom free. Of the patients who immediately redevelop their complaints, symptomatic relief can be reacquired by giving the former H2RA patients one half of their former dose, and by giving the former PPI patients a standard dose of H2RA. The duration of this maintenance dose is variable, but commonly exceeds six months. During this time, further efforts at long term life style changes should be made. Maintenance therapy with acid suppression alone is not as effective as the combination of acid suppression plus prokinetic activity<sup>[36]</sup>.

Over extended time, the reflux symptoms resolve, as shown by a study of the natural history of reflux disease<sup>[37]</sup>. Of patients thought to have severe disease, the prevalence of erosive esophagitis fell from 60% to 10% over a two-decade study. Symptoms reduced in most patients, and the use of medications ended in 68% of the patients. This suggests that reflux, even in severe patients, will slowly resolve over time.

### Summary

Approximately 85% to 90% of patients with heartburn have mild disease. This can be suspected in patients who are under the age of 50 years, have intermittent symptoms only during the waking hours, and have no warning symptoms. Other patients with mild illness may be discovered by finding normal esophageal tissue at endoscopy, or the presence of only distal esophageal erythema. These mild patients should begin therapy with H2RA<sup>[38]</sup>. The use of H2RA plus prokinetic agents is more effective than acid suppression alone<sup>[36]</sup>. The uncommon patient with more severe illness may need PPI. These medicines are continued for six to eight weeks. During this time, all necessary lifestyle changes should be attempted, particularly loss of weight and cessation of smoking. Patients who do not respond to therapy, and are not already receiving a medication with prokinetic activity, should be considered for further evaluation which may include gastric emptying time, esophageal pH probe, or manometry.

### REFERENCES

- 1 Locke GR, Talley NJ, Zinsmeister AR, Melton LJ. Prevalence and clinical spectrum of gastroesophageal reflux: a population based study in Olmsted County, Minnesota. *Gastroenterology*, 1997;112(5):1448-1456
- 2 Nebel OT, Fornes MF, Castell DO. Symptomatic gastroesophageal reflux: incidence and precipitation factors. *Am J Dig Dis*, 1976;21(11):953-956
- 3 Ofoegbu RO. Incidence, pattern and African variations of common benign disorders of the esophagus. Experience from Nigeria. *Am J Surg*, 1982; 144(2):273-276
- 4 Chen PH. Review: Barrett's oesophagus in Taiwan. *J Gastro Hepatol*, 1997; 12(6):S19-22

- 5 Penston JG, Pounder RE. A survey of dyspepsia in Great Britain. *Alim Pharm Therapeutic*, 1996;10(1):83-89
- 6 Kay L, Jorgensen T, Jensen KH. Epidemiology of abdominal symptoms in a random population: prevalence, incidence, and natural history. *Eur J Epidemiol*, 1994;10(5):559-566
- 7 Zhang TC, Zhu MZ, Geng XC, Li YJ, Cao T, Zhang LP. Endoscopic study of reflux esophagitis. *JAMA Southeast Asia*, 1996;12(2Suppl):22-24
- 8 Chang CS, Poon SK, Lien HC, Chen GH. The incidence of reflux esophagitis among the Chinese. *Am J Gastro*, 1997;92(4):668-671
- 9 Min YI, Lee SK, Kim MH, Yi SY, Jung HY. Epidemiology of reflux esophagitis in a general health screening population. *JAMA Southeast Asia*, 1996;12 (2Suppl):11-15
- 10 Wienbeck M, Barnert J. Epidemiology of reflux disease and reflux esophagitis. *Scand J Gastro*, 1989;156:7-13 supplement
- 11 Zarling EJ, Bernsen MB. The effect of gender on the rates of hospitalization for gastrointestinal illnesses. *Am J Gastro*, 1997;92(4):621-623
- 12 Voskuil JH, Cramer MJ, Breumelhof R, Timmer R, Smout AJ. Prevalence of esophageal disorders in patients with chest pain newly referred to the cardiologist. *Chest*, 1996;109(5):1210-1214
- 13 Lundell L, Ruth M, Sandberg N, Bove-Nielsen M. Does massive obesity promote abnormal gastroesophageal reflux. *Dig Dis Sci*, 1995;40(8):1632-1635
- 14 Xia HH, Talley NJ. Helicobacter pylori infection, reflux esophagitis, and atrophic gastritis: an unexplored triangle. *Am J Gastro*, 1998;93(3):394-400
- 15 DeMeester TR, Mason RJ, Ritter MP, Gadenstatter M, Bremner CG, Peters JH. Association of lower esophageal sphincter length with postprandial gastroesophageal reflux. *Gastroenterol*, 1997;112(4):A1458
- 16 Trudgill N. Familial factors and gastroesophageal reflux disease. *Gastroenterol*, 1997;112(4):A315
- 17 Rakic S, Stein HJ, DeMeester TR, Hinder RN. Role of esophageal body function in gastroesophageal reflux disease: implications for surgical management. *J Am Col Surg*, 1997;185(4):380-387
- 18 Soykan I, Lin Z, Jones S, Chen J, McCallum RW. Gastric myoelectrical activity, gastric emptying and correlations with dyspepsia symptoms in patients with gastroesophageal reflux. *J Invest Med*, 1997;45(8):483-487
- 19 Sloan S, Kahrlas PJ. Impairment of esophageal emptying with hiatal hernia. *Gastroenterol*, 1991;100(3):596-605
- 20 Vaezi MF, Richter JE. Role of acid and duodenogastroesophageal reflux in gastroesophageal reflux disease. *Gastroenterology*, 1996;111(5):1192-1199
- 21 Cameron AJ, Zinsmeister AR, Ballard DJ, Carney JA. Prevalence of columnar lined (Barrett's) esophagus. *Gastroenterology*, 1990;99(4):918-922
- 22 Eisen GM, Sandler RS, Murray S, Gottfried M. The relationship between gastroesophageal reflux disease and its complications with Barrett's esophagus. *Am J Gastro*, 1997;92(1):27-31
- 23 Mayrand S. Treatment of Barrett's esophagus. *Can J Gastro*, 1997;11 Suppl B:98B-102B
- 24 DeVault KR, Castell DO. Guidelines for the diagnosis and treatment of gastroesophageal reflux disease. *Arch Intern Med*, 1995;155:2165-2173
- 25 Longstreth GF. Long term costs after gastroenterology consultation with endoscopy versus radiography in dyspepsia. *Gastrointest Endosc*, 1992;38(1):23-26
- 26 Harvey RF, Cordon PC, Hadley N, Long DE, Gill TR, MacPherson RI, Beats BC, Tottle AJ. Effects of sleeping with the head raised and of ranitidine in patients with severe peptic oesophagitis. *Lancet*, 1987;ii:1200-1203
- 27 Rex D. Gastroesophageal reflux disease in adults: pathophysiology, diagnosis, and management. *J Fam Pract*, 1992;35(6):673-681
- 28 Robinson M. Gastroesophageal reflux disease. *Postgrad Med*, 1994;95(2):88-102
- 29 Kim SL, Goldschmid S. A survey of community practice patterns for refractory gastroesophageal reflux disease. *Gastroenterology*, 1997;112(4):A175
- 30 Ueki S, Seiki M, Yoneta T, Aita H, Chaki K, Hori Y, Morita H, Tagashira E, Itoh Z. Gastroprokinetic activity of nizatidine, a new H-2 receptor antagonist, and its possible mechanism of action in dogs and rats. *J Pharm Exp Therap*, 1993;264(1):152-157
- 31 Kaneko H, Mitsuma T, Uchida K, Nagai H, Harada M, Kotera H. Nizatidine accelerates gastric emptying of a solid meal in rats. *Dig Dis Sci*, 1995;40(9):2043-2051
- 32 Harasawa S, Miwa T. The effect of nizatidine on gastric emptying and plasma gastrin concentrations in patients with peptic ulcer. *Jpn Pharmacol Ther*, 1993;21(3):327
- 33 Mee AS, Rowley JL. Rapid symptom relief in reflux oesophagitis: a comparison of lansoprazole and omeprazole. *Aliment Pharm Ther*, 1996;10(5):757-763
- 34 Hinder RA, Perdakis G, Klinger PJ, DeVault KR. The surgical option for gastroesophageal reflux disease. *Am J Med*, 1997;103(5A):144S-148S
- 35 Jobe BA, Wallace J, Hansen PD, Swanstrom LL. Evaluation of laparoscopic Toupet fundoplication as a primary repair for all patients with medically resistant gastroesophageal reflux. *Surg Endosc*, 1997;11(11):1080-1083
- 36 Vigneri S, Termini R, Leandro G, Badalamenti S, Pantalena M, Savarino V, DiMario F, Battaglia G, Sandro G, Pilotto A, Plebani M, Davi G. A comparison of five maintenance therapies for reflux esophagitis. *New Engl J Med*, 1995;333(17):1106-1110
- 37 Isolauri J, Luostarinen M, Isolauri E, Reinikainen P, Vijacka M, Keyrilainen O. Natural course of gastroesophageal reflux disease: 17-22 year follow up of 60 patients. *Am J Gastro*, 1997;92(1):37-41
- 38 Beck LT, Champion MC, Lemire S, Thomson ABR. The second Canadian consensus conference on the investigation and treatment of gastroesophageal reflux disease. *Gastroenterology*, 1997;112(4):A3
- 39 Corder AP, Jones RH, Sadler GH, Daniels P, Johnson CD. Heartburn, esophagitis and Barrett's esophagus in self medicating patients in general practice. *Br J Clin Pract*, 1996;50(5):245-248
- 40 Isolauri J, Jaipala P. Prevalence of symptoms suggestive of gastro-esophageal reflux disease in an adult population. *Ann Med*, 1995;27(1):67-70
- 41 Physicians' Desk Reference, 52nd edition. Montvale NJ:Medical Economics Company, 1998

# Therapy of acute severe pancreatitis awaits further improvement

WU Xian-Zhong

**Subject headings** pancreatitis/therapy; pancreatitis/drug therapy; pancreatitis/mortality; pancreatitis/complication; TCM-WM therapy

Being one of the highly fatal diseases, severe acute pancreatitis (SAP) had a mortality of over 30% before the 90s as documented at home and abroad<sup>[1,2]</sup>, with large divergence between various therapeutic viewpoints and methods. However, over the recent decades, great changes have taken place in the knowledge of the disease, the prognostic factors become basically clear, thus providing favorable conditions for improving therapeutic methods as well as clinical results. The mortality has been reduced to around 20%<sup>[3]</sup>.

With pathologic morphological, pathophysiological, biochemical, immunologic and imaging live observations, adequate evidences have revealed that SAP is a systemic rather than a local critical condition. So the prognosis can hardly be changed by merely treating the organ. However, the concrete situation of the individual patient should be considered seriously, be it systemic treatment or local management of the pancreas or peritoneum. What ought to be stressed is the individualized treatment but not a standardized or regularized one.

The clarification of the pathophysiological role of cytokines and other inflammatory media, and the continuous maturation of the SIRS/MODS hypothesis are helpful in the explanation of the natural course of SAP. During the clinical course of most SAP patients, two MODS peaks can be seen. The first one appears in a week after the onset, being nonseptic MODS, and can be recovered through appropriate treatment. The mortality in this stage is reduced significantly in comparison with the past. The second peak usually appears 10 days to two weeks, being septic MODS, with rather high

morbidity and mortality. This is due to necrotizing pancreatic tissue and intraperitoneal infection which is caused by translocation of intestinal flora. As the two MODS events develop on different backgrounds, different methods of treatment should be instituted. Operative intervention will not be beneficial to the first peak, while in the second peak, accurate evaluation of the liquidifying sequestration degree of necrotizing pancreatic tissue, definite location of abscess formation and prompt drainage, and prevention of other complications are of great significance in patient's recovery. The systemic treatment of SAP is actually the treatment of MODS, that should be carried out under intensive monitoring and care, including supportive systemic treatment, prevention and treatment of infection, keeping water, electrolyte and acid-base balance. Trypsin inhibitor can be given earlier, and gastric acid secretion inhibitor given to prevent stress ulcer bleeding. In case of certain organ deficiency, corresponding supportive treatment is mandatory.

Early in the mid 80s, Beger pointed out four prognostic factors of SAP, i.e., the extent of pancreatic necrosis, presence or absence of retroperitoneum invasion, presence or absence of ascites and infection or not of the necrotic pancreatic tissue<sup>[4]</sup>. Active and aggressive therapeutic measures pointing to these factors which have been proved by later documents would help increase the cure rate of SAP.

Since the mid 80s extensive researches on the treatment of SAP by integrating traditional Chinese and western medicine has been carried out in this country with significant advances. Systematic reports have been presented by West China Medical University, Dalian Medical University, Harbin Medical University and Tianjin Institute of Acute Abdominal Diseases<sup>[5-7]</sup>. Extensive clinical investigations and experimental studies revealed the therapeutic mechanisms of traditional Chinese medicine and the medicinal herbs were mainly the actions on SIRS/MODS and the control by the herbs on peritoneal exudation and absorption<sup>[8,9]</sup>. These can be summarized as follows:

1. MODS can be definitely prevented by early use of purgative herbs such as Dachengqi Decoction. Since ileus is very frequently seen in SAP, not only interfering with the evacuation of intestinal contents but also causing multiple reproduction of intestinal

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Received 1998-02-06

flora and expansion of endotoxin pools. Restoration of the intestinal motor function through the action of purgative herbs is helpful in reducing the risks of enterogenic infections and enterogenic endotoxemia, the effects and the results have been proved by clinical and experimental studies.

2. Purgative herbs have a significant protection on the intestinal barrier. In all the three types of MODS rat models, i.e., bacterial infection of the peritoneum, mesenteric artery ischemia and reperfusion, and zymogen peritonitis, obvious failure of intestinal barrier could be seen. When *E. coli* labeled with 35S were injected into the intestinal lumen, translocation of the labeled *E. coli* to various viscerae could be detected. If the animal was given Dachengqi Decoction in advance through the gastric tube, translocation of flora would be markedly inhibited.

3. Experimental studies proved the pharmacological actions such as improving the blood supply of intra-abdominal viscerae, reducing inflammatory exudation, promoting remission of inflammation and minimizing abscess formation, of the medicinal herbs with effects of “clearing heat and detoxicating” and of “activating blood circulation and dispersing stasis”.

4. One hundred and forty-six patients with SAP were treated with integrated traditional Chinese and western medicine in Tianjin Institute of Acute Abdominal Diseases. Besides the conventional supportive treatment in western medicine, purgative herbs were administered early; when the abdominal distention was diminished with bowel movements, herbs with effects of “clearing heat and detoxicating” and of “activating blood circulation and dispersing stasis” were used. For patients with surgical indications, corresponding operative procedures were performed. The results of clinical observation revealed that herbal treatment lowered

the incidence of injury of extrapancreatic organs, markedly decreased the incidence of late infections of SAP, and finally reduced the mortality to 16.6%, which is significantly superior to the control group of western medical therapy alone.

In recent years although progresses have been made in the treatment of severe acute pancreatitis, the mortality remains as high as nearly 20%; for a few specially critical cases, effective therapeutic strategies are still lacking; in those cured cases the course was lengthy, usually over 2-3 months, with many complications, thus causing heavy medical expenses; treatment by integrated medicine is at present in the testing stage, more experience in reasonable application of Chinese herbs need to be accumulated.

Facing the forthcoming twenty-first century, I believe that it will be an era of rapid development of medical sciences, in which a new hope will be brought to patients under critical care including those with severe acute pancreatitis, with increase of cure rate and amelioration of sufferings of patients.

## REFERENCES

- 1 Banks PA. Acute pancreatitis: medical and surgical management. *Am J Gastroenterol*, 1994;89(8):S78-S85
- 2 Qi QH, Zhang WG, Lu SY. Risk factors and prognosis of severe Pancreatitis. *Chin J Dig*, 1995;15(6):364-366
- 3 Beger HG, Rau B, Mager J, Pralle U. Natural course of acute pancreatitis. *World J Surg*, 1997;21(2):130-135
- 4 Beger HG, Krautg Beger W, Bittner R, BLOCK S, Buchler M. Results of surgical treatment necrotizing pancreatitis. *World J Surg*, 1985;9(5):972-979
- 5 Ni H, Cui NQ, Li JK, Wu XZ. The change of TNF and amylase in early phase of different acute experimental pancreatitis. *Chin J Surg Integrated*, 1997;3(2):115-118
- 6 Yao GQ, Wu XZ. Clinical study of treatment of severe acute pancreatitis with Qinyi decoction. *Chin J Surg Integrated*, 1997;3(4):244-246
- 7 Song AN, Wei JJ, Jiang JM, Zhang ZD, Jin LR, Shen WJ *et al*. Compared effectiveness of therapies with surgery and non surgery for severe acute pancreatitis. *Chin J Surg Integrated*, 1998;4(1):14-16
- 8 Wu XZ, Tian ZS. Research on the symptom complex and therapeutic methods. *Tianjin J TCM*, 1996;13(5):32-35
- 9 Cui NQ, Zhao Q, Ge ZH, Yin ZF, Jin DG, Sun XM *et al*. Effective observation on treatment of MODS with the therapeutic method of Tongli Gongxia. *Chin J Surg Integrated*, 1996;2(5):315-320

# Apoptosis and its relationship with cell proliferation, p53, Waf1p21, bcl-2 and c-myc in esophageal carcinogenesis studied with a high-risk population in northern China

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**Subject headings** esophageal neoplasms; apoptosis; precancerous lesions; Waf1 p21 gene; p53 gene; bcl-2 gene; c-myc gene

## Abstract

**AIM** To determine the extent of apoptosis and its possible relationship with the changes of p53, Waf1p21, bcl-2, and c-myc at different stages of esophageal carcinogenesis.

**METHODS** Two hundred and forty-one esophageal biopsy samples from symptom-free subjects and 38 surgically resected esophageal carcinoma tissues from a high-risk population for esophageal cancer in Henan, China were used in this study. Apoptotic cells and apoptotic bodies were identified by well-established morphological criteria. The extent of apoptosis and its possible relationship with the rate of cell proliferation (PCNA) and changes of p53, Waf1p21, bcl-2, and c-myc were analyzed in samples with esophageal precancerous and cancerous lesions.

**RESULTS** The apoptotic cells, identified morphologically, were located in the same proliferative compartment of hyperproliferative cell population in the esophageal epithelia as the cells immunostaining-positive for p53, bcl-2, c-myc and PCNA. The apoptotic indices (total number of apoptotic cells and apoptotic bodies per mm<sup>2</sup> of the tissue section) were low in the normal epithelia, and increased significantly as the lesions progressed from BCH to DYS and to SCC. The extent of apoptosis correlated well with the cell proliferation indices based on PCNA. The total number of positive cells for p53 stain was much higher than that of apoptotic cells. No difference in apoptotic indices was found between p53-positive and p53-negative samples. Waf1p21-positive cells resided in

cell layers were higher in number than p53 and PCNA-positive cells. The number of immunostaining positive cells for Waf1p21 increased slightly from normal to BCH, but decreased in DYS and SCC. Positive staining samples for bcl-2 and c-myc increased as the lesions progressed from BCH to DYS and to SCC. No apparent correlation between apoptosis and Waf1p21, bcl-2 or c-myc expression was observed.

**CONCLUSION** The extent of apoptosis was low in normal esophageal epithelium and increased as the lesions progressed. The apoptotic cells were located in the same hyperproliferative cell compartment as cells immunostaining-positive for p53, bcl-2, c-myc and PCNA, but no apparent correlation between apoptosis and these parameters was observed, possibly due to the complexities of molecular changes in esophageal carcinogenesis.

## INTRODUCTION

Carcinoma of the esophagus (EC) is a widely occurring disease in Huixian and Linxian counties of Henan Province in northern China and remains a leading cause of cancer-related death<sup>[1,2]</sup>. The development of human esophageal squamous cell carcinoma (SCC) is a multistage progressive process<sup>[3-6]</sup>. An early indicator of abnormality in persons predisposed to EC is the increased proliferation of esophageal epithelial cells, morphologically manifested as basal cell hyperplasia (BCH), dysplasia (DYS) and carcinoma in situ (CIS), which could be considered precancerous lesions of EC. Changes in cell proliferation and cell death may be a key factor contributing to the rate of neoplastic progression and tumor growth. Although previous studies on the subjects from these high incidence areas have suggested the importance of epithelial cell hyperproliferation in human esophageal carcinogenesis, little information is available on cell death and the molecular mechanisms involved.

Recent evidence suggests that apoptosis, a genetically programmed cell death, is of fundamental importance in tumorigenesis<sup>[7,8]</sup>. There

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Received 1998-07-28

are several genes which participate in the regulation of apoptosis, and *bcl-2* has been identified as an apoptosis inhibitor<sup>[9-11]</sup>. In spite of a lack of detectable autonomous transforming activity, *bcl-2* has been shown to cooperate with *c-myc* in immortalizing cells<sup>[12]</sup>. Under certain conditions, constitutive expression of *c-myc* induces apoptosis and it can be suppressed by *bcl-2*. It appears that the *c-myc*-cooperating oncogenic activity of *bcl-2* is related to its inhibition of apoptotic pathways<sup>[9]</sup>.

The tumor suppressor gene *p53*, a transcription factor, has been identified as a participant in the cellular DNA damage response. Upon DNA damage, *p53* up regulates *Waf1p21* to cause G1 arrest, which allows time for damaged DNA to be repaired, or for triggered apoptosis to eliminate genetically damaged cells<sup>[13-16]</sup>. The apoptosis promoting capacity of *p53* is presumably due to its ability to activate *bax*, a gene that encodes an inhibitor of *bcl-2*<sup>[15]</sup>. *Bax* prote in inhibits the function of *bcl-2* leading to increased apoptosis. Recent reports indicate that *Waf1p21* can also induce apoptosis<sup>[17]</sup>. As the *Waf1p21* is up-regulated by wild type *p53* protein, the levels of *Waf1p21* protein may reflect the functional status of *p53*.

Our previous studies indicated that accumulation of *p53* protein occurred at early stages of human esophageal carcinogenesis, even in histopathologically normal epithelium<sup>[18-19]</sup>; *p53* gene mutations were observed in some of these samples<sup>[20-22]</sup>. The results indicated that *p53* protein accumulation and gene mutation could be an early event in esophageal carcinogenesis. The loss of normal *p53* function, for instance due to mutations, could delay apoptosis<sup>[15]</sup>.

Quantitative analysis of both apoptosis and proliferation should provide important insights into cancer development, but such analysis has not been made in samples at different stages of esophageal carcinogenesis. In this study, we have investigated the extent of apoptosis and its relationship with cell proliferation and changes of *p53*, *Waf1p21*, *bcl-2* and *c-myc* in human esophageal epithelia with different severities of precancerous and cancerous lesions from subjects in Henan, China.

## MATERIALS AND METHODS

### *Tissue collection and processing*

Esophageal biopsy tissues were collected from 241 symptom-free subjects, and surgically-resected EC specimens were collected from 38 patients in Huixian and Linxian, China. Of these 279 subjects, there were 160 males (20 to 71 years of age with a mean±SD of 48±14 years) and 119 females (20 to 79 years of age with a mean±SD of 47±16 years). None of these cancer patients received any treatment of chemotherapy or radiotherapy before

the operation. All specimens were fixed with 85% alcohol, embedded with paraffin, and serially sectioned at 5 μm. The sections were mounted onto histostick-coated slides. Three or 4 adjacent ribbons were collected for histopathological analysis (hematoxylin and eosin stain), for apoptosis analysis, and for immunohistochemical staining.

### *Histopathology analysis*

Histopathological diagnosis for esophageal epithelia were made according to cellular morphological changes and tissue architecture using previously established criteria<sup>[23]</sup>. In brief, the normal esophageal epithelium contained 1-3 proliferating basal cell layers; the papillae were confined to the lower half of the epithelium. In BCH, the proliferating basal cells were increased to more than 3 cell layers and less than half of the whole epithelial thickness. DYS was characterized by the partial loss of cell polarity and cell nuclear atypia. SCC was characterized by confluent and invasive sheets of cohesive, polymorphous cells with hyperchromatic nuclei.

### *Immunohistochemical staining for PCNA, p53, Waf1p21, bcl-2 and c-myc*

The avidin-biotin-peroxidase complex (ABC) method was used for PCNA, *p53*, *Waf1p21*, *bcl-2* and *c-myc* antigen detection (Oncogene Science, Inc., Manhasset, NY). In brief, after dewaxing, inactivating endogenous peroxidase activity, and blocking cross-reactivity with normal serum, the sections were incubated overnight at 4 °C with a diluted solution of the primary antibodies (1:200 for PCNA, 1:1 000 for *p53*, 1:20 for *Waf1p21* and *bcl-2* and 1:150 for *c-myc*). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit). Normal serum blocking and omission of the primary antibody were used as negative controls<sup>[18,19]</sup>.

### *Quantitative analysis of immunostaining results*

Quantitative analysis of nuclear immunostaining results was recorded as the number of positive cells per mm<sup>2</sup> of the tissue section as described previously<sup>[19]</sup>. This was done by counting all the positive stained cells in the whole piece of tissue under a microscope with magnification ×400; usually 24 fields for biopsy and 50 fields for surgically resected specimens were counted.

### *Analysis of apoptotic index*

Apoptotic cells and apoptotic bodies were identified by well-established morphological criteria<sup>[7,24-26]</sup>. In

general, the morphologic characteristics attributed to apoptotic cells include cell shrinkage, disconnection with neighboring cells, nuclear chromatin condensation, maintenance of cytoplasmic membrane integrity, strong eosinophilic cytoplasm and lack of an inflammatory reaction. Apoptotic bodies were defined as small, roughly spherical or ovoid cytoplasmic fragments, some of which contain nuclear fragments. Apoptosis was scored in coded slides by microscopic examination of H & E-stained sections at 400X. Quantitation of apoptotic cells and bodies was performed in the following manner<sup>[25]</sup>. The serial sections of the tissues were first scanned under a microscope with low magnification, and the tissue slides were selected based on the following criteria: flat with good orientation, clear cell and tissue structure, and uniform staining. All identified apoptotic cells and bodies in the slide were counted. In SCC, only fields with the least amount of stroma were selected for counting. Apoptotic cells or bodies were not evaluated from the vicinity of necrotic areas. In cases in which many apoptotic bodies were found clearly in one cell, the group of apoptotic bodies was counted as one. Quantitative analyses of apoptotic changes were recorded as apoptotic cell indices (ACI, the number of apoptotic cells per mm<sup>2</sup> of the tissue), apoptotic body indices (ABI, the number of apoptotic bodies per mm<sup>2</sup> of the tissue) and apoptotic indices (AI, the combined number of apoptotic cells and bodies per mm<sup>2</sup> of the tissue).

#### **Statistical analysis**

The mean±SE of the PCNA, p53 and Waf1p21 immunostaining positive cells/mm<sup>2</sup> and the AI in the esophageal biopsy and surgically resected EC samples in each histologic category were calculated by univariate analysis, compared by the Wilcoxon rank-sum test for unpaired data and correlated by Pearson's correlation. The Chi-squared test was used for the percentage of samples with immunostaining positivity.

## **RESULTS**

### **Apoptotic indices in esophageal precancerous and cancerous lesions**

Both apoptotic cells and apoptotic bodies were observed in tissues with different severities of lesions (Table 1). The apoptotic cells usually had small nuclei and condensed eosinophilic cytoplasm. In normal epithelia and epithelia with BCH and DYS, apoptotic cells were located among the hyperproliferative basal cells (Figure 1). In SCC, most of the apoptotic cells were seen among the peripheral cancer cells, but not in the keratinized

areas of the tumor. The apoptotic cells were usually scattered single cells which had highly condensed nuclear chromatin and cytoplasm as well as strong eosinophilic staining of the cytoplasm. No inflammatory response was observed around these apoptotic cells. The apoptotic bodies most frequently appeared as a single structure separated from the surrounding intact cells by a clear halo (Figure 1). The AI and the frequency of apoptosis occurrence was low in normal epithelia, but increased significantly from BCH to DYS and to SCC ( $P < 0.01$ , Table 1). The ratio of ACI and ABI reversed from 1.5:1 in normal epithelia to 1:1.7 in SCC, suggesting a higher turnover of apoptosis activity in cancers than in normal tissues.

### **Immunoreactivity for PCNA, p53, Waf1p21, bcl-2 and c-myc in different stages of esophageal carcinogenesis**

Intense immunostaining of p53 protein and PCNA was observed in the cell nuclei of tissues with different lesions. In brief, similar immunostaining patterns were observed for PCNA and p53, but the number of PCNA-positive cells was higher than that of p53 in the same histological category (3- to 4-fold, Tables 1 and 2). As the esophageal tissue progressed from BCH to DYS and to SCC, the PCNA and p53 immunostaining positive cells increased significantly in number and expanded upwards in the epithelium. This finding is consistent with our previous report<sup>[19]</sup>.

The Waf1p21 positive cells were located at the third and fourth cell layers which was 2 to 3 cell layers higher than those staining positive for PCNA and p53 in esophageal epithelia (Figure 2). Waf1p21 immunostaining was confined to the cell nucleus. The percentage of samples with Waf1p21 positive immunostaining was similar in normal and BCH esophageal epithelia, but the number of Waf1p21 immunostaining positive cells was slightly higher in BCH than in normal (Table 2). As the lesions progressed to DYS and to SCC, the percentage of samples with Waf1p21 immunoreactivity and the number of immunostaining positive cells decreased. The total number of Waf1p21 positive cells appeared to be lower than that of p53 in normal and BCH esophageal epithelia, much lower in DYS (24 fold) and markedly lower in SCC (162 fold).

Bcl-2 immunoreactivity was observed only in the cytoplasm of the cells. Bcl-2 positivity was seen in none of the 23 morphologically normal esophageal epithelial samples but was observed in 16% (5/31) of the BCH, 40% (4/10) of the DYS and 86% (6/7) of the SCC samples (Figure 3). C-myc immunoreactivity was observed in both the

nuclei and the cytoplasm of the cells. The percentage of the samples with positive immunostaining for c-myc was 13% (4/31) in normal epithelia, increased to 25% (15/61) in BCH and markedly increased to 54% (7/13) in DYS, and 59% (16/27) in SCC (Figure 3). Strong nuclear staining for c-myc was noted in esophageal glandular cells in submucosal tissues.

**Apoptosis in relation to cell proliferation**

Both AI and the number of PCNA positive cells increased significantly as the lesions progressed from BCH to DYS and to SCC ( $P < 0.01$ , Table 1, Figure 4). Pearson's correlation analysis showed a good correlation between AI and the proliferation index in esophageal carcinogenesis ( $r = 0.363$ ,  $P < 0.001$ ). In the same category of lesions, the number of PCNA positive cells was several hundred fold higher than that of AI (Table 1).

**Apoptosis in relation to p53, Waf1p21, bcl-2 and c-myc**

In samples with p53 positive staining, the AI was similar in the normal epithelia and BCH (Table 3). All 31 samples with DYS lesions were p53-positive. The AI for samples with p53-positive stain appeared to be slightly higher than those with p53-negative stain, but this difference was not statistically significant (Table 3). The total number of p53 positive cells per mm<sup>2</sup> was higher than that of apoptotic cells in morphologically normal esophageal epithelia, BCH and DYS, and much higher in SCC (Tables 1 and 2). In bcl-2 staining positive samples, the AI was low in BCH, but increased in DYS and SCC. The AI in BCH, DYS and SCC with bcl-2 positive stain was not significantly different from the corresponding samples with bcl-2 negative stain (Table 3). The AI in normal, BCH, DYS and SCC with c-myc positive staining were also not significantly different from those with c-myc negative stain (Table 3).

**Table 1 Apoptosis and cell proliferation in esophageal epithelia with different severities of lesions**

Histology	Apoptosis		Apoptotic indexa ( $\bar{x} \pm s$ )			PCNA immunostaining			
			Number of samples examined	Percent of samples with apoptosis	AI	ACI	ABI	Number of samples examined	Number of positive cells/mm <sup>2</sup> ( $\bar{x} \pm s$ )
BCH	156	32	0.6±0.9	0.3±0.6 <sup>b</sup>	0.2±0.5	106	286±150 <sup>b</sup>		
DYS	47	79 <sup>c</sup>	1.8±1.5 <sup>b</sup>	0.9±0.9 <sup>b</sup>	0.8±1.0 <sup>b</sup>	31	719±389 <sup>b</sup>		
SCC	38	100 <sup>c</sup>	2.5±2.1 <sup>b</sup>	0.9±0.9	1.6±1.5 <sup>b</sup>	26	1 261±45 <sup>b</sup>		

<sup>a</sup>ACI, the number of apoptotic cells per mm<sup>2</sup>; ABI, the number of apoptotic bodies per mm<sup>2</sup>; AI, the number of both apoptotic cells and apoptotic bodies per mm<sup>2</sup>. <sup>b</sup>Statistically different from the adjacent low-grade lesion by Wilcoxon rank-sum test, <sup>c</sup> $P < 0.01$ .

**Table 2 Changes of p53 and Waf1p21 in esophageal epithelia with different severities of lesions**

Histology	p53 immunostaining		Waf1p21 immunostaining	
	% of samples with positive stain % (n/N) <sup>a</sup>	Number of positive cells/mm <sup>2</sup> ( $\bar{x} \pm s$ )	% of samples with positive stain % (n/N) <sup>a</sup>	Number of positive cells/mm <sup>2</sup> ( $\bar{x} \pm s$ )
Normal	74 ( 23/31)	53±7839	(11/28)	34±72
BCH	87 (102/117)	74±66	38 (14/37)	53±10 <sup>b</sup>
DYS	100 ( 31/31)	262±341 <sup>b</sup>	27 ( 3/11)	11±20 <sup>b</sup>
SCC	89 ( 23/26)	1 297±1110 <sup>b</sup>	14 ( 1/7)	8±22

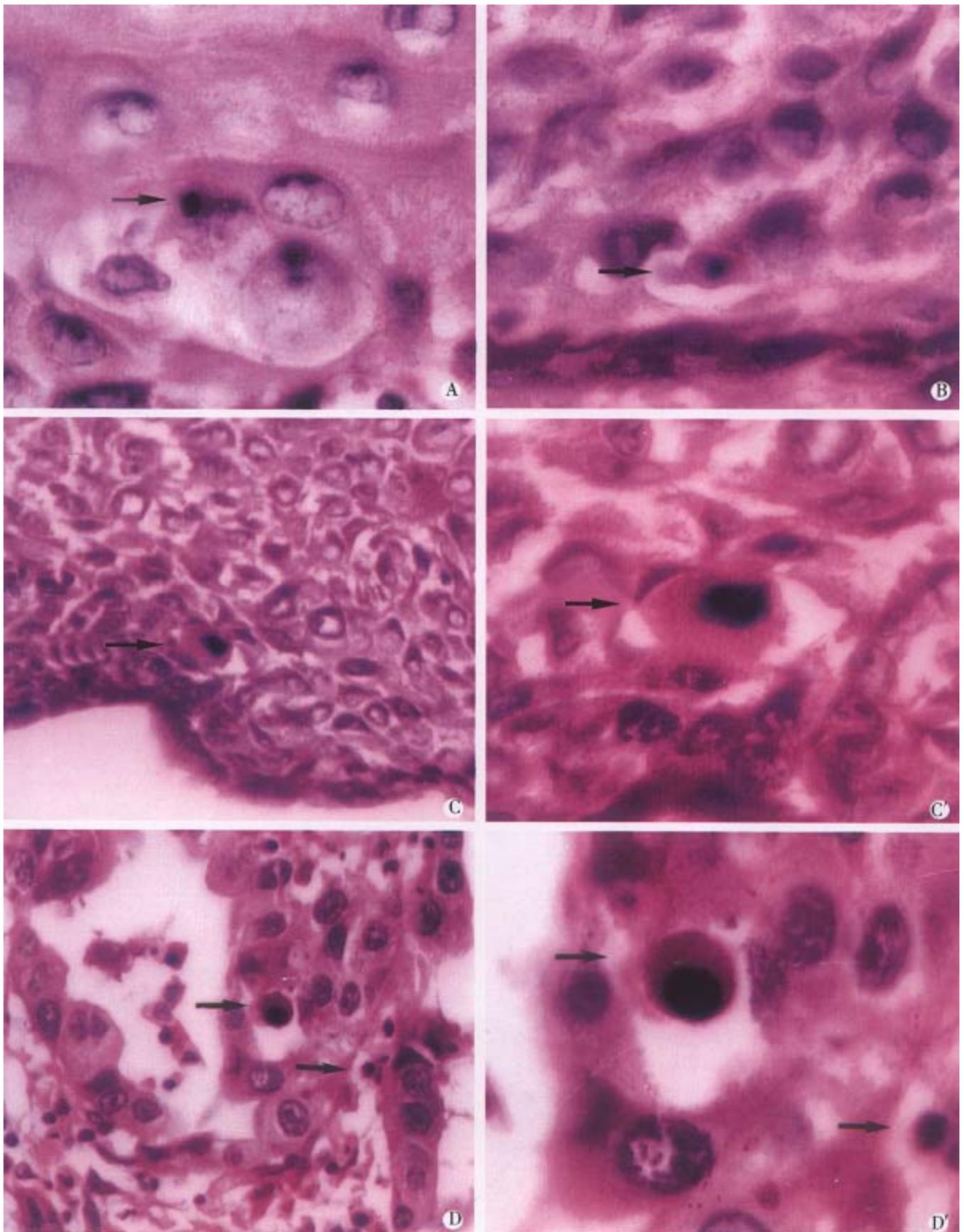
<sup>a</sup>n, positive cases; N, total cases examined. <sup>b</sup>Statistically different from the adjacent low-grade lesion by Wilcoxon rank-sum test,  $P < 0.01$ .

**Table 3 Apoptotic indexes in samples with positive and negative immunoreactivity for p53, bcl-2 and c-myc in esophageal epithelia with different severities of lesions**

Histology	p53 immunostaining				bcl-2 immunostaining				c-myc immunostaining			
	Positive		Negative		Positive		Negative		Positive		Negative	
	Na	AIb	N	AI	N	AI	N	AI	N	AI	N	AI
Normal	23	0.4±0.8	8	0.2±0.4	0	0	23	0.2±0.6	4	0.6±1.2	27	0.3±0.6
BCH	102	0.4±0.8	15	0.3±0.6	5	0.1±0.3	26	0.5±1.1	15	0.8±1.1	46	0.5±1.1
DYS	31	1.8±1.5	0		4	1.7±1.3	6	1.9±0.4	7	2.0±1.7	6	0.7±0.7
SCC	23	2.3±2.1	3	1.1±0.4	6	2.0±0.9	1	2.8	16	2.2±2.2	11	2.0±1.8

<sup>a</sup>N, Number of samples examined.

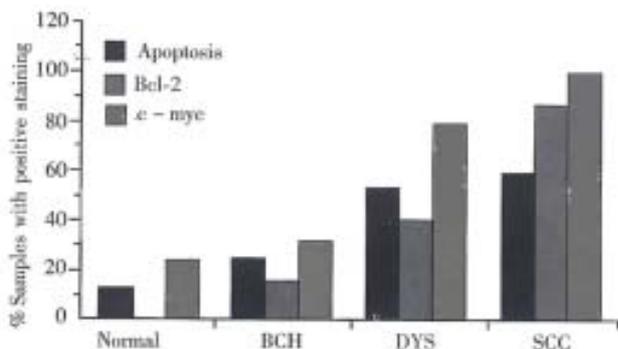
<sup>b</sup>AI, apoptotic index, i.e., the number of apoptotic cells and apoptotic bodies per mm<sup>2</sup>.



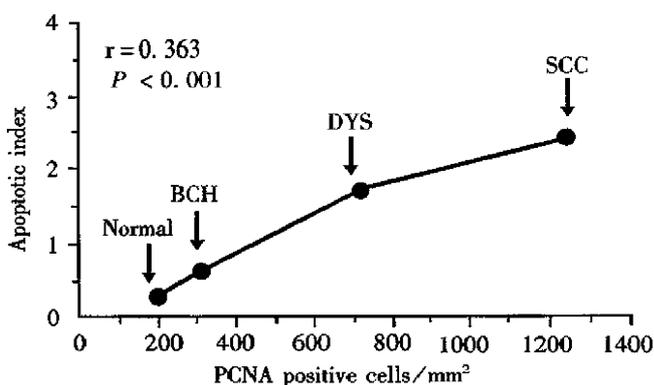
**Figure 1** Apoptotic cells in human esophageal epithelia (H & E staining). Micrographs illustrate the apoptotic cells in normal tissue (A) and the tissues with lesions of BCH (B), DYS (C) and SCC (D).  $\times 400$ . C' and D' were the higher magnification of C and D.  $\times 1\ 000$ . Scattered apoptotic cells (arrow) were located among the compartments of dysplastic and hyperproliferative cells. The apoptotic bodies most often appeared as a single structure separated from the surrounding intact cells by a clear halo (C).



**Figure 2** Immunostaining of Waf1p21 in biopsy samples of esophageal BCH. The Waf1p21-positive immunostaining cells were located at the third and fourth cell layers of the epithelium (arrow).  $\times 400$



**Figure 3** Changes of apoptosis and immunoreactivity of bcl-2 and c-myc in esophageal normal epithelia and epithelia with different severities of lesions.



**Figure 4** Changes of apoptosis and cell proliferation determined by PCNA in esophageal carcinogenesis.

**DISCUSSION**

The detection of apoptotic cells and apoptotic bodies by normal light microscopy is based on several well-established morphological features<sup>[7,24-26]</sup>. These features include condensation of chromatin and cytoplasm as well as fragmentation of the cells

which leads to the appearance of membrane-bound apoptotic bodies.

An interesting observation in this study was that apoptotic cells were located in the same compartment of hyperproliferative cell population in the esophageal epithelium as the cells staining positive for p53, bcl-2, c-myc and PCNA. This finding was consistent with the recent report on human lung and breast tumors<sup>[25,27]</sup>, but not consistent with the report on apoptosis detection by terminal deoxynucleotide transferase mediated UTP nick end labeling (TUNEL) method<sup>[28]</sup>. In our samples the apoptotic cells were also found at the superficial layers of the esophageal epithelium by TUNEL (data not shown). The superficial cells of the epithelium are usually at the late stage of differentiation, and the cells gradually die with DNA fragmentation which may be detected by the TUNEL method. TUNEL has been used to detect apoptotic cells in many studies<sup>[27,29]</sup>, but different opinions exist concerning the interpretation of the results<sup>[30]</sup>. It has been reported that apoptotic cells were not detected by TUNEL, but were observed on routine H&E stained sections in colorectal carcinomas<sup>[31,32]</sup>. The most meaningful standard of apoptotic cell death in studying carcinogenesis, we believe, is the characteristic morphological changes.

Another observation is that the number of cells undergoing apoptosis was low in esophageal epithelia with different lesions. p53 is known to act as a cell cycle check-point protein and to induce apoptosis following DNA damage<sup>[14,33,34]</sup>. Inactivation of wild-type p53 function, frequently reflected by the accumulation of mutated p53 protein, could delay apoptosis<sup>[35]</sup>. In the present study, no distinctive difference was noted in the level of AI between the p53 positive and p53-negative lesions. It is possible that the effect of p53 inactivation on apoptosis in these cases is masked by other dominant regulators.

Expression of bcl-2 oncoprotein has been shown to block apoptosis in various experimental systems<sup>[36]</sup>. In the present study, we observed a low level of apoptosis and high frequency of bcl-2 and c-myc expression in DYS and SCC, which is in keeping with the role of bcl-2 in blocking apoptosis. The lack of difference in AI between the bcl-2 positive and negative groups suggest that other factors may dominate the regulation of apoptosis and override the inhibitory effects of bcl-2.

A third observation is the inverse correlation between Waf1p21 and p53 and PCNA. In this study a low number of Waf1p21-positive cells, much lower than that of p53 and PCNA, in DYS and SCC was observed. Waf1p21 has been shown to bind to Cdk2 and to be a potent Cdk inhibitor, and also has been shown to bind to PCNA and to inhibit DNA

replication *in vitro*. In esophageal DYS, p53 mutation has been identified frequently<sup>[20-22]</sup>. The low levels of Waf1p21 at the stage of DYS may be related to the functional loss of p53. Other mechanisms may also be responsible for the lack of Waf1p21 expression in DYS and SCC<sup>[37]</sup>. The distribution of Waf1p21-positive cells was different from that of p53 and PCNA in that Waf1p21-positive cells reside in the middle third of epithelium, a better differentiated area, and are usually located 2 to 4 cell layers higher than that of p53- and PCNA-positive cells, suggesting that the expression of Waf1p21 is related to the presence of wild type p53 or with cell differentiation.

In summary, we found that AI was at a low level in normal esophageal epithelia and increased as the lesions progressed. The low number of Waf1p21-positive cells may be due to functional loss of p53 by mutations. The relationship between Waf1p21 and bcl-2 positivity and apoptosis is not clear, possibly due to the complexities of molecular changes in esophageal carcinogenesis.

**Acknowledgments** We would like to thank Dr. Guang Yu Yang for his helpful discussions, Mr. Darren Seril for technical help, and Ms. Dorothy Wong for the preparation of this manuscript.

## REFERENCES

- 1 Yang CS. Research on esophageal cancer in China: a review. *Cancer Res*, 1980; 40(7):2633-2644
- 2 Lu JB, Yang WX, Zu SK, Zchang QL, Sun XB, Lu WQ *et al.* Cancer mortality and mortality trends in Henan, China, 1974-1985. *Cancer Detect Prev*. 1988; 13(2):167-173
- 3 Correa P. Precursors of gastric and esophageal cancer. *Cancer (Phila.)*, 1982; 50(11):2554-2565
- 4 Munoz N, Crespi M, Grassi A, Wang GQ, Shen Q, Li ZC. Precursor lesions of oesophageal cancer in high risk population in Iran and China. *Lancet*, 1982; 1(5):876-879
- 5 Qiu SL, Yang GR. Precursor lesions of esophageal cancer in high-risk populations in Henan Province, China. *Cancer (Phila.)*, 1988;62(3):551-557
- 6 Wang LD, Lipkin M, Qiu SL, Yang GR, Yang CS, Newmark HL. Labeling index and labeling distribution of cells in esophageal epithelium of individuals at increased risk for esophageal cancer in Huixian, China. *Cancer Res*, 1990;50(9):2651-2653<sup>9a</sup>
- 7 Kerr JFR, Winterford CM, Harmon BV. Apoptosis: its significance in cancer and cancer therapy. *Cancer*, 1994;73(8):2013-2026
- 8 Kastan MB, Canman CE, Leonard CJ. p53, cell cycle control and apoptosis: implications for cancer. *Cancer Metas Rev*, 1995;14(1)3-15
- 9 Uhlmanu EJ, D'sa Eipper C, Subramanian T, Wagner AJ, Hay N, Chinnadurai G. Deletion of a nonconserved region of Bcl-2 confers a novel gain of function: suppression of apoptosis with concomitant cell proliferation. *Cancer Res*, 1996;56(10):2506-2509
- 10 Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*, 1992;80(5):879-886
- 11 Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol*, 1994; 124(1):1-6
- 12 Vaux DL, Cory S, Adams JM. Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature (Lond)*, 1988;335(3):440-442
- 13 Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB. Wild type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA*, 1992;89(16):7491-7495
- 14 Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, 1991; 51(23):6304-6311
- 15 Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science (Washington DC)*, 1994;266(13):1821-1828
- 16 Shimamura A, Fisher DE. p53 in life and death. *Clinical Cancer Res*, 1996;2(3):435-440
- 17 Sheikh MS, Rochefort H, Garcia M. Overexpression of p21 Waf1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. *Oncogene*, 1995;11(9):1899-1905
- 18 Wang LD, Hong JY, Qiu SL, Gao HK, Yang CS. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Res*, 1993;53(8):1783-1787
- 19 Wang LD, Shi ST, Zhou Q, Goldstein S, Hong JY, Shao P *et al.* Changes in p53 and cyclin D1 protein levels and cell proliferation in different stages of human esophageal and gastric cardia carcinogenesis. *Int J Cancer*, 1994;59(4):514-519
- 20 Gao HK, Wang LD, Zhou Q, Hong JY, Huang TY, Yang CS. p53 tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high risk populations in Henan, China. *Cancer Res*, 1994;54(16):4342-4346
- 21 Wang LD, Zhou Q, Hong JY, Qiu SL, Yang CS. p53 protein accumulation and gene mutations in multifocal esophageal precancerous lesions from symptom free subjects in a high incidence area for esophageal carcinoma in Henan, China. *Cancer*, 1996;77(7):1244-1249
- 22 Shi ST, Feng B, Yang GY, Wang LD, Yang CS. Immunohistochemical sequencing (IHSS) of p53 tumor suppressor gene in human oesophageal precancerous lesions. *Carcinogenesis*, 1996;17(1):101-106
- 23 Wang LD, Qiu SL, Yang GR, Lipkin M, Newmark HL, Yang CS. A randomized double blind intervention study on the effect of calcium supplementation on esophageal precancerous lesions in a high risk population in China. *Cancer Epidemiol Biomarkers Prev*, 1993;2(1):71-78
- 24 Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer*, 1972;26(2): 239-257
- 25 Meyn RE, Stephens LC, Mason KA, Medina D. Radiation-induced apoptosis in normal and pre-neoplastic mammary glands *in vivo*: significance of gland differentiation and p53 status. *Int J Cancer*, 1996;65(3):466-472
- 26 Ei-Labban NG, Osorio-Herrera E. Apoptotic bodies and abnormally dividing epithelial cells in squamous cell carcinoma. *Histopathology*, 1986;10(5): 921-931
- 27 Tormanen U, Eerola AK, Rainio P, Vahakangas K, Soini Y, Sormunen R *et al.* Enhanced apoptosis predicts shortened survival in non-small cell lung carcinoma. *Cancer Res*, 1995;55(23):5595-5602
- 28 Ohbu M, Saegusa M, Okayasu I. Apoptosis and cellular proliferation in oesophageal squamous cell carcinomas: differences between keratinizing and nonkeratinizing types. *Virchows Arch*, 1995;427(3):271-276
- 29 Tatebe S, Ishida M, Kasagi N, Tsujitani S, Kaibara N, Ito H. Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal-deoxynucleotide-transferase-mediated dUTP-biotin nick end labeling. *Int J Cancer*, 1996;65(2):173-177
- 30 Mundle SD, Gao XZ, Khan S, Gregory SA, Preisler, HD, Raza A. Two in situ labeling techniques reveal different patterns of DNA fragmentation during spontaneous apoptosis *in vivo* and induced apoptosis *in vitro*. *Anticancer Res*, 1995;15(4):1895-1904
- 31 Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM *et al.* Inhibition of apoptosis during development of colorectal cancer. *Cancer Res*, 1995;55(5):1811-1861
- 32 Staunton MJ, Gaffney EF. Tumor type is a determinant of susceptibility to apoptosis. *Am J Clin Pathol*, 1995;103(3):300-307
- 33 Marx J. Cell death studies yield cancer clues. *Science*, 1993;259(5):760-761
- 34 Lane DP. p53, guardian of the genome. *Nature*, 1992;358(6381):15-16
- 35 Bartek J, Bartkova J, Vojtesek B. Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene*, 1991;6(9):1699-1703
- 36 Bissonnette RP, Echeverri F, Mahboubi A, Green DR. Apoptotic cell death induced by c-mys is inhibited by bcl-2. *Nature*, 1992;359(6395):552-554
- 37 Barbareschi M, Caffo O, Doglioni C, Fina P, Marchetti A, Buttitta F *et al.* P21Waf1 immunohistochemical expression in breast carcinoma: correlations with clinicopathological data, oestrogen receptor status, MIB1 expression, p53 gene and protein alterations and relapse free survival. *Br J Cancer*, 1996;74(2):208-215

# Expression of alpha fetoprotein messenger RNA in BEL-7404 human hepatoma cells and effect of L-4-oxalysine on the expression \*

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**Subject headings** oxalysine; liver neoplasms; fetoprotein; tumor cell, cultured; RNA, messenger; gene expression; in situ hybridization; immunohistochemistry

## Abstract

**AIM** To investigate alpha-fetoprotein (AFP) mRNA expression in BEL-7404 human hepatoma cells and the effect of L-4-oxalysine (OXL) on the expression.

**METHODS** Bel-7404 human hepatoma cells were maintained in RPMI 1640 media. Human AFP cDNA probe was labelled with digoxigenin-11-dUTP by the random primer labelling method. The expression of AFP mRNA in Bel-7404 cells was determined by an in situ hybridization technique with digoxigenin-labelled human AFP cDNA probe. The positive intensities of AFP mRNA in cells were analyzed by microspectrophotometer and expressed as absorbance at 470nm. For the experiment with OXL, cells were incubated with various concentrations of the agent for 72h.

**RESULTS** Essentially all the hepatoma cells contained AFP mRNA in the cytoplasm, although in various amounts. The specificity of the hybridization reaction was confirmed by control experiments in which the use of Rnase-treated BEL-7404 cells, non-AFP producing cells (HL-60 human leukemia cells) or a nonspecific cDNA probe resulted in negative hybridization. When the cells were

treated with OXL (25, 50mg/L), the content of AFP mRNA in the cytoplasm was decreased with the inhibition percentages of 34.3% and 70.1%, respectively ( $P < 0.05$ ).

**CONCLUSION** AFP mRNA was expressed in BEL-7404 human hepatoma cells and OXL suppressed AFP mRNA expression in the cells.

## INTRODUCTION

Serum alpha-fetoprotein (AFP) has been widely detected as a marker for primary hepatocellular carcinoma (PHC). However, the relationship between AFP and PHC is still unclear. We found recently that AFP directly stimulated the growth of mouse ascites hepatoma-22 cells and inhibited the immune responses<sup>[1-4]</sup>. It is suggested that AFP contributes the generation and development of PHC and is an important target of anti-hepatoma drugs<sup>[5-7]</sup>. L-4-oxalysine (OXL) is a natural product isolated from a new species of *Streptomyces roseo viridofuscus* n. sp. in China. Previous studies indicated that OXL exhibited marked antiproliferative activity against several animal tumors. The antimetastatic influence of OXL was also detected in mice bearing Lewis lung carcinoma<sup>[8-10]</sup>. OXL also exhibited immunoregulatory activity<sup>[11]</sup>. Preliminary clinical studies suggested that oral treatment with OXL induced an improvement in the symptoms of PHC patients, and no serious side effects were observed<sup>[12]</sup>. Recently, our laboratory also found that OXL antagonized the biological activities of AFP<sup>[13]</sup>. AFP content in human BEL-7404 hepatoma cells and cultured media was obviously decreased after the OXL treatment<sup>[14]</sup>. It is inferred that OXL has anti-AFP activities. In this report, an in situ-hybridization (ISH) technique was used to study the level of AFP mRNA expression in BEL-7404 human hepatoma cells. The effect of OXL on AFP mRNA expression was also observed.

## MATERIALS AND METHODS

### Cell culture

A human hepatoma cell line, BEL-7404, was maintained in RPMI 1640 media (Gibco)

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**Project supported by the National Natural Science Foundation of China, No.39570824**

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**Received** 1998-03-08

supplemented with 10% calf serum, 100kU/L of penicillin and 100mg/L of streptomycin, at 37°C, 5% CO<sub>2</sub> and 100% humidity. The RPMI 1640 media was replaced with fresh media every three to four days. For in vitro experiment with OXL, cells at a density of 5×10<sup>6</sup> cells/L were grown on circular coverslip in each well of 24-well culture plate. Twenty-four hours later, various concentrations of OXL (Department of Antibiotics of this Institute) were added, and cells were again incubated for 72h. Control group contained cells alone. After incubation, adhesive cells were directly used for ISH assay.

### Labelling of probes

The probes used in this experiment are shown in Table 1<sup>[15,16]</sup>. Recombinant plasmids pHAF-2 containing human AFP cDNA and phalb-7 containing human serum albumin (HSA) cDNA were kind gifts of Drs Yoshitake Hayashi and Kyosuke Ohta at University of Kobe, Japan. Plasmids were grown in bulk in *Escherichia coli*- HB101, extracted by the alkaline procedure, purified by phenol and two ethanol precipitations. Plasmids were digested with restrictive enzymes (Promega). The digests were then electrophoresed in 1% preparative agarose gels to separate the purified inserted gene sequences from the residual linearized plasmid band<sup>[17]</sup>.

**Table 1 Probes used in this experiments**

Plasmids	Carrier	Gene contented	Inserted	Restrictive enzyme	Resistance
pHAF-2	pBR322	AFP	900bp	Pst I+Hap II	Tetracycline
phalb-7	pBR322	Albumin	727bp	Pst I+Hind III	Tetracycline

A 900bp Pst I-Hap II fragment from the plasmid pHAF-2 and a 727bp Pst I-Hind III fragment from the plasmid phalb-7 were labelled with digoxigenin (Dig)11-dUTP by random primer labelling method with Dig DNA labelling kit (Boehringer Mannheim Company) and used as AFP and albumin probes, respectively<sup>[18]</sup>. Briefly, the cDNA was denatured by heating for 5min at 100°C and then quickly chilled on ice. The following reagents were added to an Eppendorf tube on ice: 4 µl freshly denatured cDNA (1 µg), 2 µl hexanucleotide mixture, 2 µl- dNTP labelling mixture, 11 µl- sterile water and 1 µl Klenow enzyme, mixed and incubated at 37°C for at least 1h (usually incubated 2h-3h). Ten µl of 2g/L yeast tRNA (Sigma) was added and the probes were precipitated with the addition of 4 µl of 3mol/L NaAc (pH 5.2) and 3 volumes of 95% prechilled ethanol (-20°C) at -70°C for at least 30min. The supernatant was discarded by centrifugation and the

probes were stored in 50 µl of TE (10mmol/L Tris HCl, 1mmol/L EDTA, pH 8.0) at -20°C. The yield of labelled probes in this reaction was 250ng (5ng/µl). To test the sensitivity of each probe, dot-blot hybridization was carried out with Dig-labelled cDNA probe. The size of the probes was 50 to 250bp as estimated by polyacrylamide gel electrophoresis.

### In situ hybridization (ISH)

ISH was done essentially according to the procedure of Breborowicz *et al*<sup>[19]</sup> with some modifications. Briefly, adhesive cells were fixed in 4% paraformaldehyde for 5min-8min at room temperature. The coverslips were serially washed with the following solutions at room temperature: 0.05mol/L Tris-buffered saline (TBS, pH 7.2) three times, 5min each; 100mmol/L glycine once, 15min; TBS three times, 5min each; 0.4% Triton X100 in TBS once, 15min; and TBS three times, 5min each. The coverslips were then treated with 1mg/L of proteinase K (Sigma) in 20mmol/L Tris-HCl (pH 7.4) and 2mmol/L CaCl<sub>2</sub> for 15min at 37°C, washed with TBS three times for 5min each, air dried, postfixed in 4% paraformaldehyde for 5min at room temperature, and washed with TBS three times for 5min each. The coverslips were finally washed with 2×SSC (1×SSC: 150mmol/L NaCl, 15mmol/L sodium citrate) or treated with RNase (Sigma, 100mg/L in 2×SSC) for 30min at 37°C.

The hybridization mixture contained 50% deionized formamide, 5×SSC, 10% dextran sulfate (Sigma), 5×Denhardt's solution, 2% sodium dodecyl sulfate, 100mg/L of salmon sperm DNA (Sigma) denatured at 100°C and 25mg/L-50mg/L of Dig-labelled probe denatured at 100°C. 100×Denhardt's solution contained 2% Ficoll 400 (Sigma), 2% polyvinylpyrrolidone (Sigma) and 2% bovine serum albumin (BSA, Sigma). Two hundred µl of the hybridization mixture was added to each well of 24-well plate. Cells were incubated in a humidified atmosphere for 18h at 37°C. The coverslips were washed at 37°C in 4×SSC three times for 5min each, and then sequentially immersed in 2×SSC, 1×SSC, 0.5×SSC and 0.1×SSC at 37°C for 30min each. The coverslips were then washed in TBS containing 1% BSA and 0.4% Triton X-100 for 30min at room temperature. Sheep anti-Dig antibody conjugated to alkaline phosphatase (AP, Boehringer Mannheim Company) was diluted 1:500 with TBS containing 1% BSA and 0.4% Triton X-100, and applied to specimens. Cells were incubated for 2h at 37°C, then washed in buffer I (100mmol/L Tris-HCl, 100mmol/L NaCl, 10mmol/L MgCl<sub>2</sub>, pH 8.0) and II (100mmol/L Tris-HCl, 100mmol/L NaCl, 50mmol/L MgCl<sub>2</sub>, pH 9.5), respectively, for 10min each at room temperature. Development

reagent contained 33  $\mu$ l of nitroblue tetrazolium salt (NBT, 75  $\mu$ g/L in 70% dimethylformamide) and 25  $\mu$ l of 5-bromo 4 chloro 3 indocyl phosphate (BCIP, X-phosphate, 50  $\mu$ g/L in dimethylformamide) in 7.5ml of buffer II. Cells were incubated in the color solution at 37°C for up to 4h in the dark. Color development was periodically checked and reaction was stopped by washing the coverslips for 5min in TE at room temperature. The coverslips were rinsed well in distilled water, air-dried, cleared in xylene and mounted with glycerin jelly.

The following controls were performed. Just before the hybridization, cells were incubated with 2 $\times$ SSC containing RNase 100mg/L for 30min at 37°C, then were processed for ISH as above; Diglabelled AFP cDNA probe was replaced by Diglabelled albumin cDNA probe; Nonhepatoma cells (human leukemia HL-60 cells) were incubated with hybridization buffer containing the Diglabelled AFP cDNA probe. The positive intensities of AFP mRNA in hepatoma cells were analyzed by microspectrophotometer (Leitz MPV-3) and expressed as absorbancy at 470nm (A470).

#### Statistical analysis

The statistical significance of differences was evaluated using analysis of variance (ANOVA).

## RESULTS

#### Detection of AFP mRNA by ISH

It was demonstrated with dot-blot hybridization that the sensitivity of the Diglabelled probes was 1.0pg. When Diglabelled AFP cDNA probe was used, purple grains were present in the cytoplasm of BEL-7404 human hepatoma cells, with fewer grains seen in cell nuclei. The number of grains in the cytoplasm varied, but essentially all the hepatoma cells, including those undergoing mitotic division, were considered to contain AFP mRNA -(Figure 1).

In order to establish the specificity of hybridization, BEL-7404 cells were incubated with Diglabelled albumin cDNA probe under the same hybridization conditions. No accumulation of grains over the cells was observed (Figure 2). Pretreatment of BEL-7404 cells with RNase abolished the formation of grains. Other cell lines not producing AFP, such as HL-60 human leukemia cells, also gave negative results.

#### Influence of OXL on AFP mRNA expression in BEL-7404 human hepatoma cells

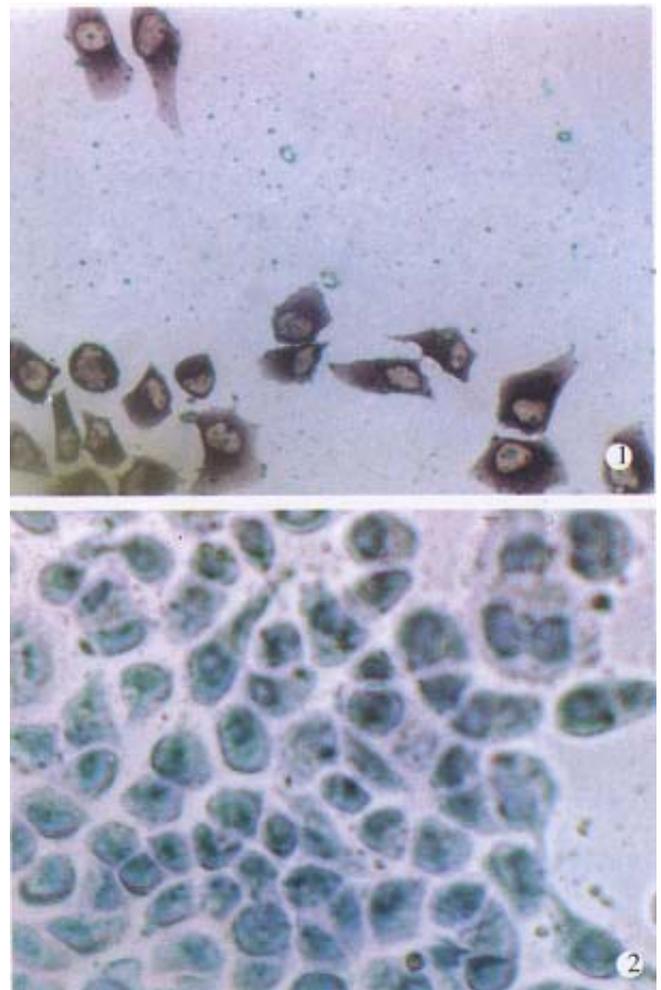
Hepatoma cells were incubated with OXL 25, 50mg/L. Cell viability was greater than 95% using trypan blue exclusion. Seventy-two hours later, the change in AFP mRNA content in hepatoma cells during OXL treatment was determined by ISH. It

was found that AFP mRNA content in hepatoma cells was significantly decreased by OXL; the effect of higher concentration was more obvious. It is suggested that OXL inhibits AFP mRNA expression in hepatoma cells (Table 2).

**Table 2 Influence of L-4-oxalysine (OXL) on AFP mRNA expression in BEL-7404 human hepatoma cells**

Treatment	Concentration (mg/L)	Positive intensities of AFP mRNA (A470 $\pm$ S)	Inhibition (%)
Control		0.67 $\pm$ 0.08	
OXL	25	0.44 $\pm$ 0.06 <sup>a</sup>	34.3
OXL	50	0.20 $\pm$ 0.05 <sup>b</sup>	70.1

BEL-7404 human hepatoma cells (5 $\times$ 10<sup>6</sup> cells/L) were cultivated for 72h in absence or presence of various concentrations of OXL. The results were expressed as absorbance at 470nm (A470). The percentage of inhibition was calculated in the cultures in absence of OXL. <sup>a</sup>P<0.05; <sup>b</sup>P<0.01.



**Figure 1** Detection of alpha-fetoprotein (AFP) mRNA in human BEL-7404 hepatoma cells. In A, BEL-7404 cells were hybridized *in situ* with digoxigenin (Dig)-labelled AFP cDNA probes. Reaction products were visible in almost all cells.

**Figure 2** Same as in Figure 1, except that the Diglabelled albumin cDNA was used as probes. Cells were counterstained with hematoxylin.  $\times$ 400

## DISCUSSION

Various amounts of AFP were present in almost all the BEL-7404 hepatoma cells shown by the avidin-biotin-peroxidase complex (ABC) method<sup>[14]</sup>. We got the similar result in our study. The specificity of the hybridization reaction was confirmed by control experiments in which the use of RNase-treated BEL-7404 cells, non-AFP-producing cells or a nonspecific cDNA probe resulted in negative hybridization. We presume that ISH method has the following advantages: It permits to study the expression of genes qualitatively and quantitatively in an individual cell; it is not necessary to purify RNA, fewer instrument and equipment are needed, and only a small quantity of specimen is required; the samples can be preserved for a long time and can be reviewed if necessary; it needs only a small amount of DNA probes; and Dig-labelled probes cause no radioactive contamination.

It has been known that the synthesis of AFP is often elevated to a significant level in association with development of PHC. Hence, the in situ detection of AFP mRNA may help in histopathological diagnosis of PHC which does not secrete AFP in amounts detectable by immunological means. Moreover, ISH also provides a very useful tool for investigating the effect of some drugs on AFP mRNA expression<sup>[20]</sup>. Present results indicated that OXL suppressed, to a certain extent, AFP mRNA expression in hepatoma cells. Taking our investigation on the whole, it is strongly inferred that OXL exhibits significant anti-AFP activities, which may be one of the mechanisms of anti-tumor action of OXL. Such findings could also lead to the development of new anti-PHC drugs based on AFP target.

**Acknowledgments:** The plasmids containing human AFP cDNA and albumin cDNA were kindly provided by Drs Yoshitake Hayashi and Kyosuke Ohta at University of Kobe,

Japan. We sincerely thank Dr Wang Yao, Australian National University, Australia; Dr Zhang Da-Bing, Shanghai Institute of Plant Physiology, Chinese Academy of Sciences, Shanghai; and Dr Xiang Zheng Hua, the Second Military Medical University, Shanghai for their assistance.

## REFERENCES

- 1 Wang XW, Xu B. Effect of alpha-fetoprotein on the growth of mouse ascites hepatoma-22 cells in vitro. *Acta Oncol Sin*, 1995;5(4):229-231
- 2 Wang XW, Xu B. Influence of alpha-fetoprotein on the growth of tumor cells in vitro. *Chin J Cancer Res*, 1997;9(2):79-82
- 3 Wang XW, Xu B. Effect of alpha-fetoprotein (AFP) on splenocyte proliferation of mice bearing ascites hepatoma-22 in vitro. *Shanghai J Immunol*, 1995; 15(6):327-329
- 4 Wang XW, Xu B. Effect of human alpha-fetoprotein on immunological functions in mice bearing ascites hepatoma-22 in vitro. *Shanghai J Immunol*, 1997; 17(4):224-226
- 5 Wang XW, Xu B. Effect of some drugs on alpha-fetoprotein and treatment of primary hepatocellular carcinoma. *Chin Oncol*, 1995;4(12):24-26
- 6 Wang XW, Xu B. Several new targets of antitumor agents. *Acta Pharmacol Sin*, 1997;18(4):289-292
- 7 Wang XW, Xu B. Alpha-fetoprotein, primary hepatocellular carcinoma and anticancer drugs. In: Cao SR, ed. New theory and technology of oncology. Shanghai: *Shanghai Science and Technology Education Press*, 1997:485-497
- 8 Wang XW, Xu B. L-4-oxalysine, a new antitumor agent of natural origin. *Med Chem Res*, 1996;6(4):225-232
- 9 Wang XW, Xu B. Mechanisms of antitumor action of L-4-oxalysine, a new natural product. *Med Chem Res*, 1996;6(4):233-247
- 10 Wang XW, Xu B. Antitumor and immunological activities of oxalysine. *Chin J Oncol*, 1997;19(2):115-117
- 11 Wang XW, Xu B. Immunoregulatory activity of L-4-oxalysine: an in vitro study. *Meth Find Exp Clin Pharmacol*, 1997;19(7):437-442
- 12 Wang XW, Xu B. L-4-oxalysine: Its antitumor activities and mechanisms of action. *Drugs Fut*, 1996;21(7):727-731
- 13 Wang XW, Xu B. Anti-alpha-fetoprotein activity of L-4-oxalysine. *Asia Pacific J Pharmacol*, 1996;11(2):25-28
- 14 Wang XW, Xu B. Effect of L-4-oxalysine on alpha-fetoprotein gene expression in human BEL-7404 hepatoma cells. *Asia Pacific J Pharmacol*, 1997;12(1-2):37-40
- 15 Urano Y, Sakai M, Watanabe K, Tamaoki T. Tandem arrangement of the albumin and alpha-fetoprotein genes in human genome. *Gene*, 1984;32(11): 255-261
- 16 Morinaga T, Sakai M, Wegmann TG, Tamaoki T. Primary structures of human alpha fetoprotein and its mRNA. *Proc Natl Acad Sci USA*, 1983;80(8):4604-4608
- 17 Sambrook J, Fritsch EF, Mantiatis T. Molecular cloning: A laboratory manual. New York: *Cold Spring Hober Laboratory Press*, 1989:304-316
- 18 Furuta Y, Shinohara T, Sano K, Meguro M, Nagashima K. In situ hybridization with digoxigenin labelled DNA probes for detection of viral genomes. *J Clin Pathol*, 1990;43(3):806-809
- 19 Breborowicz J, Tamaoki T. Detection of messenger RNAs of alpha-fetoprotein and albumin in a human hepatoma cell line by in situ hybridization. *Cancer Res*, 1985;45(4):1730-1736
- 20 Wang XW, Xu B. Research advances of research of alpha-fetoprotein gene expression and the expression regulation. *Acta Oncol Sin*, 1996;6(4):281-284

# Anal cancer in Chinese: human papillomavirus infection and altered expression of p53 \*

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**Subject headings** anus neoplasms; papillomavirus infection; DNA, viral; p53 gene; gene expression; polymerase chain reaction

## Abstract

**AIM** To detect the presence of HPV DNA and study the alteration of p53 expression in anal cancers in Chinese.

**METHODS** HPV DNA was amplified by PCR. The amplified HPV DNA was classified by DBH. HPV antigen and p53 expression were respectively detected by immunohistochemistry.

**RESULTS** HPV DNA was amplified only in one case of squamous cell carcinoma of the 72 Chinese anal cancers and further classified as HPV type 16. Others were all HPV negative. HPV antigen and p53 expression were also detected in this case. Positive stainings with anti-p53 antibody were seen in 61.2% anal cancers. There were no statistically significant differences between anal squamous cell carcinomas and adenocarcinomas and between anal adenocarcinomas and rectal adenocarcinomas. p53 protein expression was observed in the basal cells of squamous epithelium of condyloma acuminatum and morphologically normal squamous epithelium in 2 cases invaded by anal adenocarcinoma.

**CONCLUSION** HPV infection was not associated with these cases of anal cancer. p53 alteration was a common event. Positive p53 immunostaining can not be regarded as a marker for differentiating benign from malignant lesions.

## INTRODUCTION

Anal cancer is an uncommon tumor accounting for 2%-3% of all anorectal tumors. The association between human papillomavirus (HPVs) and lesions of anus has been reported, but the reported incidence of HPV infection in anal lesions varies in different studies. HPVs are infectious agents with tropism for cutaneous or mucosal epithelium. Infection with high-risk HPV types 16, 18 and 33 has been detected in a high percentage of patients with several types of cancers including anal cancers, suggesting that it may be a risk factor for some carcinomas<sup>[1]</sup>.

The oncogene products of several DNA tumor viruses appear to target the retinoblastoma gene (Rb) product and p53 protein. The E6 and E7 oncoproteins encoded by HPV respectively bind to p53 protein and to Rb product, resulting in inactivation of them<sup>[2]</sup>. The binding of E6 protein encoded by HPV16 and 18 rapidly degrades p53 via the ubiquitin-directed pathway<sup>[3]</sup>. Therefore, it is obvious that the role of the E6 oncoprotein is to eliminate or inactivate p53 as a tumor suppressor, although the functional consequences of p53 bound and degraded by E6 in HPV-induced cancers remain unclear. It is often found that there is an inverse relationship between p53 mutation and HPV infection in HPV-related tumors, but the actual correlation between HPV infection and p53 mutation needs to be clarified.

In this study, anal cancers in Chinese were screened for the presence of HPV DNA and for the alteration of p53 expression.

## MATERIALS AND METHODS

### Materials

Tumor samples. Archival, formaldehyde-fixed, paraffin-embedded tissue blocks of anal carcinomas were obtained from the First Affiliated Hospital of Zhejiang Medical University from 1979 to 1984. The term "anal cancer" is defined according to the WHO 1989 standard as the cancer occurring in the anal canal, "which extends from the upper to the lower border of the internal anal sphincter (from pelvic floor to anal verge)". The anal cancer in this series include 19 squamous cell carcinomas, 8 cloacogenic carcinomas, 23 adenocarcinomas, 6 adenosquamous cell carcinomas and 16 other tumors

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**Project supported by the National Natural Science Foundation of China, No.39300050.**

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Received 1998-03-08

of the anus. In addition, 23 rectal adenocarcinomas were also tested. In each case, all the available hematoxylin and eosin-stained sections were reviewed, and a representative block was chosen for further studies. Altered expression of p53 was immunohistochemically detected in most of the above samples because there were not enough tissues in some cases, although all the above cases were detected for HPV infection.

### Methods

Polymerase chain reaction (PCR) amplification was used with confirmation of the products by dot blot hybridization (DBH). DNA was extracted from paraffin-embedded tissues as standard procedures using phenol and chloroform. HPV target sequences were amplified for 40 cycles using HPV L1 consensus sequence primers (MY11 and MY09) (Shanghai Institute of Cell Biology). Reaction volume of 100  $\mu$ l included 50mmol/L KCl, 4mmol/L MgCl<sub>2</sub>, 10mmol/L Tris-HCL pH 8.5, 0.2mmol/L each of dNTPs, 2.5 unit Taq DNA polymerase (Perkin-Elmer/Cetus), 0.5mmol/L each of the primer sequences and 10  $\mu$ l purified DNA sample. Positive control sample (Hela cell DNA) and negative control sample (distilled water) were run simultaneously with the test specimens. Each cycle consisted of 94°C for 1 minute, annealed at 52°C for 1 minute and extended at 72°C for 1 minute. After the last cycle of amplification, 10  $\mu$ l of product was analyzed by agarose gel electrophoresis, ethidium bromide staining and visualization under UV light. The amplification product was confirmed using dot blot hybridization by five biotinylated type specific probes as follows:

HPV L1 MY11(+): GCMCAGGGWCATAAYAATGG  
MY09(-): CGTCCMARRGGAWACTGATC  
M=A+C, R=A+C, W=A+T, Y=C+T

MY12HPV6: CATAAGTAACTACATCTTCCA  
MY13HPV11: TCTGTGTCTAAATCTGCTACA  
MY14HPV16: CATAACCTCCAGCACCTAA  
MY74HPV18: GGATGCTGCACCGGCTGA  
MY16HPV33: CACACAAGTAACTAGTGACAG

DBH procedure. In brief, 5  $\mu$ l amplified product was first heat-denatured, then directly spotted to the nitrocellulose membrane and air dried. The membrane was put into denatured solution for 10 minutes, then neutralizing solution for 1 minute, air-dried and baked for 60 minutes in an 80°C vacuum oven. The membrane was put into prehybridization solution at 65°C for 2 hours. The

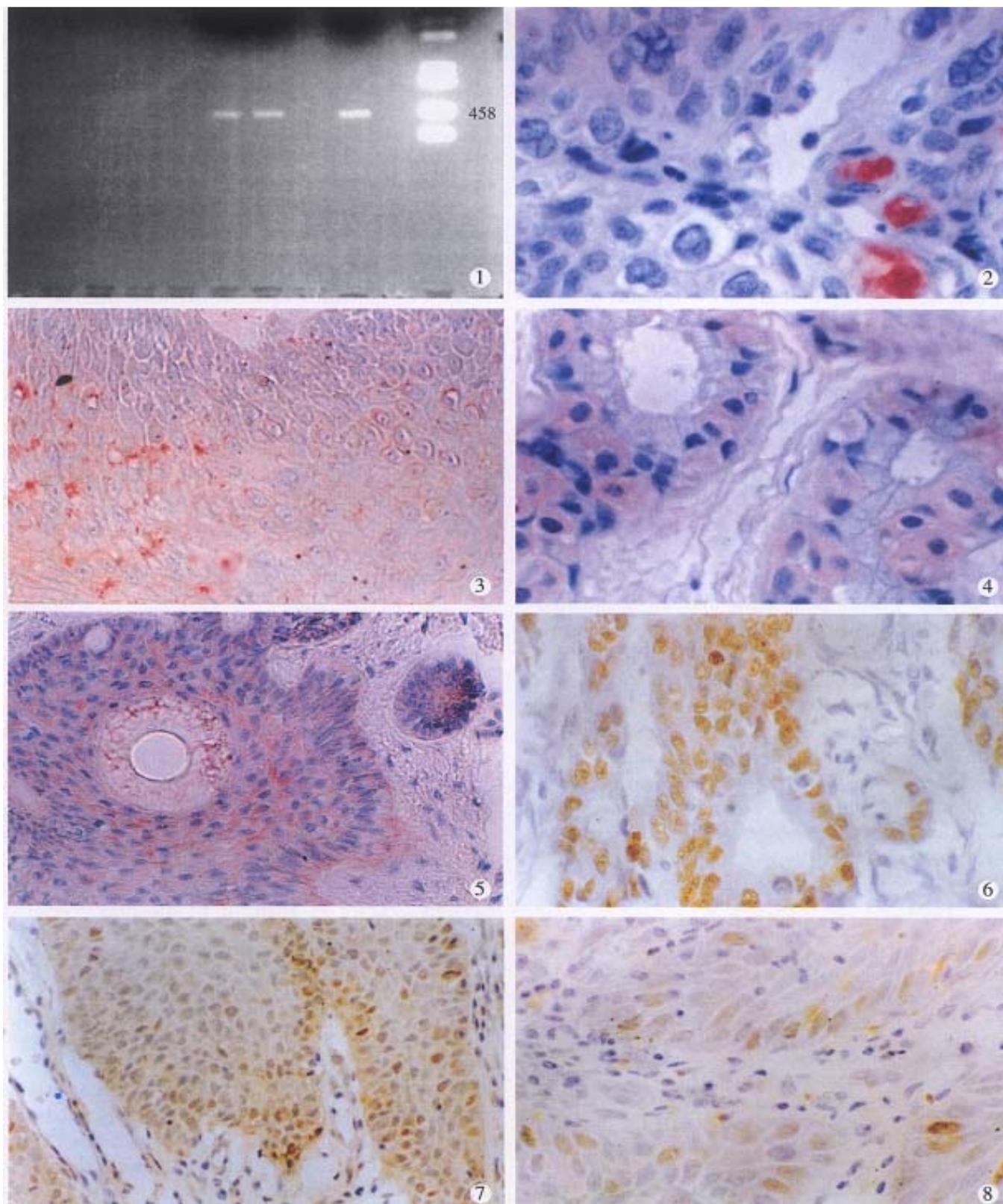
bionylated probe was added to the above solution at the last concentration of 2ng/L. Hybridization was performed at 65°C overnight. The membranes were washed at high stringency and incubated at 63°C for 60 minutes with 3% (W/V) bovine serum albumin (BSA). Treated membranes were incubated with streptavidin (4ng/L) at 37°C for 60 minutes and washed and finally incubated with biotinylated alkaline phosphatase (2ng/L) at 37°C for 60 minutes. Substrate was naphol As-MX, stained with fast blue. Hela cell DNA was used as positive control and distilled water at negative control.

Immunohistochemistry. HPV antigen was assessed by immunohistochemical examination with rabbit anti-bovine papillomavirus (Dako, dilution 1:100), detected by goat antibody against rabbit (Dako, dilution 1:50), then by rabbit PAP (Dako, dilution 1:200), and stained by AEC. P53 protein expression was examined with a monoclonal antibody (D0-M7001), detected by SABC (streptavidin-biotin complex peroxidase kit, 1:100) and stained by DAB. Antibodies were checked by positive and negative controls.

### RESULTS

HPV DNA was amplified only in one squamous cell carcinoma (SCC) (Figure 1), representing 5% of anal SCC (1/19). The amplified HPV DNA was further classified as HPV16 by DBH. Others were all HPV negative. HPV antigen was also immunohistochemically detected in this case. HPV antigen positive cells were not only distributed in cancer cells (Figure 2), but also found in nonneoplastic superficial epithelium (Figure 3), sebaceous glands, sweat glands (Figure 4) and hair follicles (Figure 5) adjacent to the tumor positive for HPV DNA.

Positive stainings with the anti-p53 antibody were detected in 61.2% anal cancers (30/49) (Figure 6). The positive rates in different histological types of anal cancers were 71.4% (10/14) in SCC, 57.1% (16/28) in adenocarcinomas, 50% (3/6) in cloacogenic carcinomas, 100% (1/1) in malignant melanoma and 60% (9/15) in rectal adenocarcinomas. There were no statistically significant differences between anal SCC and adenocarcinomas and between anal adenocarcinomas and rectal adenocarcinomas. P53 protein expression was also found in the SCC case with positive HPV DNA and antigen and the basal cells of squamous epithelium of two cases of condyloma acuminatum (Figure 7) and morphologically normal squamous epithelium invaded by anal adenocarcinoma (Figure 8).



**Figure 1** Detection of PCR products by agarose gel electrophoresis. Lane A: DNA size marker (kb), Lane B: negative control, Lane C: HeLa cell DNA amplified as a positive control, Lane D/E: the sample containing HPV DNA.

**Figure 2** Immunohistochemical staining for HPV antigen in squamous cell carcinoma.  $\times 400$

**Figure 3** Immunohistochemical staining for HPV antigen in nonneoplastic superficial epithelium.  $\times 400$

**Figure 4** Immunohistochemical staining for HPV antigen in sweat gland.  $\times 400$

**Figure 5** Immunohistochemical staining for HPV antigen in hair follicles.  $\times 400$

**Figure 6** Immunohistochemical staining for p53 in anal adenocarcinoma.  $\times 400$

**Figure 7** Immunohistochemical staining for p53 in condyloma acuminatum.  $\times 200$

**Figure 8** Immunohistochemical staining for p53 in morphologically normal epithelium invaded by anal adenocarcinoma (left above).  $\times 400$

## DISCUSSION

More and more evidence have shown that HPV plays a causal role in benign and malignant lesions of the urogenital tract and the head and neck region. Carcinoma of the cervix, aerodigestive tract, lung and esophagus have been proved to be related to HPV. HPV DNA has been found in up to 90% of cases of cervical dysplasia and 100% of SCC of the cervix<sup>[4]</sup>. Sixteen HPV types are involved in genital tract lesions. HPV was epidemiologically implicated in the pathogenesis of anal cancers. HPV antigen has been immunohistochemically found in both biopsy specimens of condyloma acuminatum and at least half of the biopsy specimens of anal squamous cell carcinomas<sup>[5]</sup>. HPV16 or 18 DNA were detected in more than half of the cases (Palmer, *et al.*, 1989) by southern blot or dot blot hybridization, and HPV 16 was found positive in 49 of 207 cases of anal SCC (Scholefield, *et al.*, 1990) by alkaline hydrolysis. Histochemical localization of the virus by in situ hybridization was demonstrated in 22%-67% of anal SCC. HPV types 6/11, 16/18, 31, or 33 were identified in up to 100% of cases of invasive anal SCC by PCR<sup>[6]</sup>. Using immunohistochemical staining and PCR techniques, HPV DNA and antigen were found in only one of 19 SCC cases in our series (5%) and HPV type 16 was further confirmed by DBH. It is considered that the pathogenesis of this group of anal carcinoma is not associated with HPV infection.

It has been suggested that multiple factors may be operative in the pathogenesis of anal carcinoma. Risk factors for anal cancer include cigarette smoking, anorectal chronic inflammation, radiation of anorectal area, virus infections such as herpes simplex virus-2, increasing number of sexual partners and poor sexual hygiene, immunosuppression, etc<sup>[1]</sup>. Scholefield *et al.*<sup>[7]</sup> have shown that positive rate of HPV DNA in SCC was much higher in Sweden and Brazil than in South African and India, that there may exist demographic differences in the prevalence of HPV infection. An increasing prevalence of HPV infection in homosexual men is associated with an increasing incidence of anal cancer. Therefore, prevalences of HPV infection vary in different ethnic population and areas. It was reported that 25% of American women had experienced heterosexual anal intercourse and it occurred frequently in 8%. The low HPV infection in our series may be related to different sexual habit from that in western countries. Anal intercourse may contribute to both anal condyloma and carcinoma. It has been suggested that anal cancer in older group, especially older women, is not associated

with HPV infection<sup>[4]</sup>. The average age of our patients with SCC is 61 years (ranging from 50-81) and 60% were women.

It was suggested that there were HPV-positive and HPV-negative subgroups of HPV-related tumors. In HPV-negative subgroup, mutation of p53 often occurred, while in HPV-positive tumor it did not. E6 protein of HPV bound and degraded wild p53 protein by ubiquitin-directed pathway<sup>[3]</sup>. The wild-type p53 protein, a product of the p53 gene located at 17p13, is a normal growth control protein. Mutation of the p53 gene produced a mutant p53 protein which promoted tumor formation through loss of growth suppression. The deletion, mutation and inactivation of the p53 gene were present in the majority of solid tumors. In our study 61.2% of total anal carcinomas and 71.4% of SCC were immunohistochemically p53 positive. This result indicated that the alteration of the p53 gene is a common phenomenon. Auvinen *et al.*<sup>[8]</sup> reported that 69% of 144 cases of colorectal carcinomas were p53 positive. Jakate *et al.*<sup>[9]</sup> demonstrated that mutant p53 protein was present in 58.6% of anal cancers, in 85.7% of anal adenocarcinoma and 42.1% of SCC, and coexpression of both mutant p53 and E6 protein was seen in three cases (10.3%). p53 overexpression also existed in the SCC with HPV infection in our group. The above results indicated that HPV infection and p53 mutation are two independent genetic events in anal cancers.

It has been generally accepted that the p53 protein detected immunohistochemically is a mutant p53 protein and is seen only in malignant cells<sup>[10]</sup>, but accumulating evidences deny this concept. Overexpression of wild p53 gene may also be detected by immunohistochemistry and identified in benign lesions<sup>[11-13]</sup>. Two cases of normal squamous epithelium adjacent to anal carcinomas and 2 cases of anal condyloma acuminatum had p53 overexpression and immunohistochemical positivity of p53 in our series. Weaker staining signals were seen in the basal cells of epithelium. Combined with the fact that almost 40% of anal cancers were p53 negative, immunohistochemical staining of p53 protein can not be regarded as a marker for differentiating benign from malignant, although p53 protein detected in benign lesions can not be exclusively ruled out to be a mutant protein.

## REFERENCES

- 1 Noffsinger A, Witte D, Fenolio Preiseo CM. The relationship of human papillomavirus to anorectal neoplasia. *Cancer*, 1992;70(5):1276-1287
- 2 Werness BA, Levine AJ, Howley PM. Association of human papillomavirus type 16 and 18 E6 protein with p53. *Science*, 1990(4951);248:76-79
- 3 Scheffner M, Hulbregtse JM, Vierstra RD, Howley PM. The HPV 16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53.

- Cell*, 1993;75(3):495-505
- 4 Richart RM. Causes and management of cervical intraepithelial neoplasia. *Cancer*, 1987;60(8):1951-1959
- 5 Gal AA, Meyer PR, Taylor CR. Papillomavirus antigens in anorectal condyloma and carcinoma in homosexual man. *JAMA*, 1987;257(3):337-340
- 6 Zaki SR, Judd R, Coffield LM, Greer P, Rolston F, Evatt BL. Human papillomavirus infection and anal carcinoma. Retrospective analysis by in situ hybridization and the polymerase chain reaction. *Am J Pathol*, 1992;140(6):1345-1355
- 7 Scholefield JH, Kerr IB, Shepherd NA, Miller KJ, Bloomfield R, Northover JMA. Human papillomavirus type 16 DNA in anal cancers from six different countries. *GUT*, 1991;32(6):674-676
- 8 Auvinen A, Isola J, Visakorpi T, Koivula T, Virtanen S, Hakama M. Overexpression of p53 and long term survival in colon carcinoma. *Br J Cancer*, 1994;70(2):293-296
- 9 Jakate SM, Saclarides TJ. Immunohistochemical detection of mutant p53 protein and human papillomavirus-related E6 in anal cancer. *Dis Colon Rectum*, 1993;36(11):1026-1029
- 10 Elina T, Eriksson MD, Hendrik-Schimmelpenninck MD, Aspenblad U, Zetterberg A, Auer GU. Immunohistochemical expression of the mutant p53 protein and nuclear DNA content during the transition from benign to malignant breast tissue. *Hum Pathol*, 1994;25(11):1228-1233
- 11 Kennedy SM, Macgeogh C, Jaffe R, Spurr NK. Overexpression of the oncoprotein p53 in primary hepatic tumors of childhood does not correlate with gene mutations. *Hum Pathol*, 1994;25(5):438-442
- 12 Busby-Earle RMC, Steel CM, Williams ARW, Cohen B, Bird CC. p53 mutations in cervical carcinogenesis-low frequency and lack of correlation with human papillomavirus status. *Br J Cancer*, 1994;69(4):732-737
- 13 Cooper K, Herington SC, Mark FE, Evans MF, Gatter KC, Mcgee JO'D. p53 antigen in cervical condylomata, intraepithelial neoplasia and carcinoma: relationship to HPV infection and integration. *J Pathol*, 1993;171(1):27-34

# Radioimmunoimaging of colorectal cancer using $^{99m}\text{Tc}$ -labeled monoclonal antibody \*

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**Subject headings** colorectal neoplasms/radioimmunoimaging; antibody, monoclonal; isotope labeling; technetium/diagnostic use

## Abstract

**AIM** To determine whether Hb3 and its fragment  $\text{F(ab')}_2$  have practical value in radioimmunoimaging of colorectal cancer.

**METHODS** Intact Hb3 was purified by hydroxylapatite chromatography. The fragment  $\text{F(ab')}_2$  was prepared by cold digestion and purified as intact Hb3. Hb3 and its fragment  $\text{F(ab')}_2$  were labeled with  $^{99m}\text{Tc}$  by direct labeling method using  $\text{SnCl}_2$  as reducing agent. The radioactive doses ranged from 15 to 40 mCi. The imaging was accomplished by single photon emission computerized tomograph (SPECT) with imaging time ranging from 2.5 to 48 hours. In this study, 10 patients were selected. Among them, 7 were administered with intact Hb3, and 3 with  $\text{F(ab')}_2$  fragment. All the patients were diagnosed as having colorectal adenocarcinoma.

**RESULTS** After purification, intact Hb3 and its fragment  $\text{F(ab')}_2$  were fit for radioimmunoimaging. The percentage of labeling of  $^{99m}\text{Tc}$  to Hb3 or  $\text{F(ab')}_2$  was 80.6%-91.5%. Among the 10 patients, 3 of 7 patients administered with intact Hb3 had positive scans, the other 4 had negative scans, and 2 of 3 patients administered with  $\text{F(ab')}_2$  had positive scans, the other 1 had negative scans.

**CONCLUSION** The results showed that both intact Hb3 and its  $\text{F(ab')}_2$  have some practical value in radioimmunoimaging of colorectal cancer, and the effects of imaging with  $\text{F(ab')}_2$  was better than that with intact Hb3.

## INTRODUCTION

Colorectal cancer is a common malignant disease. In recent years, there have been many reports about radioimmunoimaging used to diagnose the colorectal cancer, but antibodies used were mainly the anti-CEA monoclonal antibodies. Hb3 is an anti-colorectal cancer monoclonal antibody produced by our laboratory, its sensitivity and specificity are superior to that of anti-CEA<sup>[1]</sup>. The positive rate of radioimmunoimaging with  $^{131}\text{I}$  labeled Hb3 in nude mice model of colorectal cancer was 92.4% (13/14)<sup>[2]</sup>. This result shows that Hb3 may be useful in clinical diagnosis of colorectal cancer. Based on the preparation of intact Hb3 and its fragments, clinical radioimmunoimaging has been done in 10 patients, the results showed that Hb3 is valuable in clinical practice.

## MATERIALS AND METHODS

### *Preparation and purification of Hb3*

The Hb3 ascites was prepared routinely and collected under aseptic condition. Hb3 was purified by hydroxylapatite chromatography (Sigma)<sup>[3]</sup>. In the process of purification, aseptic condition and pyrogen free condition were maintained.

### *Digestion of Hb3 to prepare $\text{F(ab')}_2$*

Cold digestion was performed by modified Ballou method. Briefly, intact Hb3 solution (5  $\mu\text{g/L}$ -10  $\mu\text{g/L}$ ), was adjusted to pH 4.0-4.2 with 0.3N acetate buffer, and pepsin (Sigma) in 0.3N acetate buffer (pH 4.0) was added to a final ratio of 1:10 (pepsin: Hb3, Wt/Wt). The digestion was allowed to proceed for 24-34 hours at 4°C. The reaction was terminated by adding 1M NaOH to bring the pH to 7.8-8.0. The  $\text{F(ab')}_2$  fragment was purified using hydroxylapatite chromatography, and the yield of  $\text{F(ab')}_2$  was calculated (Wt/Wt).

### *Detection of purity of Hb3 and $\text{F(ab')}_2$*

The purity of Hb3 and its fragment  $\text{F(ab')}_2$  were detected by SDS-PAGE using Multiphor II Electrophoresis System (Pharmacia). The concentration of stacking gel was 3%, and resolving gel 7.5%. The constant current was 50mA, the temperature 15°C, and the running time 2 hours. After electrophoresis, the gel was stained immediately using Coomassie Blue and dried in vacuum condition and reserved in room temperature. Before electrophoresis, 2-

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**Supported by the National "Eighth Five-Year Plan" Program, No.85-722-18-02.**

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**Received** 1998-01-20

mercaptoethanol was added to the sample solution, and the samples then boiled in water bath for 3 minutes.

#### **Labeling of Hb3 and its fragment F(ab')<sub>2</sub> with <sup>99m</sup>Tc**

The modified Paik's direct labeling method was used<sup>[5]</sup>. Briefly, monoclonal antibody Hb3 and its F(ab')<sub>2</sub> (2mg-8mg) were adjusted to pH 4.0-4.2 with 0.3N acetate buffer (pH 4.0), then added with freshly prepared SnCl<sub>2</sub>/HCl and mixed, the antibody-SnCl<sub>2</sub> mixture was incubated for 15-30 minutes at room temperature, then freshly prepared 15mCi-40mCi <sup>99m</sup>Tc was added, reaction of antibody with technium was maintained for 0.5-1.0 hour at room temperature.

#### **Detection of labeling efficiency**

The free-form and combined <sup>99m</sup>Tc was detected with G-50 chromatography (0.9cm×40cm, Sephadex), and the radioactivity of free-form <sup>99m</sup>Tc (FT), combined <sup>99m</sup>Tc (CT), and G-50 column absorbed <sup>99m</sup>Tc (AT) was measured with radioactivity meter. The percentage of labeling and radiochemical purity of antibody were calculated (because the labeled antibody was used directly without further purification, percentage of labeling was equal to radiochemical purity). The immunoreactivity of antibody before and after labeling and the fraction of immunoreactivity in G-50 chromatography were detected. Percentage of labeling = radiochemical purity = CT/CT+FT+AT

#### **Indirect ELISA to detect specific antibody Hb3 and its F(ab')<sub>2</sub>**

Wells of plate were coated with 50 μg colorectal cancer crude antigen. The antibodies to be detected were Hb3 and its fragment F(ab')<sub>2</sub>. The anti-Ig was rabbit anti-mouse IgG+IgM+IgA conjugated with horseradish peroxidase (diluted 1:1 000). The substrate was ABTS. OD<sub>405</sub> value was obtained using Microplate Autoreader (EL309, BIO-TEK).

#### **Clinical cases and imaging**

Ten patients (6 males, 4 females, aged from 36 to 74 years, averaging 54.2) were selected (Table 1). Seven patients suffered from rectal cancer, and one of them was complicated with liver metastasis. Three patients had colonic cancer. All of the patients proved to have adenocarcinoma by histopathology and the results of immunohistochemistry of biopsies with Hb3 were positive. The amount of antibodies used were 2mg-8mg, and the radioactivity of <sup>99m</sup>Tc used was 15mCi-40mCi.

Radioimmunoimaging was performed with GE STARCAM 3 000/4 000 SPECT system. <sup>99m</sup>Tc labeled antibodies were diluted in 100ml 0.9%

NaCl solution, then injected into the patients iv. The anterior, posterior, left, and right side planar scanings were taken 3, 6, 24 and 48 hours later. Tomography was performed if the planar imaging is questionable. Thirty-two or 64 angles were recorded in each tomogram.

## **RESULTS**

#### **Preparation of Hb3 and its fragment**

The hydroxylapatite chromatogram of Hb3 ascite showed three peaks. The third peak was IgM and possessed immunoreactivity<sup>[3]</sup>. After digestion, the chromatogram showed three peaks as well. The first was low molecular weight peptides, the second was F(ab')<sub>2</sub>, and the third was intact IgM. Among them, the second and the third peaks possessed immunoreactivity (Figure 1). After digestion, the F(ab')<sub>2</sub> yield was 25.0%-36.6%, and the immunoreactivity of F(ab')<sub>2</sub> was lower than that of intact Hb3.

#### **SDS-PAGE**

As showed in Figure 2, there were two bands in the lane of intact Hb3 denaturated and broken, their molecular weights were about *Mr* 72 000 and *Mr* 23 000, and they represented H chain and L chain, respectively (lane 2-5, the bands with molecular weight higher than *Mr* 94 000 were contaminated proteins). In the lane of F(ab')<sub>2</sub> (unbroken, lane 7), two bands were found, and their molecular weights were about *Mr* 130 000 and *Mr* 65 000, and represented F(ab')<sub>2</sub> and Fab' respectively. In the lane of F(ab')<sub>2</sub> (broken, lane 6), there were two bands as well, and their molecular weight were about *Mr* 43 000 and *Mr* 23000, and represented VH+CH1 and L chain respectively.

#### **The labeling of antibody with <sup>99m</sup>Tc**

<sup>99m</sup>Tc Labeled Hb3 was analyzed by G-50 chromatography. The percentage of labeling and radiochemical purity was 80.4%-91.5%. In the chromatogram, the fractions of immunoreactivity of labeled antibody overlapped the fractions of radioactivity (Figure 3). After the antibodies were labeled, their immunoreactivity was 90% more than that of unlabeled antibodies.

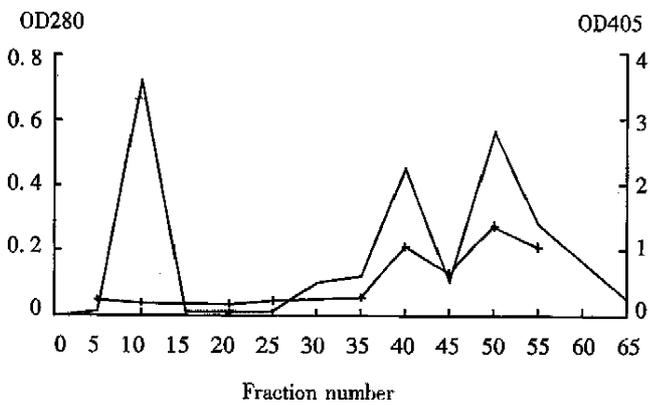
#### **Imaging**

Radiommuimaging was performed in 10 patients, 5 had positive images, and 5 negative images (Table 1), with no side effects. Patient No. 4 suffered from rectal carcinoma complicated with liver metastasis with positive images at 6 and 24 hours. Figure 4 (upper left) shows an unclear abnormal radiation concentration under bladder in 6 hour. In 24 hour scan (lower left), there was a clear abnormal radiation concentration in the left lobe of liver.

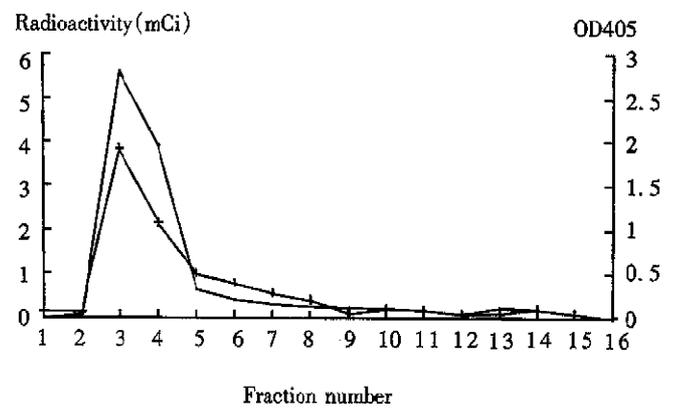
**Table 1 Clinical data and imaging results**

No.	Sex	Age (year)	Clinical diagnosis	Tumor size(cm)	Pathological diagnosis	Immunohisto chemistry	Quantity of antibody (mg)	Radioactivity of <sup>99m</sup> Tc (mCi)	Imaging
1	Male	74	Rectal carcinoma	3.0×5.0	Adenocarcinoma	+++	3.0	23	+
2	Female	47	Rectal carcinoma	1.8×2.0	Papilloadenocarcinoma	++	5.0	20	-
3	Male	35	Rectal carcinoma	3.0×4.5	Adenocarcinoma	++	7.5	19	-
4	Male	67	Rectal carcinoma	3.5×4.5	Low differentiated adenocarcinoma	+++	7.5	16	+
5	Female	54	Colonic carcinoma	4.0×5.0	Adenocarcinoma	++	8.0	15	-
6	Female	55	Rectal carcinoma	2.5×3.5	Adenocarcinoma stage II	++	5.0	19	+
7	Male	61	Colonic carcinoma	2.5×3.5	Adenocarcinoma	+	5.0	40	-
8**	Male	38	Rectal carcinoma	3.5×5.0	Adenocarcinoma	++	5.0	38	+
9**	Male	65	Colonic carcinoma	2.5×3.0	Adenocarcinoma stage II	++	2.0	15	-
10**	Female	44	Rectal carcinoma	2.5×3.0	Adenocarcinoma stage II	++	5.0	18	+

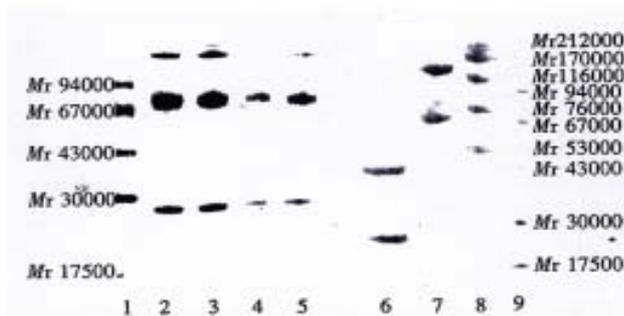
“+” partial weak staining; “++” partial strong staining or broad weak staining; “+++” broad strong staining, complicated with liver metastasis. The antibodies used were fragment F(ab')<sub>2</sub>.



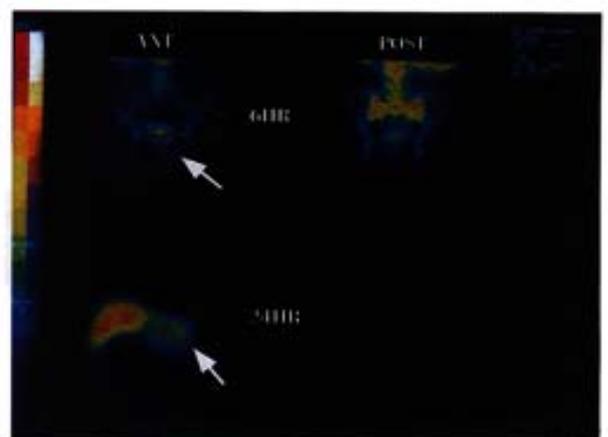
**Figure 1** Chromatogram of digested Hb3. OD<sub>280</sub> (iα - iα) shows concentration of protein. OD<sub>405</sub> (+ - +) shows immunoreactivity.



**Figure 3** Chromatogram of <sup>99m</sup>Tc labeled antibody, OD<sub>405</sub> shows immunoreactivity. iα - iα radioactivity, + - + immunoreactivity.



Lane 1: low molecular weight marker; lane 2-3: intact Hb3; lane 4-5: intact Hb3 (1/10 concentration); lane 6: Hb3 fragments F(ab')<sub>2</sub> (broken); lane 7: Hb3 fragments F(ab')<sub>2</sub> (unbroken); lane 8: high molecular weight marker; lane 9: low molecular weight marker.



**Figure 4** Images of Patient 4. Rectal cancer complicated with liver metastasis. Anterior scan (6 hour) shows an unclear abnormal radiation concentration under the bladder (upper left). Anterior scan (24 hour) shows a clear abnormal radiation concentration in the left lobe of liver (lower left).

## DISCUSSION

### *Purification of antibody and preparation of fragment*

Hydroxylapatite chromatography is simple. The column is short and is convenient for aseptic and pyrogen free treatment. The purity of antibody after purification was fit for clinical usage. As reported, antibody can be digested to  $F(ab')_2$  by cold and warm digestion. In our study we selected cold digestion to prepare  $F(ab')_2$ . The yield of  $F(ab')_2$  varied from 25.0% to 36.6%, lower than that prepared by Ballou<sup>[4]</sup> (cold digestion, 40%-60%), but higher than that prepared by Kurkela<sup>[5]</sup> (warm digestion, 24%±11%). As a matter of fact the prepared fragments contained  $F(ab')_2$  and Fab' (Figure 2), because pepsin digestion can produce these two kinds of fragments, and it is difficult for hydroxylapatite chromatography to separate them.

### *Labeling of Hb3 with <sup>99m</sup>Tc*

Direct labeling of antibody is to combine the reduced <sup>99m</sup>Tc with antibody through the disulfied linkage. Its advantage is that the labeling process can be finished in one step, which is beneficial for making a marketable radioimmunoimaging kit. In this study, we selected SnCl<sub>2</sub> as reducing agent, and labeled antibody with <sup>99m</sup>Tc using direct method. The reaction was performed in one step.

### *Imaging*

Hb3 is an IgM monoclonal antibody against CA-Hb3. The results of immunohistochemistry showed that the positive rate of Hb3 reacting with colorectal carcinoma was high, and it did not react with normal colorectal tissue<sup>[1]</sup>. The results of radioimmunoimaging in nude mice model of colorectal carcinoma also showed a higher imaging efficiency<sup>[2]</sup>. Among the 10 patients in this study, five got positive images. The reasons why the positive rate is not high may be: ① The effect of antibody molecular weight: The molecular weight of intact Hb3 is Mr950 000, its half-life and optimum imaging time are longer than 48 hours, yet the half-life of <sup>99m</sup>Tc is 6 hours, and the imaging is required to be performed within 24 hours. In his study, one of the radioimmunoimaging was performed in a transverse colon cancer patient with intact Hb3, the imaging result at 24 hour was negative. Fourty-eight hours later, the resected tumor sample was scanned by SPECT. In the circumstance of prolonging the acquisition time (30 minutes), the radioactivity of tumor was higher than that of the surrounding

normal tissues. This evidence showed that the inconsistency of half-life between antibodies and radionuclide was unfavorable to imaging. The molecular weight of  $F(ab')_2$  is 1/7 that of intact Hb3, and its half-life is 26 hours, which is more consistent with that of <sup>99m</sup>Tc, and its imaging efficiency is higher than that of intact Hb3. ② Effect of free <sup>99m</sup>Tc: Free <sup>99m</sup>Tc (5.3%-8.6%, Figure 3) can pass through gastric mucosa accompanying the secretion of gastric juice, and get into the gastrointestinal tract through the peristalsis, resulting in false positiveness<sup>[7]</sup>. Among the 10 patients in this study, negative images were found in the intestinal tract of four patients. Accumulation of Tc-Sn colloids (absorbed by column G50, 3.2%-11.0%, Figure 3) can also influence tumor imaging. ③ The effect of HAMA (human anti-mouse antibody): Antibody Hb3 is mouse-derived, it can cause HAMA after being injected into human bodies. This reaction will interfere with the combination of antibody with antigen, and then influence the imaging. In this experiment, the duration from antibody injection to imaging was short, so HAMA was not the major influencing factor.

In conclusion, we consider that Hb3 is a valuable monoclonal antibody in radioimmunoimaging of colorectal carcinoma. The imaging effect of  $F(ab')_2$  is better than that of intact Hb3. Further study is needed to optimize the labeling condition, increase the labeling rate of Hb3 with <sup>99m</sup>Tc, and finally improve the diagnostic value of radioimmunoimaging.

## REFERENCES

- 1 Sun QB, Ho JI, Kim YS. Human colonic cancer associated antigens detected by three monoclonal antibodies. *Chin Med J*, 1986;99(1):63-74
- 2 Zhang J, Wang CL, Sun QB, Pan AY, Chen SL, Liu CG *et al*. Radio immunoimaging study with <sup>131</sup>I-labeled anti-colorectal carcinoma monoclonal antibody in nude mouse model. *Bull Human Med Univ*, 1990;15(3): 235-238
- 3 Stanker LH, Vanderlaan M, Juarez-Salinas H. One-step purification of mouse monoclonal antibodies from ascites fluid by hydroxylapatite chromatography. *J Immunol Methods*, 1985;76(1):157-169
- 4 Ballou B, Reiland J, Levine G, Knowles B, Hakala TR. Tumor location using  $F(ab')_2$  from a monoclonal IgM antibody: Pharmacokinetics. *J Nucl Med*, 1985;26(3):283-192
- 5 Paik CH, Phan LNB, Hong JJ, Sahami MS, Heald SC, Reba RC *et al*. The labeling of high affinity sites of antibody with <sup>99m</sup>Tc. *Int J Nucl Med Biol*, 1985;12 (1):3-8
- 6 Kurkela R, Vuolas L, Vihko P. Preparation of  $F(ab')_2$  fragments from monoclonal mouse IgG1 suitable for use in radioimaging. *J Immunol Methods*, 1988;110(2):229-236
- 7 Rhodes BA. Direct labeling of proteins with <sup>99m</sup>Tc. *Nucl Med Biol*, 1991;18(7): 667-676
- 8 Murray JL, Rosenblum MG, Zhang HZ, Podoloff DA, Kasi LP, Curley SA *et al*. Comparative tumor localization of whole immunoglobulin G anticarcinoembryonic antigen monoclonal antibodies IMM-4 and IMM-4  $F(ab')_2$  in colorectal cancer patients. *Cancer*, 1994;73(3 Suppl):850-857

# Modification of ricin and its hepatotoxicity and activity against hepatocellular cancer in mice

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**Subject headings** ricin/chemistry; ricin/toxicity; liver/drug effects; liver neoplasms; carcinoma, hepatocellular; glutathione transferase

## Abstract

**AIM** To observe the effects of ricin (RT) with and without chemical modification on both hepatotoxicity of mice and activity against hepatocellular cancer (HCC), and evaluate the possibility to improve RT anticancer activity via chemical modification.

**METHODS** RT was modified with N-succinimidyl(3-(2-pyridyldithio) propionate (SPDP), a heterobifunctional cross-linker, and SPDP derivative of RT (PDP-R) was obtained. The serum glutathione-S-transferase (SGST) activity, as an index of liver damage, was determined in mice intoxicated with RT and PDP-R, at various doses and time. The tissue damage of HCC in the nude mice ip injected with PDP-R was compared with that with RT at the same dose by immunohistochemical method, the relative content of both RT and PDP-R in the HCC tissues was measured by computerized image-analysis.

**RESULTS** The SGST activities increased with doses or/and time intoxicated with both RT and PDP-R, and the increase in the value of RT group was more significant than that in the PDP-R group; the SGST activity of RT group was 2.8-fold ( $P < 0.01$ ) of PDP-R group at a dose of 12.5  $\mu\text{g}/\text{kg}$  for 42 h, showing the much lower toxicity of R-PDP than that of RT. Under an optical microscope, hemolysis and necrosis of massive cells in the HCC tissues of PDP-R group were observed and the ratio of necrosis mounted to 90.5% while the corresponding value of RT group only to 62.5%. With computerized image-analysis, the average relative content of RT and

PDP-R in the HCC tissues, represented as greyness, was  $140.06 \pm 3.43$  and  $169.10 \pm 2.74$ , respectively. There was significant difference between the two ( $P < 0.05$ ), indicating the higher content of PDP-R in the HCC tissue than that of RT.

**CONCLUSION** The hepatotoxicity of PDP-R to mice may be reduced by chemical modification with SPDP, but both the affinity of PDP-R to the HCC tissues and ability to kill it may be stronger than that of RT. So this might be a valuable attempt to improve the anticancer activity of RT.

## INTRODUCTION

Ricin toxin (RT), a glycoprotein with high cytotoxicity, isolated from castor beans (*Ricinus communis* beans), has received extensive interest in anticancer research. The natural RT, however, has been restrained in treatment of cancer due to its extreme toxicity. In order to overcome its side effect, various attempts have been made by investigators<sup>[1,2]</sup>, but no report so far has been presented in direct use as anticancer agent. We modified RT with N-succinimidyl(3-(2-pyridyldithio) propionate (SPDP), a heterobifunctional cross-linker, and toxicity of the resultant (PDP-R) to normal tissues reduced but its inhibition to some tumor cells increased<sup>[2,3]</sup>. In the present paper, we reported the preparation, determination of PDP-R and comparison between PDP-R and RT in hepatotoxicity to normal mice and activity against hepatocellular cancer (HCC) in nude mice.

## MATERIALS AND METHODS

### Materials

RT was prepared as described previously<sup>[5]</sup> and shown to be a single band by SDS-PAGE, Mr 65 000. SPDP was synthesized by a modified method<sup>[6]</sup>, mp 80°C-81°C. Glutathione reduced form (GSH, BRITISH) was freshly made into 10.0 mmol/L (pH 6.5) solution in 1.0 mol/L of PB prior to use. 1-chloro-2,4-dinitrobenzene (CDNB, Xi'an Chemical Reagent Factory) was made into 1.11 mmol/L solution in 1.0 mol/L of PB containing 40 ml/L ethanol after second recrystallization in absolute ethanol, m.p 53°C-54°C. Kunming mice, weighing 17.5g-22.5g and nude mice weighing 18.0g, with human HCC were supplied by The Research Center

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**Project supported by the National Natural Science Foundation of China, No.3870638**

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**Received** 1998-03-20

of Experimental Animals in our university.

### Methods

**Preparation and determination of PDP-R RT** of a 4.2mg in 1.0ml of PBS (0.1mol/L, pH 7.5, containing 1.5mol/L of NaCl) was added under stirring, 20 $\mu$ l solution of N, N-dimethylformamide containing 0.25 mg of SPDP and reacted for 30 min at 23 $^{\circ}$ C. The solution of reacted mixture was thoroughly dialyzed against saline at 4 $^{\circ}$ C. PDP-R in the solution and the number (n) of PDP groups bonding to molecules of RT were determined by ultraviolet spectrophotometry<sup>[7]</sup>.

### Effects of PDP-R and RT on SGST activity in normal mice.

**The effect of dosage** Fifty Kunming mice were randomly divided into PDP-R group and RT group. Twenty-five mice in each group were equally subdivided into 5 groups, of which each mouse was injected ip with 2.5, 5.0, 7.5, 10.0 and 12.25  $\mu$ g/kg body weight respectively. Ten mice served as control group, of which each mouse was ip injected with the same volume of saline. The mice intoxicated were continuously observed for 42 h, blood was collected from fundus veniplex, and serum was separated and SGST activity of each specimen measured by dynamics method<sup>[8]</sup>.

**Effect of intoxication time** Eighty Kunming mice were randomly divided into PDP-R group and RT group. Forty mice in each group were equally subdivided into 8 groups, of which each mouse was ip injected with 12.5  $\mu$ g/kg body weight and the intoxication time was 6, 12, 18, 24, 30, 36, 42 and 48 h, respectively. Then the procedure was followed as mentioned above.

**Comparison between PDP-R and RT in hepatotoxicity to normal mice and activity against HCC in nude mice** Eighteen nude mice with human-HCC were randomly divided into PDP-R group, RT group and control group. Six mice of each group were ip injected with the same dose of PDP-R, RT and saline, respectively. The HCC tissue of mouse was taken out 4 h later and was treated by routine procedure to obtain the pathological slices with thickness of 4  $\mu$ m-6  $\mu$ m, which were stained immunohistochemically (ABC method). The slices were observed under the optical microscope and the relative content of both RT and PDP-R in the HCC tissues was measured by computerized image-analysis.

## RESULTS

### Determination of PDP-R and the number of bonding PDP (n) in each molecule of RT

A small amount of PDP-R solution was added to

dithiothiol (DTT) to a final concentration of 40 mmol/L and treated for 30 min at room temperature. The ultraviolet absorption spectrum of the reaction solution was determined in spectrophotometer. After thorough dialysis of the reaction solution against PB, the ultraviolet absorption spectrum was determined again and compared with those of both solution of PDP-R and RT. The results are shown in Figure 1 and the absorbance (A) of the four solutions at 280 nm and 343 nm are shown in Table 1. The A value of PDP-R solution at 280 nm is 0.19 more than that of RT (Table 1). The number (n) of PDP bonding to each molecule of RT was 6.02 according to both molecule absorption coefficient of SPDP at 280 nm and the concentration of RT. On the other hand, based on both molecular absorption coefficient of  $\alpha$ -mercaptopyridine ( $\alpha$ -MP) and the concentration of RT, the value was calculated to be 5.8 because when the solution of PDP-R was treated with excess of DTT, it results in equimolecular  $\alpha$ -MP to PDP and the  $\alpha$ -MP in the solution had a strong absorption at 343 nm. The n value obtained from the two ways is close.

**Table 1 Absorbance of the four kinds of solution at 280nm and 343nm**

$\lambda$ / nm	Absorbance			
	RT	PDP-R	PDP-R+DTT	(PDP-R+DTT) dialyzed
280	0.476	0.665	0.700	0.484
343	0.007	0.065	0.345	0.066

\*The four kinds of solution were ricin (RT), SPDP derivative of RT (PDP-R), PDP-R treated with dithiothiol (PDP-R+DTT) and dialyzed (PDP-R+DTT).

### Effect of PDP-R and RT on the SGST activity of mice

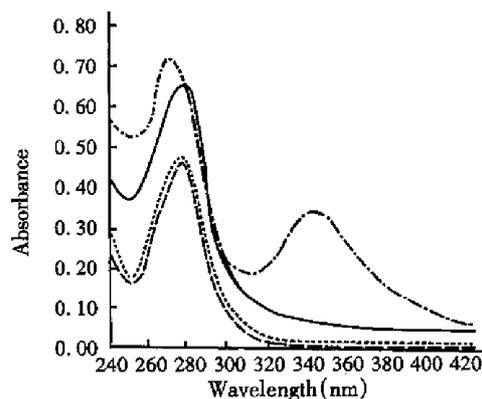
**Effect of dose** Under the same conditions, the SGST activities increased with doses intoxicated with both RT and PDP-R, but the increase in the value of RT group was more significant than that in the PDP-R group (Figure 2). When the dose increased to 12.5  $\mu$ g/kg death in the mice of RT group occurred, the survivor's blood volume decreased and the blood viscosity increased with apparent symptoms of intoxication. The average SGST activity of RT group was as high as 1.797  $\mu$ m/s $\pm$ 0.087  $\mu$ mol/s, being 5.3-fold that of the control group (0.340  $\mu$ m/s $\pm$ 0.025  $\mu$ m/s). However, it seems that the symptoms of PDP-R group resulting from intoxication with the same dose were not at all serious, no death for 42 h, no difference between PDP-R group and control group in the blood volume and viscosity in these mice, their average SGST activity being only 0.642  $\mu$ m/s $\pm$ 0.054  $\mu$ m/s, 36% ( $P$ <0.01) of that of RT group at a dose of 12.5  $\mu$ g/kg for 42 h, which showed much lower toxicity of R-PDP than that of RT.

**Effect of intoxication time** The SGST activities of mice of both PDP-R and RT group increased with intoxication time extended, although within 10 h difference between the two were insignificant (Figure 3). After 10 h, however, increase of RT group was gradually getting more quick and difference between the two at 42 h was up to a maximum measurable value. After 42 h this value was unable to measure due to increased number of death of mice in RT group, while the SGST activity of PDP-R group smoothly decreased.

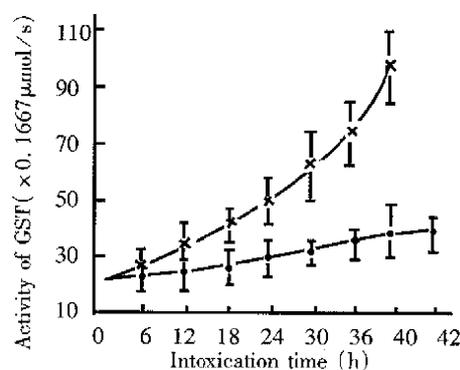
**Comparison between PDP-R and RT in activity against hepatocellular cancer** Under an optical microscope, hemolysis and necrosis of massive cells in the HCC tissue of PDP-R group was observed and the ratio of necrosis mounted to 90.5%, while it was only to 62.5% in RT group. With computerized image-analysis, the average relative content of RT and PDP-R in the HCC tissue, represented as greyness, was  $140.06 \pm 3.43$  and  $169.10 \pm 2.74$ , respectively. There was significant difference between the two ( $P < 0.05$ ), indicating the higher content of PDP-R in the HCC tissue than that of RT and PDP-R has stronger affinity and activity against HCC than that RT.

## DISCUSSION

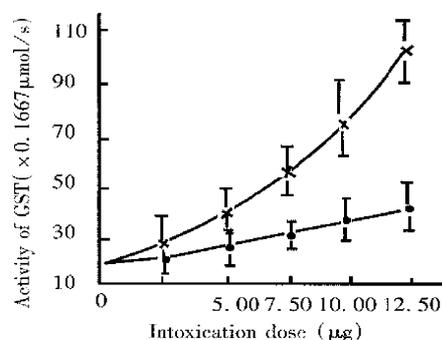
Reaction of RT with SPDP and determination of PDP-R The molecular structure of SPDP and its reaction with RT is shown in Figure 4. There were 13 amino groups ( $^{15}\text{N}$ ) in each molecule of RT which may be acylated by SPDP. The experiment showed that acylation ratio of  $-\text{NH}_2$  in RT molecule is not high under general conditions, possibly due to that some  $-\text{NH}_2$  was located at hydrophobic domain of RT molecule and being not easy to get access to SPDP. In this experiment 26 equimolecular SPDP was used to react with RT to increase the acylation ratio. The resultant, PDP-R, was determined by two ways: first, based on the number ( $n$ ) of PDP bonding to each molecule of RT to be positively proportional to increased value in absorbance of PDP-R at 280nm, so the  $n$  value can be calculated from absorbance of solution, the molecular absorption coefficient of SPDP at 280 nm and the concentration of RT. Second, when the solution of PDP-R was treated with excessive DTT, it resulted in equimolecular  $\alpha$ -MP to PDP and the  $\alpha$ -MP in the solution had a strong absorption at 343 nm. So the  $n$  can be calculated from the absorbance of reacted solution at 343 nm, molecular absorption coefficient of  $\alpha$ -MP and the concentration of RT. The  $n$  value is close by the two ways and it can be used as the evidence for each other. After dialysis of the reaction solution, its absorption spectrum was the same as that of RT, which showed that the absorption at 343 nm was caused by  $\alpha$ -MP.



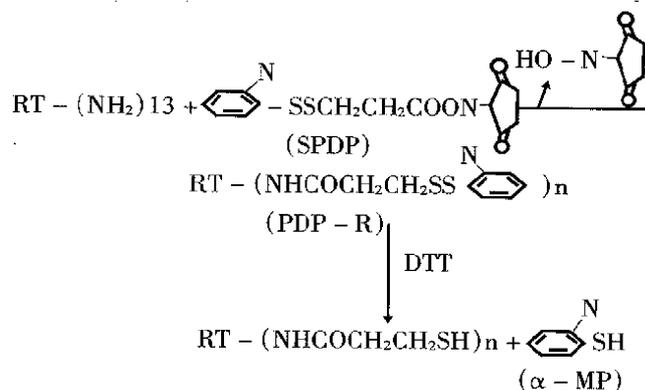
**Figure 1** Ultraviolet absorption spectrum of four kinds of solution.



**Figure 2** The effects of dosage of ricin and its derivative (PDP-R) on serum glutathione-s-transferase activity in mice.



**Figure 3** The time effect of intoxication time with ricin and its derivative (PDP-R) on serum glutathione-s-transferase activity in mice.



**Figure 4** Reaction of ricin with N-succinimidyl13-(2-pyridyldithio) propionate and determination of resultant.

### Comparison between PDP-R and RT in effect on SGST activity of mice

The liver injury caused from intoxication may lead to release of GST to blood flow, resulting in the significant increase of SGST activity and this has been a sensitive index to detect liver injury<sup>[9]</sup>. We compared the hepatotoxicity of PDP-R with that of RT by determination of SGST activity of intoxicated mice. The results were identical with that obtained by pathomorphological method<sup>[3]</sup>. The experiment showed, in view of effects of either dose or time, that the increase of SGST activity caused by PDP-R apparently lower than that by RT, indicating that the hepatotoxicity of PDP-R was significantly lower than that of RT, other symptoms intoxicated with PDP-R was less serious than that with RT and the mice had stronger tolerance to PDP-R than to RT. These differences may imply that the mechanism of intoxication of PDP-R differs from that of RT.

### Some biological activities of PDP-R different from that of RT

It is reported that after RT entered into a mouse body for 0.5h almost 50% of it was located in the liver, showing very strong hepatotoxicity<sup>[10]</sup>. Therefore, to determine the hepatotoxicity of PDP-R is of a typical significance to know its systemic toxicity. The experiment showed that the ability of RT to bind galactose (G) and the residue containing G will be decreased by reaction of RT with SPDP<sup>[11]</sup>. It may be inferred that the toxicity to normal cells including hepatocytes might be weakened because the interaction between RT and the receptor containing G on cellular surface can be decreased by modification of RT with SPDP. On the other hand, the PDP in PDP-R molecules were very sensitive to hydrosulfury (-SH) on the cellular surface of some tumors and easy to bind to these

cellular surface. It is reported that the content of SH on cellular surface of some tumors was ten times more than that on normal cells<sup>[12]</sup>. PDP-R molecules may have a higher affinity to some cancers and stronger ability to kill them. These suggest that PDP-R molecules might have biological activities different from that of RT and might be a anticarcinogen worth investigating further. Modification of RT with heterobifunctional linker containing group able to bind -SH groups, such as SPDP and so forth, might be a new way to improve activity of RT against cancer including hepatocellular cancer and related investigations are underway.

### REFERENCES

- 1 Lambert JK, McIntyre G, Gauthier MN, Zullo D, Rao V, Steeves RM *et al.* The galactose-binding sites of the cytotoxic lectin ricin can be chemically blocked in high yield with reactive ligands prepared by chemical modification of glycopeptides containing triantennary N linked oligosaccharides. *Biochemistry*, 1991;30(13):3234-3247
- 2 Lambert JK, Goldmacher VS, Collinson AR, Nadler LM, Balattler WA. An immunotoxin prepared with blocked: a natural plant toxin adapted for therapeutic use. *Cancer Res*, 1991;51(23):6236-6242
- 3 Dong JY, Wang WX, Cao YX, Li YS, Zou BY. Study on comparison between ricin and modified it in localization at major viscera of mice. *J 4th Military Medical University*, 1995;16(1):17-20
- 4 Li L, Wang WX, Zou BY. The preparation of modified ricin and its cytotoxicity. *J 4th Military Medical University*, 1996;17(3):178-180
- 5 Wang WX, Guo ZR. Isolation of ricin using simple and economical method. *J 4th Military Medical University*, 1987;6(2):66-69
- 6 Wang WX. Synthesis of heterobifunctional cross linking agent SPDP using modified method. *J 4th Military Medical University*, 1987;8(1):66-69
- 7 Drevin CJ, Axen R. Protein thiolation and reversible protein-protein conjugation. *Biochem J*, 1978;173(3):732-737
- 8 Chen GZ, Li YH. Determination of activity of serum glutathione-S-transferase and its clinical practices. *Shaanxi J of Lab Med*, 1994;9(1):8-10 (in Chinese)
- 9 Adachi Y, Horil K, Takahashi Y, Tanihata M, Ohba Y, Yamamoto T. Serum glutathione-S-transferase activity in liver diseases. *Clin Chem Acta*, 1980; 106(3):243-255
- 10 Fodstad O, Olsnes S, Pihl A. Toxicity, distribution and elimination of the cancerostatic lectins abrin and ricin after parenteral injection into mice. *Br J Cancer*, 1976;34(4):418-425
- 11 Wang WX, Dong JY, Li L, Yang HX, Zou BY. Change in bonding of modified ricin to galactose and hemagglutination. *Med Info PLA*, 1994;8(5):269
- 12 Mehishi JN, Grassetti DR. Hydrosulfuryl on the surface of intact Ehrlich ascites tumor cells, human blood platelets and lymphocytes. *Nature*, 1969;224(8):563-564

# Effect of cytokines on liver necrosis \*

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**Subject headings** hepatitis; liver necrosis; tumor necrosis factor; interleukins; interferon

## Abstract

**AIM** To investigate the effect of cytokines on the liver necrosis.

**METHODS** rIL (interleukin)-1, rIL-6, rIFN (interferon) $\gamma$ , rTNF (tumor necrosis factor) $\alpha$  with or without D-galactosamine (D-GAL) were injected into the abdominal cavity of mice separately. ALT, TBIL (total bilirubin) and histological changes were observed.

**RESULTS** There was no effect on hepatocyte of normal mice after injection of rIL-1, rIL-6, rIFN alone or together. The serum total bilirubin (TBIL) and liver necrosis of mice increased after rTNF $\alpha$ , rIL-6 or rIFN $\gamma$  were used separately with D-GAL. The TBIL level ( $\mu\text{mol/L}$ ) was  $46.19 \pm 10.62$ ,  $44.55 \pm 12.9$  and  $41.94 \pm 14.9$ , higher than that caused by D-GAL alone (TBIL,  $26.67 \mu\text{mol/L}$   $\Delta 11.14 \mu\text{mol/L}$ ). The serum TBIL of mice and the degree of liver necrosis increased after injection of IL-1, IL-6 with D-GAL and rTNF $\alpha$ .

**CONCLUSION** Cytokines, like IL-1, IL-6, IFN $\gamma$  and TNF $\alpha$  joined in the process of hepatocyte necrosis. They can enhance the degree of liver necrosis induced by D-GAL.

## INTRODUCTION

Immune system in human body is a net system and so are the effects of cytokines, which can be affected by other cytokines. They promote or inhibit each other, TNF $\alpha$  can aggravate hepatic necrosis<sup>[1]</sup>, and its effect is also influenced by other cytokines. The levels of IL-1, IL-6 and IFN $\gamma$  in plasma of hepatitis patients were increased after infection<sup>[2-6]</sup>. We investigated the effect of multicytokines to understand the synergistic action of multicytokines.

## MATERIALS AND METHODS

### Material and animal

**Animal** Ninety-six Balb/c male mice weighing 20g each were used. They were provided by the Center of Experiment Animal of Chinese Academy of Medical Sciences.

**Reagent** rIL-1 ( $10^5\text{U/ml}$ ), rIL-6 ( $10^5\text{u/ml}$ ), rIFN ( $5 \times 10^5\text{U/ml}$ ) and rTNF $\alpha$  ( $5 \times 10^5\text{U/ml}$ ) were provided by the Chinese Academy of Military Medical Sciences. D-GAL were derived from Department of Chemistry, Chongqing Medical University.

### Method

**The effect of rIL-1, rIL-6, rTNF $\alpha$  and rIFN $\gamma$  on normal mice** Thirty mice were selected and divided into 5 groups. rIL-1 ( $10^6\text{U/kg}$ ), rIL-6 ( $10^6\text{U/kg}$ ), rIFN $\gamma$  ( $5 \times 10^6\text{U/kg}$ ) and rTNF $\alpha$  ( $5 \times 10^5\text{U/kg}$ ) were injected into the abdominal cavity of mice separately and normal saline was used for the control. TBIL and ALT were detected 48 hours after the injection. The pathologic changes of the liver were observed.

**The combined effect of TNF $\alpha$  with IL-1 or IL-6 or IFN $\gamma$  on liver** Eighteen mice were selected and divided into 3 groups. TBIL and ALT were detected 48 hours after injection of rTNF $\alpha$  with rIL-1, or rIL-6, or rIFN $\gamma$  separately into the abdominal cavity. Liver histological changes were observed at the same time.

**The aggravating effect of rIL-1, rIL-6, rIFN $\gamma$  and rTNF $\alpha$  separately in liver necrosis** Thirty mice were divided into 5 groups. D-GAL ( $1.5\text{g/kg}$ ), or D-GAL with rIL-1 ( $5 \times 10^4\text{U/kg}$ ) or rIL-6 ( $5 \times 10^4\text{U/kg}$ ) or rIFN $\gamma$  ( $2.5 \times 10^5\text{U/kg}$ ) or rTNF $\alpha$  ( $1 \times 10^4\text{U/kg}$ ) were injected separately into abdominal cavity of

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**Supported by National Natural Science Foundation of China, No.39170704.**

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**Received** 1998-05-18

mice. Serum TBIL and pathologic changes in liver were compared with the control after 48 hours.

The rest 18 mice were divided into 3 groups. D-GAL and rTNF $\alpha$  with rIL-1 or rIL-6 or rIFN $\gamma$  (the same dose as above) were injected into the abdominal cavity of mice separately.

#### ALT and TBIL detection Beckman Biochemistry Machine was used to detect the serum ALT and TBIL.

Methods of liver biopsy<sup>[7]</sup> Hepatic tissues were taken and fixed in Bovin's fluid. After dehydration they were embedded in paraffin wax, and sections were made, and stained by HE. We marked mild degeneration as - or  $\pm$ , spot necrosis as +, small piece necrosis as ++, sublarge necrosis as +++, large necrosis as ++++.

**Statistical analysis**  $\chi^2$  test was used to compare the difference among groups.

## RESULTS

### The effect of multicytokines on normal liver of mice

rTNF $\alpha$ , rIL-1, rIL-6 and rIFN $\gamma$  were injected into the abdominal cavity of normal mice separately. The changes of their hepatic function are shown in Table 1. And the results of their hepatic histologic examination were - or  $\pm$ . There was no difference from normal control. There was no difference in TBIL and ALT either between each group, indicating that the injection of rTNF $\alpha$ , rIL-1, rIL-6 and rIFN $\gamma$  have no effects on liver function and liver histology of normal mice.

**Table 1** The changes of liver function after cytokines injection in mice ( $\bar{x}\pm s$ )

	n	TBIL( $\mu\text{mol/L}$ )	ALT(IU/L)	AST(IU/L)
Control	6	7.28 $\pm$ 4.82	46 $\pm$ 4	187 $\pm$ 28
rTNF $\alpha$	3	8.70 $\pm$ 5.34	50 $\pm$ 11	89 $\pm$ 27
rIL-1	3	7.23 $\pm$ 0.87	40 $\pm$ 5	76 $\pm$ 10
rIL-6	3	11.77 $\pm$ 3.51	31 $\pm$ 1	62 $\pm$ 2
rIFN $\gamma$	3	9.30 $\pm$ 2.7	31 $\pm$ 5	89 $\pm$ 13

F = 08 between groups, P>0.05.

### The effect of rTNF $\alpha$ with rIL-1 or rIL-6 or rIFN $\gamma$ on normal mice

rTNF $\alpha$  with rIL-1, or rIL-6, or rIFN $\gamma$  were injected into the abdominal cavity of normal mice. The changes of their liver function are shown in Table 2. There was no difference in TBIL and ALT among the groups, and no difference in liver histology compared with normal control. So, it is obvious that rTNF $\alpha$  with rIL-1 or rIL-6 or rIFN $\gamma$  had no effect on liver function and liver histology of normal mice.

**Table 2** The combined effect of rTNF $\alpha$  with rIL-1 or rIL-6 or rIFN $\gamma$  on liver function of normal mice ( $\bar{x}\pm s$ )

	n	TBIL( $\mu\text{mol/L}$ )	ALT(IU/L)	AST(IU/L)
Control	6	7.28 $\pm$ 4.82	46 $\pm$ 4	187 $\pm$ 28
rTNF $\alpha$	3	8.70 $\pm$ 5.34	50 $\pm$ 11	89 $\pm$ 27
rTNF $\alpha$ +rIFN $\gamma$	3	7.38 $\pm$ 6.01	32 $\pm$ 3	100 $\pm$ 14
rTNF $\alpha$ +rIL-1	3	8.59 $\pm$ 5.9	42 $\pm$ 11	128 $\pm$ 19
rTNF $\alpha$ +rIL-6	3	10.85 $\pm$ 3.25	36 $\pm$ 5	115 $\pm$ 12

F = 0.8, between groups, P>0.05.

### The effect of cytokines on mice liver

DGal combined with cytokines were injected separately into the abdominal cavity of mice. The results of liver function and hepatic histology are shown in Table 3. The level of TBIL in different groups of cytokine was higher than that of controls. The TBIL level in groups of D + rIL-6, D + rIFN and D + rTNF $\alpha$  rose significantly compared with group D, and hepatic necrosis worsened. There was no difference in TBIL level between group D+rIL-1 and group D.

**Table 3** The effect of D-GAL with cytokines on TBIL and histological changes of liver

Group	n	TBIL( $\mu\text{mol/L}$ )	Histological changes (n)		
			$\pm$	+	++
Control	6	7.28 $\pm$ 4.82	6		
D-GAL	6	26.67 $\pm$ 11.14		6	
D+rIL-1	6	29.69 $\pm$ 9.6		6	
D+rIL-6	6	45.55 $\pm$ 12.9 <sup>a</sup>		1	5
D+rIFN $\gamma$	6	41.94 $\pm$ 14.9 <sup>a</sup>		2	4
D+rTNF $\alpha$	6	46.19 $\pm$ 10.62 <sup>a</sup>		2	4

<sup>a</sup>P<0.05, vs group D-GAL.

**Table 4** The effects of DGal and rTNF $\alpha$  with rIL-1, rIL-6, rIFN $\gamma$  separately on TBIL and hepatic cell of mice

Group	n	BIL( $\mu\text{mol/L}$ ) ( $\bar{x}\pm s$ )	Histological changes				
			-or $\pm$	+	++	+++	++++
Control	6	7.28 $\pm$ 4.8	6				
D+rTNF $\alpha$	6	46.19 $\pm$ 10.6		2	4		
D+rTNF $\alpha$ +rIL-1	6	66.27 $\pm$ 18.7 <sup>a</sup>				3	3 <sup>a</sup>
D+rTNF $\alpha$ +rIL-6	6	69.28 $\pm$ 18.9 <sup>a</sup>				2	4 <sup>a</sup>
D+rTNF $\alpha$ +rIFN $\gamma$	6	36.75 $\pm$ 14.5		2	4		

<sup>a</sup>P<0.05, vs group of D+rTNF $\alpha$ .

DGal and rTNF $\alpha$  with rIL-1, or rIL-6 or rIFN $\gamma$  were injected into the abdominal cavity of mice. The results of liver function and hepatic histology are shown in Table 4. The degree of hepatic necrosis was more severe and the level of TBIL was higher in group D+T+1 and group D+T+6 than in group D+T. There was no significant difference between group D+T+IFN $\gamma$  and group D+T. It is suggested that D+T can raise the level of TBIL and the degree of hepatic necrosis. And adding, rIL-1

and rIL-6 can increase the level of TBIL and ALT and enhance the degree of liver necrosis, but no difference with rIFN $\gamma$ .

## DISCUSSION

Our experimental results showed that various cytokines, such as TNF $\alpha$ , IL-1, IL-6 and rIFN $\gamma$  alone or together have no effect on liver. But TNF $\alpha$ , rIL-6 and rIFN $\gamma$  can enhance the degree of liver lesions caused by D-Gal and rIL-1 and rIL-6 can aggravate liver necrosis caused by TNF $\alpha$ .

Lipopolysaccharide (LPS) is a very strong inducer of many cytokines. It can induce IL-1, IL-6, TNF $\alpha$  and IFN $\gamma$  and so on,<sup>[8,9]</sup> e.g., hepatitis B can induce TNF $\alpha$  in hepatocytes<sup>[10]</sup>. Besides increased TNF $\alpha$  in patients with viral hepatitis, the level of many other cytokines was elevated, for example IL-1, IL-6 and IFN $\gamma$ . The effect of any cytokine could be affected by other cytokines. IFN $\gamma$  can induce the production of TNF $\alpha$ <sup>[11]</sup>, which produced cytokines including IL-1 and IL-6. So, not only TNF $\alpha$ , but many other cytokines affect the liver function when infections appear. Our results demonstrated the role of multiple cytokines in hepatic necrosis. There might be many routes for multiple cytokines to worsen the hepatic damage in hepatitis. One of the routes is local Shwartzman reaction. TNF $\alpha$  and LPS can induce Shwartzman as either sensitizing agent or stimulating agent<sup>[12]</sup>. TNF $\alpha$  together with IL-1, IL-6 can aggravate hepatic necrosis and bleeding. They can spoil the cell membrane by inducing indirectly serine protease and by activating phosphatase A<sub>2</sub><sup>[13]</sup>. Cytokines could not induce hepatic lesions in mice with normal liver function. The susceptibility to cytokines was increased after injection of D-GAL. D-GAL, actimycin D and mitomycin C are hepatocyte-specific inhibitors of RNA synthesis. The toxic effect of cytokine can increase dramatically when

cytokines injected with DGAL<sup>[14]</sup>. The catabolism and anabolism of protein in hepatocytes are affected in hepatopathy and the toxic effect of cytokine can reveal in hepatitis.

It can be supposed that viral infection and bacteriotoxemia in patients with hepatitis can induce a large amount of cytokines and aggravate liver necrosis through their interaction, resulting in severe hepatitis. To study the effect of multicytokines in patients with hepatitis is of great importance to understand the mechanism of hepatic necrosis and to search for effective means for prevention and treatment of liver necrosis.

## REFERENCES

- 1 Yu YY, Si CW, Lang ZW, Tian XL, He Q, Xue HP. The effect of tumor necrosis factor on viral hepatitis. *Chin J Intern Med*, 1996;(35)1:28-31
- 2 Anastassakos C, Alexander GTM, Worstencroft RA. Interleukin-1 and interleukin-2 activity in chronic hepatitis B virus infection. *Gastroenterology*, 1988;94(4):999-1005
- 3 Shinichi Kakumu, Tadashi Shinagawa, Tetsuya Ishikawa. Serum Interleukin 6 level in patients with chronic hepatitis B. *Am J Gastroenterol*, 1991;86(12):1804-1808
- 4 Khoruts A, Stahnke L, McClain CJ, Logan G, Allen JH. Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholics. *Hepatology*, 1991;13(2):267-276
- 5 McClain CJ, Hill DB and Schmid J. Cytokines and alcoholic liver diseases. *Semin Liver Dis*, 1993;13(1):170-182
- 6 Yeqing S, Katsutoshi T, Etsuko I, Katsumi Y, Hiroshi O. Elevated serum interleukin-6 level in patients with acute hepatitis. *J Clin Immunol*, 1992;12(3):197-200
- 7 Huang F, Wang JY, Gao H, Dong XJ. The level of tumor necrosis factor in plasma of mice and the protection effect of HCG and PGE. *J Chin Med Univ*, 1993,22(4):275-277
- 8 Blanchard DK, Djeu JY, Klein TW, Priedmen H, Stewart WE. Interferon induction by Lipopolysaccharide, dependence on interleukin 1 and macrophage. *J Immunol*, 1986;136(3):963-976
- 9 Lehmann V, Freudenberg MA, Galanos C. Lethal toxicity of LPS and TNF in normal and D-GAL treated mice. *J Exp Med*, 1987;165(3):657-663
- 10 Gozalez AR, Garcia MC, Garcia BL, Moreno OR, Alonso JL, Yaque E. Induction of tumor necrosis factor  $\alpha$  production by human hepatocytes in chronic viral hepatitis. *J Exp Med*, 1994;179(3):841-848
- 11 Marshall JS, Dolovich J, and Wasserman S. The induction of peripheral blood mononuclear cell TNF $\alpha$  production by IFN $\gamma$  can be inhibited by budesonide. *J Aller Clin Immunol*, 1995;95(2):365
- 12 Bian ZQ, Wang WY, Qin YZ, Qin RM, Wang GZ, Qin HY. Experimented study of Tumor-Necrosis Factor induced acute liver necrosis in Duckling infected with DHBV. *J Chin Infect Dis*, 1992,10(2):88-92
- 13 Suffys P, Beyaert R, Vanvov F and Fiers W. Involvement of a serine protease in tumor-necrosis factor mediated cytotoxicity. *Eur J Biochem*, 1988;178(1):257-265
- 14 Alcorn JM, Fierer J, Chojkier M. The acute phase response protects mice from D-GAL sensitization to endotoxin and tumor necrosis factor- $\alpha$ . *Hepatology*, 1992;15(1):122-129

# Phase tissue intercellular adhesion molecule-1 expression in nude mice human liver cancer metastasis model \*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; neoplasm metastasis; intercellular adhesion molecule-1; disease models, animal

## Abstract

**AIM** To study the phase cancer tissue intercellular adhesion molecule-1 (ICAM-1) expression of human cancer metastasis model in nude mice, and to analyze the relationship between ICAM-1 expression and the metastasis and recurrence of hepatocellular carcinoma (HCC).

**METHODS** HCC tissues from liver cancer metastasis model in nude mice (LCI-D20) was orthotopically implanted, and ICAM-1 expression in HCC tissues at different growing time were detected by immunodot blot. Tumor size, intrahepatic and extrahepatic metastasis foci were observed by naked eyes and under light microscope.

**RESULTS** ICAM-1 was positively correlated to the tumor growing time ( $r = 0.88$ ,  $P < 0.01$ ) and tumor size  $r = 0.5$ ,  $P < 0.05$ ). It was higher in metastatic HCC than in nonmetastatic HCC ( $8.24 \pm 0.95$  vs  $3.03 \pm 0.51$ ,  $P < 0.01$ ). ICAM-1 content in cancer tissues increased suddenly after metastasis occurred and then maintained in a high level. ICAM-1 was also higher in multimetastasis group than in monometastasis group ( $10.05 \pm 1.17$  vs  $5.48 \pm 0.49$ ,  $P < 0.05$ ).

**CONCLUSION** Tissue ICAM-1 could predict not only the metastasis of human liver cancer metastasis model in nude mice early and sensitively, but also the metastasis degree. So tissue ICAM-1 may be a potential index indicating the status of metastasis of HCC patients.

## INTRODUCTION

Although many methods had been used to prohibit posthepatectomic metastasis of liver cancer in clinic, but the 5-year postoperative metastasis rate was still up to 61.9% in general, and 45.3% in small HCC<sup>[1]</sup>. Metastasis and recurrence have become the main obstacle in HCC patients to gain better outcome and longer survival. Up to now we can not diagnose or predict it before the formation of metastasis node. In the pervious studies, we found ICAM-1 was related to liver cancer and its metastasis, and tissue and serum ICAM-1 could predict the status of HCC metastasis<sup>[2,3]</sup>. It was unclear whether the ICAM-1 could reflect the HCC metastasis early and sensitively, and how ICAM-1 changed during the HCC growing time. So in this experiment, we observed the phase tissue ICAM-1 expression of human liver cancer high metastasis model in nude mice (LCI-D20) at different time period from tumor implantation to metastasis and telophase.

## MATERIALS AND METHODS

### Reagents

Monoclonal antibody of ICAM-1 (1g/L) was purchased from R & D Company, Britain. AKP-rabbit-anti-mouse IgG was from Sino-American Company, China. NC membrane, NBT and BCIP were bought from Sigma Company.

### Mice

BALB/cA male nude mice (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China) aged 4 to 6 weeks and the nude mice model of human hepatocellular carcinoma with high metastatic potential (LCI-D20)<sup>[4]</sup>, which were established in our institute in 1996, were used in this study.

### Animal model

Tumor block of LCI-D20 nude mice human liver cancer metastasis model was implanted into the left lobe of the nude mouse liver according to the routine procedure in our laboratories. Briefly, a left upper abdominal transverse incision was made under anaesthesia; the left lobe of the liver was exposed

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Supported by Grant from Leading Specialty by the Shanghai Health Bureau, The Ninth 5-year Plan of National Natural Science and Technology 96-906-01-15, National Natural Science Foundation of China, No.396707 and China Medical Board, No. 93-583, New York, NY, USA.

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Received 1998-07-06

and a part of the liver surface were mechanically injured with scissors. Then, a tumor block of 0.2cm×0.2cm×0.2cm was fixed within the liver tissue. After operation, mice were kept in laminar-flow cabinets under specific-pathogen-free conditions.

Mice were killed at day 7, 10, 13, 16, 19, 21, 25, 32 and 35 postimplantation, and tumor size, intrahepatic, lung, intra-abdominal metastasis nodes were found. The tumors were resected and frozen, and then stored at -70°C for further use. The residual liver and lung were also resected and processed for routine gross and finding metastasis node under microscope.

#### Immuno-dot blot

Three hundredmg tissue was homogenized in 1.5ml suspending buffer (0.1mol/L NaCl, 0.01mol/L Tris.Cl, pH 7.6, 0.001mol/L EDTA pH 8.9, 1% Txiton-X100), the protein concentration was determined by Hartree method<sup>[5]</sup>. Thirty μl supernatant or 50 μl serum was applied onto the nitrocellulose membrane in a dot blot format, and physiological saline was used as control. After non-specific blocking with 5% lipid-free milk, the blots were incubated with ICAM-1 antibody (1:500) at room temperature for 2 h, followed by incubation with AKP-conjugated rabbit-anti-mouse-IgG (1:200) for 2 h at room temperature, then stained with NBT/BCIP (2:1v/v). The integrated optical density (IOD) of each blot was measured by MIAS-300 automatic image analyzer. Tissue ICAM-1 = [sample IOD-background IOD] × sample area/μg protein concentration/1 000.

#### Data analysis

Student's *t* test and linear correlation analysis were used statistically.

#### RESULTS

At the 7 th day postimplantation, tumors were too small, only 0.2-0.3 in diameter, to take sample for ICAM-1 detection. At the 10th and 13 th day, tumor in only one mouse was big enough for ICAM-1 detection. No metastasis node was found before the 19 th day. At the 19 th day, metastasis nodes were found in 2 of the 3 killed mice, 1 with metastasis node in lung under optical microscope, 1 mouse with visible metastasis node in iliac fossa lymphnode. Later, metastasis nodes increased, which could be found in liver, lung, iliac fossa, inferior kidney, pelvic cavity, lymphnode of mesentery, para-aorta retroperitoneum, and at last bloody ascites.

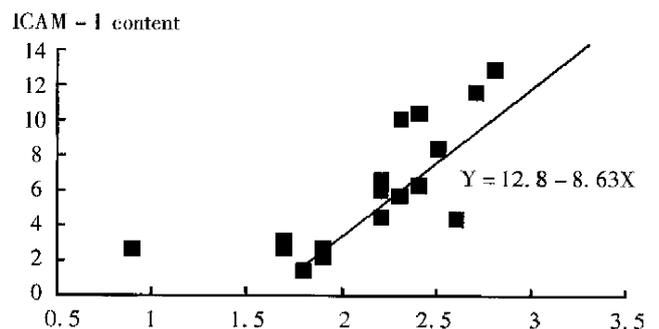
#### Tissue ICAM-1 at different tumor growing time (Table 1)

**Table 1** Tissue ICAM-1 expression at different tumor growing time

Tumor growing time (day)	Cases	ICAM-1 ( $\bar{x}\pm s$ )
10	1	2.71±0.00
13	1	2.78±0.00
16	3	2.53±0.53
19	3	5.30±0.81
22	3	7.77±1.21
32	4	8.69±2.01
36	2	6.57±1.95

Tissue ICAM-1 was highly correlated to tumor growing time, the correlation coefficient( $\gamma$ ) was 0.88( $P<0.01$ ). The time course of tissue ICAM-1 indicated that there was a plateau stage at day 10, 13 and 16 after implantation, then suddenly reached a higher level at day 19, meanwhile the metastatic node were discovered in lung and iliac fossa lymphnode. At this time ICAM-1 expression was nearly two times higher than that before day 19. Thereafter ICAM-1 expression in HCC continued and maintained in a high level until day 36. It became lower at day 36, probably because at this terminal HCC some necroses occurred inside the tumor. It was suggested that tissue ICAM-1 expression could act as an indicator of liver cancer metastasis in LCI-D20 model.

#### Tissue ICAM-1 expression and tumor size



**Figure 1** ICAM-1 content in liver cancer tissue of different tumor sizes.

According to Figure 1, ICAM-1 increased as the tumor were growing larger. Statistical analysis revealed that ICAM-1 content in tissue was correlated to tumor size ( $\gamma = 0.5$ ,  $P<0.05$ ). This suggested that tissue ICAM-1 could reflect the growing status of liver cancer.

### Tissue ICAM-1 and tumor metastasis

Tissue ICAM-1 was significantly different between before metastasis ( $3.03 \pm 0.51$ ) and after metastasis ( $8.24 \pm 0.95$ ,  $P < 0.01$ ), and between monosite metastasis ( $5.48 \pm 0.49$ ) and multisite metastasis ( $10.05 \pm 1.17$ ,  $P < 0.05$ ). These data indicated that ICAM-1 might reflect not only the status but also the degree of the metastasis of LDI-20D liver cancer metastasis model.

### DISCUSSION

The process of tumor metastasis is very complex, including the tumor cell dissociating from the primary locus, invading across the surrounding tissue, entering and extravasation from the circulation, and growing in distant organs<sup>[6]</sup>. Tumor angiogenesis, matrix degradation, cell adhesive molecule, oncogene, signal transduction, factors like IGF, ect are involved in this procedure<sup>[7]</sup>. Intercellular adhesion molecule-1 (ICAM-1), so called CD54, is a member of the immunoglobulin superfamily of adhesive molecule. It was found that ICAM related to cancer and cancer metastasis. Serum SICAM-1 (soluble ICAM-1) in patients with advanced stage (II-IV) or recurrent cervical uterine cancer increased significantly<sup>[8]</sup>.

SICAM-1 was also related to lesion thickness, staging, recurrence probability of melonoma<sup>[9]</sup>. In lung cancer patients, SICAM-1 showed a significantly positive correlation with primary tumor size, and the overall survival of patients with low serum ICAM-1 concentration tend to be longer than that of patients with high serum ICAM-1 concentration<sup>[10]</sup>. Renal cancer recurrence was related to ICAM-1 expression in cancer tissues, patients with <50% of ICAM-1 positive cell in cancer tissues showed improved disease-free survival after a median follow-up duration of 60 months<sup>[11]</sup>. But no study has been found on if serum or tissue ICAM-1 could indicate the early cancer metastasis and recurrence sensitively up to date according to

our knowledge, neither the articles about phase change of ICAM-1 during tumor formation to tumor metastasis and terminal stage.

In this experiment, we analyzed the tissue ICAM-1 expression of LCI-D20 liver cancer metastasis model of different time after implantation, and found that tissue ICAM-1 expression was positively correlated to tumor size and tumor growing time. It is interesting that ICAM-1 content increased suddenly. It was also higher in HCC with multimetastasis nodes than in HCC with monometastasis node.

With this point of view, we conclude that tissue ICAM-1 could reflect both the metastasis of LCI-D20 human liver cancer metastasis model sensitively and the degree of metastasis early. ICAM-1 might be an index indicating the status of liver cancer metastasis clinically.

### REFERENCES

- 1 Zhou XD, Tang ZY, Yu YQ, Yang BH, Lu JZ, Li ZY *et al*. Recurrence after resection of  $\alpha$ -fetoprotein-positive hepatocellular carcinoma. *J Cancer Res Clin Oncol*, 1994;120(6):369-373
- 2 Sun JJ, Zhou XD, Liu YK, Tang ZY. Study on the relationship between intercellular adhesion molecule-1 expression and the metastasis and recurrence of liver cancer. *China Oncol*, 1997;7(3):161-164
- 3 Sun JJ, Zhou XD, Zhou G, Liu YK, Tang ZY. Serum intercellular adhesion molecule-1 and liver cancer metastasis and recurrence. *Natl Med J China*, 1998;4(5):383
- 4 Sun FX, Tang ZY, Liu KD, Ye SL, Xue Q, Gao DM *et al*. Establishment of a metastatic model of human hepatocellular carcinoma in nude mice via orthotopic implantation of histologically intact tissues. *Int J Cancer*, 1996;66(2):239-243
- 5 Hartree EF. Determination of protein: a modification of Lowry method that gives a linear photometric response. *Anal Biochem*, 1972;48(3):422-427
- 6 Saiki I, Iida J, Murata J, Ogawa R, Nishi N, Sugimura K *et al*. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. *Cancer Res*, 1989;49(15):3815-3819
- 7 Hart IR, Saini A. Biology of tumor metastasis. *Lancet*, 1992;339(13):1453-1457
- 8 Nasu K, Narahara H, Etoh Y, Kawano Y, Hirota Y, Miyakawa I. Serum levels of soluble intercellular adhesion molecule-1 (ICAM-1) and the expression of ICAM-1 mRNA in uterine cancer. *Gynecol Oncol*, 1997;65(2):304-308
- 9 Natali PG, Hamby CV, Felding HB, Liang B, Nicotra MR, Di FF *et al*. Clinical significance of alpha(v)beta3 integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesion. *Cancer Res*, 1997;57(15):1554-1560
- 10 Osaki T, Mitsudomi T, Yoshida Y, Oyama T, Dhgami A, Nakanishi K *et al*. Increased levels of serum intercellular adhesion molecule-1 (ICAM-1) in patients with non-small cell lung cancer. *Surg Oncol*, 1996;5(3):107-113
- 11 Santarose M, Fanaro D, Quaia M, Spada A, Talamini R, Galligioni E. Expression and release of intercellular adhesion molecule-1 in renal-cancer patients. *Int J Cancer*, 1995;62(2):271-275

# Reversing effect of Tanshinone on malignant phenotypes of human hepatocarcinoma cell line

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**Subject headings** tanshinone; liver neoplasms; carcinoma, hepatocellular; tumor cell, cultured; cell line; immunohistochemistry; proliferation cell nuclear antigen; flow cytometry

## Abstract

**AIM** To study the reversing effect of Chinese drug tanshinone on malignant phenotype of cancer cells.

**METHODS** Human hepatocarcinoma cell line (SMMC-7721) was treated in vitro with 0.5 mg/L tanshinone for 4 days, and variation in cell differentiation was detected.

**RESULTS** The morphology of cancer cells was tended toward well differentiation and cell growth was markedly inhibited. BrdU uptake assay and immunohistochemical stain of PCNA showed that the BrdU labeling rate and PCNA positive rate were lower than the controls, but no difference was found statistically as compared with all transretinoic acid. Flow cytometric assay demonstrated that S phase cells decreased and G0/G1 phase cells increased. Expression of c-myc oncogene protein decreased but the c-fos oncogene protein markedly increased.

**CONCLUSION** Tanshinone could reverse the inducing differentiation in human hepatocarcinoma cells (SMMC-7721). It may become a new prospective inducer of cell differentiation to treat cancers.

## INTRODUCTION

Study on reversion of cancer cells is an important field of tumor molecular biology at present. The application of all transretinoic acids to treating leukemia is a successful example of differentiation inducing therapy. But attention was also been paid to screening highly effective and low toxicant inducer of differentiation to treat the solid tumors. Tanshinone is an alcohol extract from the root of the traditional Chinese herbal medicine -*Salvia Miltiorrhiza* Bunge. Previous studies in vitro have shown that tanshinone possessed cytotoxic effect on cancer cells and induced differentiation of cervical carcinoma cells<sup>[1,2]</sup>. Zhang ZW, *et al.* reported that all transretinoic acid can reverse malignant phenotypes of human hepatocarcinoma cells, and promote carcinoma cells to differentiate toward normal cells<sup>[3]</sup>. We used transretinoic acids as positive control to study the reversing effect of tanshinone on malignant phenotypes of human hepatocarcinoma cells in cellular morphology, cellular properties of growth and proliferation, cell cycle and expression of oncogene.

## MATERIALS AND METHODS

### Cell and culture

Human hepatocarcinoma cell line (SMMC-7721) was derived from Shanghai Institute of Cancer Research<sup>[4]</sup>. Cells were cultured in RPMI1640 medium that contained 15% inactivated bovine serum and 100 mg/L ampicillin, 100 mg/L streptomycin at 37°C in a 5% CO<sub>2</sub> incubator.

### Drugs and treatment

Tanshinone II A (Tan) was provided by the Chinese Institute for Drug and Biological Assay (pure product). It was dissolved in DMSO (final concentration is 0.22% V/V). The solution was filtered through a 0.22 μm microporefilter, then stored at 4°C for use. SMMC-7721 cells were seeded, and treated with 0.5 mg/L Tan or 0.5 mg/L ATRA. The control cells were added equal amount of DMSO for negative control experiment. The cells were treated with drugs or DMSO for 4 days continuously. The usage of all transretinoic acid (ATRA, Sigma's product) was equal to tanshinone.

### Reagents

5-Bro-mo-2'-deoxyuridine labeling and detection kit II is a product of Boehringer Mannheim Company, Germany. Anti-proliferation cell nuclear antigen

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\* Subject supported by the Science Fund of Chinese Ministry of Public Health, No.1994-1-240 and China Medical Board of New York, No.9408.

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Received 1998-03-08

(PCNA) monoclonal antibody PC10, c-myc and c-fos monoclonal antibody were purchased from Denmark Dako Company and S-P kit from American Maxim Company.

#### **Cellular morphological observation**

The treated cells were observed under inverse and light microscopy after HE stain. On the other hand, the cells were made into ultra-slice by routine method, and observed under transmission electron microscopy.

#### **Cellular growth measurement**

The cells treated with drugs and control cells were harvested by trypsinization and the total number of cells was counted by trypan blue exclusion daily for 4 days.

#### **BrdU labeling rate measurement**

**BrdU labeling** The cells treated with drugs for 4 days were continuously cultured with BrdU labelling culture liquid (final concentration of 10  $\mu\text{mol/L}$ ) for 30 min, centrifuged and washed with PBS, added 5% albumin PBS to make the cell smears. The diluted 1:10 BrdU monoclonal antibody (mouse IgG1) on the smears was added with 1:10 anti-mouse-IgAP, and developing dye (NBT, X-phosphate).

**Result observation** The distracts of more positive cells were chosen under low power light microscopy. The positive cells of 2000 cells were counted under oil immersion lens. The labeling rate and positive rate were calculated.

#### **Immunohistochemical detection**

LSAB method was used for immunohistochemical staining of PCNA according to SP-kit instruction. PC10 was diluted 1:200. Human tonsil tissues served as positive control and PBS substituted PC10 for negative control. Observation of result is similar to above.

#### **Flow cytometry measurement**

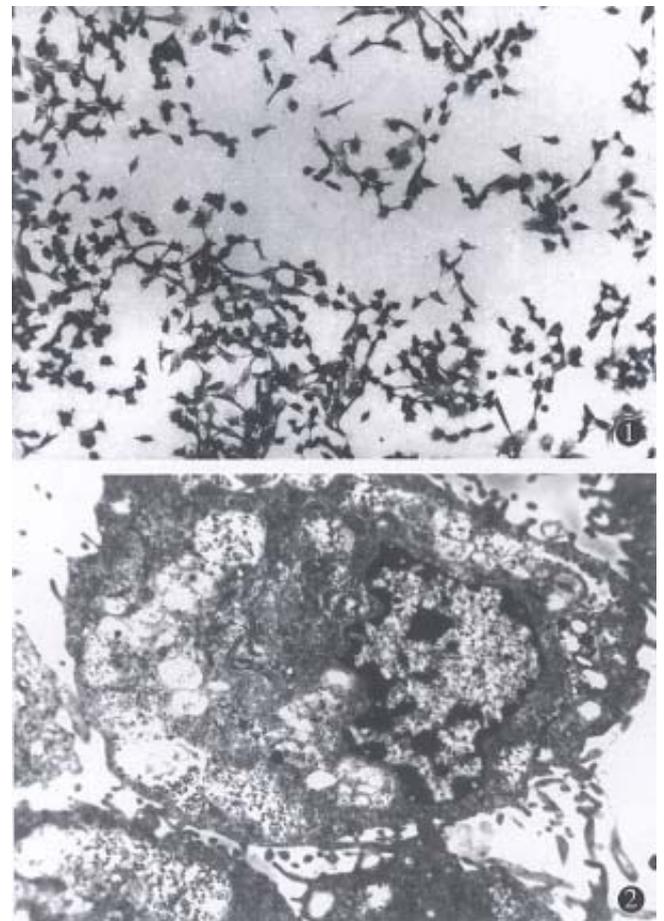
The sample preparation and measurement followed the method described in reference<sup>[5]</sup>. The cells were harvested, counted and fixed. The cell concentration was adjusted to 10<sup>5</sup>/ml. According to the routine method, using FACS-420 FCM, cell frequency distribution of each phase in cell cycle was measured, and by combined with immunohistochemical method, cell c-myc, c-fos gene and their protein expression were detected. The results were shown with scanning figure and date.

## **RESULTS**

### **Effect of Tan and ATRA on morphology of SMMC-7721 cells**

Light and electron microscopy observation. In

control group, the cells arranged in aggregation, the cellular shape was polygon or spindle, with different volumes, more tumor giant cells, large karyon and more nucleus. The karyon/cytoplasm ratio rose, with few organelle. In Tan or ATRA group, the cellular arrangement was scattered, the cells became thin and long, the cellular volume became accordant, with less tumor giant-cells (Figure 1). The karyon became small and the nucleus became scarce. The karyon/cytoplasm ratio fell, the well-differentiated organelle such as microfilament and Golgi complex occurred. Morphology of the cells tended towards well-differentiation as compared with the control cells (Figure 2).



**Figure 1** Tan group, morphology of SMMC-7721 cells under light microscopy. HE $\times$ 100

**Figure 2** Tan group, morphology of SMMC-7721 cells under transmission electron microscopy.  $\times$ 12 000

### **Effect of Tan and ATRA on growth curve of SMMC-7721 cells**

Cellular growth was measured by growth curve. The cells treated with Tan and ATRA were markedly inhibited in logarithmic growth phase (2-4 days after treatment with drugs). On day 4 after treatment with drugs, the inhibition rates of growth

were 58.1% and 52.2%. No cells died by trypan blue exclusion.

### Effect of Tan and ATRA on proliferation of SMMC-7721 cells

The BrdU labeling rate and PCNA positive rate of the Tan and ATRA group were lower than those of control group (Table 1), and there was significant difference statistically ( $P < 0.01$ ). But, there was no significant difference between PCNA positive rate of Tan group and ATRA group ( $P > 0.05$ ).

**Table 1** Effect of Tan on BrdU labeling rate and PCNA positive rate of SMMC-7721 cells

Groups	BrdU labeling rate (%)	<i>P</i>	PCNA positive rate (%)	<i>P</i>
Control	28.0		74.3	
Tan	8.9	<0.01	57.0	<0.01
ATRA	13.6	<0.01	47.6	>0.05

\*Tan compared with ATRA group by  $\chi^2$  test.

### Effect of Tan and ATRA on cell cycle and gene expression of SMMC-7721 cells

The results are shown in Table 2. Tan or ATRA increased the number of cells in G<sub>0</sub>/G<sub>1</sub> phase, but decreased the number of cells in S phase. Tan markedly inhibited the expression of cellular c-myc oncogene protein, but enhanced the expression of cellular c-fos oncogene protein, while ATRA inhibited the c-myc gene expression, but did not enhance the c-fos gene expression.

**Table 2** Effect of Tan on cell cycle and gene expression of SMMC-7721 cells

Groups	Distribution of cell cycle (%)			Labeling rate of gene expression (%)	
	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> +M	c-myc	c-fos
Control	47.7	16.2	36.1	23.8	25.9
Tan	57.6 <sup>b</sup>	8.9 <sup>b</sup>	33.5 <sup>b</sup>	14.6 <sup>b</sup>	41.7 <sup>b</sup>
ATRA	59.5 <sup>b</sup>	8.2 <sup>b</sup>	32.3 <sup>b</sup>	13.4 <sup>b</sup>	28.5 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , vs control group; <sup>b</sup> $P < 0.01$ , vs control group.

## DISCUSSION

Human hepatocarcinoma cells were treated with non-cytotoxic dose of tanshinone. After treatment, cellular morphology and structure tended toward well-differentiation, and cellular growth and proliferation were markedly inhibited. Trypan blue exclusion demonstrated that the inhibiting effects of Tan and ATRA on the cells were not caused by cytotoxicity, but by the effect of inducing differentiation.

BrdU labeling rate and PCNA positive rate are reliable indexes for cellular proliferative ability. BrdU uptake test has many advantages compared

with <sup>3</sup>H-thymidine uptake test. BrdU was a kind of analogue of thymidine. It can incorporate the multiplying cells, and label the cells of S phase<sup>6,7</sup>. BrdU monoclonal-antibody-ligase was used as an indicator. This method can measure the cells including BrdU, get the number of multiplying cells of S phase, and show the cellular DNA synthesis ability. PCNA was an auxiliary protein of DNA polymerase  $\delta$ , directly participate in DNA duplication in the course of cellular multiplication. Its expression or content represents the multiplication activity of cells. Measured positive cells are the cells of late G<sub>1</sub> phase or early S phase<sup>8</sup>. We found that BrdU labeling rate and PCNA positive rate of SMMC-7721 treated with Tan or ATRA markedly decreased. This showed that the cellular number of S phase decreased. This result is consistent with analysis of FCM (the cells were prevented in the G<sub>0</sub>/G<sub>1</sub> phase of cell cycle). Previous studies have showed that the expression of c-myc or c-fos oncogene was closely related to cellular multiplication and differentiation. The amplification and over-expression of c-myc gene were associated with malignancy and tumorigenicity of cells, but its low-expression was related to cellular morphologic changes and differentiation<sup>9</sup>. The result of FCM showed that via the inhibition or enhancement of expression of cellular gene, Tan and ATRA inhibited the ability of DNA polymerase  $\delta$  and PCNA protein expression, inhibited the cells to enter S phase and DNA synthesis, thus inhibiting the cellular growth, multiplication, and promoted cellular differentiation.

Tanshinone, acting as an effective component of a traditional Chinese medicine to induce cellular differentiation, has many advantages. It has low toxicity and side effects, can induce tumor cells to differentiate, at the same time, exerting other pharmacological effect. So tanshinone is considered to be a new prospective, high effective and low toxicant inducer of differentiation for clinical application.

## REFERENCES

- 1 Wu WK, Chang WL, Chen CF. Cytotoxic activities of tanshinone against human carcinoma cell lines. *Am J China Med*, 1991,19(3-4):207-212
- 2 Yuan SL, Huang GQ, Wang XJ, Zhou HY, Jiang YH. The differentiation-inducing effect of tanshinone and retinoic acid on human cervical carcinoma cell line in vitro. *Chin J Oncol*, 1995;17(6):424-426
- 3 Ai ZW, Zha XL, Tang H, Chen HL. The reversing effect of retinoic acid on malignant phenotypes of human hepatocarcinoma cell line, 1991;13(1):9-12
- 4 Dong RC. Establishment and basic observation of cell biological property of human hepatocarcinoma cell line SMMC-7721. *Acad J Mil Med Univ*, 1980; 1(1):5-6
- 5 Barlogic B, Raber SJ. Flow cytometry in clinical cancer research. *Cancer Res*, 1983;43:3982-3985
- 6 Del Bino G. Morphological and clinical significance of cell kinetics in non-Hodgkin's lymphomas. *Basic Appl Histochem*. 1986;30(1):197-199
- 7 Connolly KM, Bogdanffy MS. Evaluation of proliferation cell nuclear antigen (PCNA) as endogenous marker of cell proliferation in liver. A dual-stain comparison with 5-Bromo-2'-deoxy-uridine. *J Histochem*, 1993;41(1):1-4
- 8 Barvo R, Frank R, Blundell PA, Heather MB. Cyclin/PCNA is the auxiliary protein of DNA polymerase  $\delta$ . *Nature*, 1987;326(6):515-517
- 9 Adesson LC. Gene expression during normal and malignant differentiation. New York: *Academy Press*, 1985:257-262

# HBx-DNA probe preparation and its application in study of hepatocarcinogenesis \*

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**Subject headings** hepatitis B virus; liver neoplasms/etiology; carcinoma, hepatocellular/etiology; DNA probe

## Abstract

**AIM** To study the role of HBV especially HBx Open Reading Frame (ORF) in the development of hepatocellular carcinoma (HCC).

**METHODS** HBV 3.2 kb fragment was retrieved by digesting recombinant plasmid pBR322-2HBV with EcoR I, and HBx 0.59 kb fragments by digesting HBV-DNA with BamH I and Bgl II. These fragments were labelled with digoxigenin to get HBV-DNA and HBx-DNA probes. HBV-DNA was detected in HCC by dot blot and Southern blot hybridization with HBV-DNA probe, so the positive specimens in which HBV-DNA were integrated were selected. HBx-DNA was subsequently detected in the selected specimens with HBx-DNA probe.

**RESULTS** HBV-DNA was detected in 75% HCC, among which integrated type, integrated + free type covered 63.6% and 36.4%. There was no free type. HBx-DNA was detected in 90.5% specimens of integrated type.

**CONCLUSION** Hepatocarcinogenesis was highly related to HBV-DNA integration, and HBV-DNA mainly integrated into chromosome with incomplete virus DNA fragments among which HBx fragment was the predominant one.

## INTRODUCTION

HBx gene, which is the smallest one of the four open reading frames (ORF) of HBV, has the transactivation function and is closely related to hepatocarcinogenesis<sup>[1,2]</sup>. In the study of hepatocellular carcinoma (HCC) formation, HBx-DNA probe is needed to investigate HBx gene integration and its mRNA transcription. We retrieved HBx-DNA fragment by digesting HBV-DNA and labeled it with digoxigenin. To study the HBx gene function in hepatocarcinogenesis, HBV-DNA was detected first in HCC by dot blot and Southern blot hybridization, and the positive specimens in which HBV-DNA were integrated were selected. Subsequently, integrated HBx-DNA was detected in the selected specimens with HBx-DNA probe. This study may provide some fundamental data for further research into hepatocarcinogenesis.

## MATERIALS AND METHODS

### Materials

**Subjects** HCC tissues were obtained at surgical resection from Jinan Qianfeshan Hospital, which were confirmed later by pathology, and stored at -20 °C immediately after surgical resection for use.

**Reagents** Recombinant plasmid pBR<sub>322</sub>-2HBV is provided by Professor CB and DIG Hight Prime Labeling and Detection Starter Kit is product of Boehringer Mannheim Company, Germany. Restriction Endonucleases EcoR I, BamH I, Bgl II, Proteinase K are purchased from Huamei Company.

### Methods

**HBV-DNA retrieval** Isolation of recombinant plasmid pBR<sub>322</sub>-2HBV and HBV-DNA retrieval were carried out as described by CAO *et al*<sup>[3]</sup>. pBR<sub>322</sub>-2HBV was transformed into the Escherichia coli strain HB<sub>101</sub>. A 500 mL bacterial culture was grown to OD<sub>600</sub>=1.0, chloramphenicol was added to a final concentration of 170 mg/L, and the culture was continued for another 4 hours. The extracted pBR<sub>322</sub>-2HBV was digested with EcoR I, and electrophoresis was performed in 1.2% agarose gel. HBV-DNA 0.59 kb fragment (n1398-n1992) was retrieved according to the standard method<sup>[4]</sup>.

**DIG DNA labeling** HBV and HBx-DNA labeling

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**Project supported by the Natural Science Foundation of Shandong Province, No.931237222.**

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Received 1997-12-02 Revised 1998-01-02

procedures were described before<sup>[3]</sup>. Template DNA (1.0 µg) and sterile redist water were added to a reaction vial. Denature the DNA and add DIG high prime and incubate for 20 hours at 37°C. Reaction was stopped by addition of EDTA. Quantification of labeling efficiency was performed according to instructions of the manufacturer.

**DNA extraction** Genomic DNA from the tumors were isolated according to the standard methods<sup>[5]</sup>.

**HBV-DNA detection** Denatured genomic DNA 5 µl, λ DNA 10 ng, HBV-DNA 10 pg, and 1.0 pg were heated in a boiling water bath for 5 min-10 min, and chilled quickly in an ice/ethanol bath for 5 min. They were mixed with 20×SSC and tipped to nitrocellulose filters. The filters were prehybridized for 8-12 hours, hybridized for 24-36 hours at 42°C, washed and subjected to immunodetection according to the standard dot blot hybridization procedures.

**Southern blot hybridization** Positive specimens DNA which had been verified containing HBV-DNA by dot blot hybridization was then digested completely with specific restriction endonucleases, electrophoresed in agarose gels, denatured, and transferred to nitrocellulose filters. The filters were performed with standard Southern blot procedures.

**HBx-DNA detection** Selected positive specimens which had integrated HBV-DNA only were confirmed by Southern blot hybridization and standard dot blot hybridization substituting HBV-DNA probe with HBx-DNA probe was performed.

## RESULTS

### HBx-DNA retrieval result

Retrieved HBx-DNA and marker pBR322/Hae III were subjected to electrophoresis in 1.5% agarose gels. HBx-DNA was 0.59 kb fragment and its concentration was 0.1g/L (Figure 1).

### Probe sensitivity and specificity

Confirmed with homologous DNA by dot blot hybridization, HBV-DNA probe and HBx-DNA probe all had a high sensitivity of 1.0 pg, and had high specificity confirmed with heterologous DNA.

### HBV-DNA detection of HCC

Thirty-three cases (75%) were confirmed to be infected by HBV previously with HBV-DNA probe by dot blot hybridization, and 11 cases had no evidences of HBV-DNA existence.

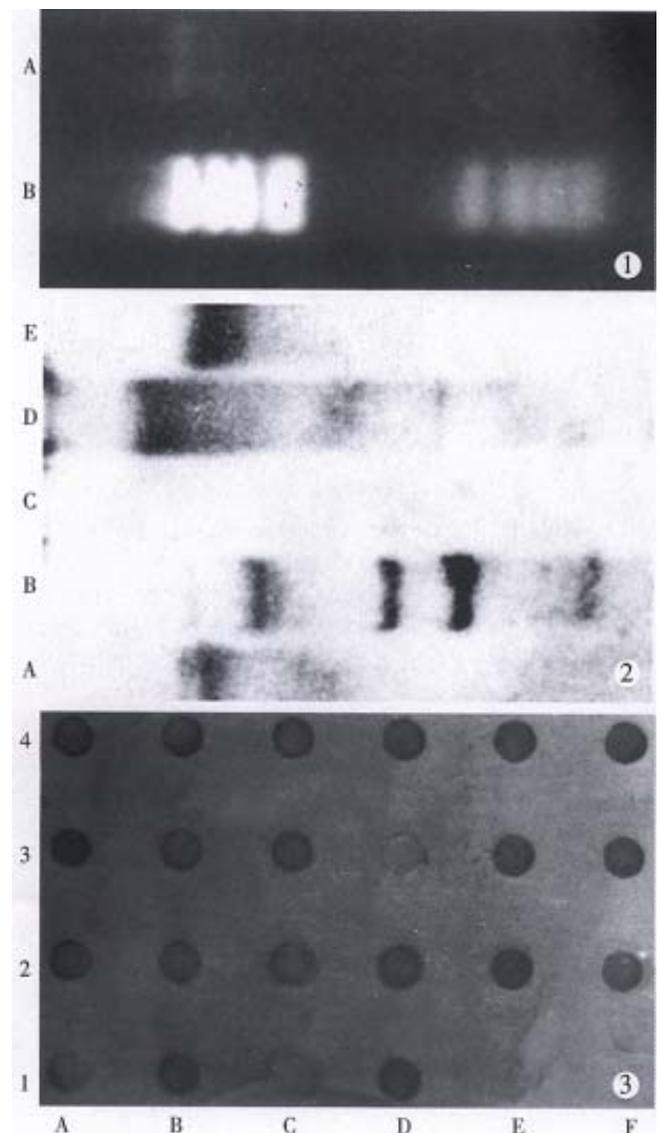
### Southern blot hybridization

Based on HBV-DNA integration into chromosome

or free existing in plasma, HBV-DNA positive HCC specimens can be divided into three types: integrated type which has integrated HBV-DNA only; free type which has free HBV-DNA in plasma; and integrated + free type which has HBV-DNA both in chromosome and in plasma. In this study, the integrated type and integrated + free type covered 63.6% and 36.4%. Free type was not found (Figure 2).

### HBx-DNA detection

Confirmed with HBx-DNA probe by dot blot hybridization, 19 (90.5%) of 21 cases of integrated type HCC specimens, showed positive HBx-DNA (Figure 3).



**Figure 1** The electrophoresis result of HBx-DNA fragment. A: HBx-DNA 0.59 kb, B: pBR322/Hae III.

**Figure 2** The result of Southern blot hybridization of liver tissues of HCC. A, C, D, E: integrated type B: both integrated and free type.

**Figure 3** The detected result of HBx fragment of integrated type HCC. 1D: HBV-DNA 1.0 pg, 1E: HBV-DNA 0.1 pg, 1F: λ DNA 10 ng, the others: specimens.

## DISCUSSION

Digoxigenin (DIG), a steroid hapten, is coupled to dUTP via an alkali-labile ester-bond. For DNA labeling, DIG-11-dUTP was labeled into HBx-DNA strands. DIG-labeled HBx-DNA probe was used for hybridization to membrane blotted nucleic acids and the hybridized probe was immunodetected with anti-digoxigenin-AP and then was visualized with the colorimetric substrates NBT/BCIP. HBx-DNA probe prepared by us can detect 1.0 pg homologous DNA and had no reaction with heterologous  $\lambda$  DNA which indicated a high sensitivity and specificity. The labeling process need no expensive equipment and without radioactive pollution. In this study, the HBx gene integration of integrated type HCC were detected using HBx-DNA probe, and HBx-DNA probe was used to observe the transcription ratio of integrated HBx-DNA. All these may provide some fundamental data for further research of hepatocarcinogenesis.

In our study, 75% HCC patients were found to be infected with HBV previously. Of all HBV-DNA positive HCC specimens, the integrated type and integrated + free type covered 63.6% and 36.4%, which indicated that HBV-DNA mainly integrated into cell chromosomes. Robinson *et al*<sup>[6]</sup> drew the similar conclusion. With PCR technique, Unsal H *et al*<sup>[7]</sup> studied the HBx existence in 80 HCC cases from Europe, Asia and Africa. The results showed that 78% cases had HBx-DNA including 28 cases from China. In this study, using intact HBV-DNA probe by Southern blot hybridization, integrated type HCC was selected first, and then integrated HBx-DNA was detected from the selected specimens. Excluding the possibility of pseudopositive results caused by free type HBV-DNA existing in plasma, our study can reflect HBx-DNA integration status exactly and efficiently. Of the integrated type HCC, 90.5% showed HBx-DNA integration, which suggest the important role of HBX gene in the development of HCC. On the other hand, the difference among HBV subgenic integration percentage may indicate that HBV-DNA integration was fragmental and always accompanied

by virus DNA deletion.

It was reported that the integrated HBx gene can be transcribed as temple and hence guided HBx protein synthesis<sup>[8]</sup>. By now, more and more evidences have demonstrated that HBx protein has a transactivation function and integrated HBx-DNA or recombinated one with flank chromosome sequences has even stronger transactivation function than wild type one. Benn J *et al*<sup>[9]</sup> discovered that HBx protein deregulated checkpoint controls and consequently promoted the G0/G1 and G2/M shift of cell cycle by activating ras or other unknown factors. Greenblatt MS *et al*<sup>[10]</sup> found that HBx protein can interact with p53 and suppress normal function of p53 protein. All these findings indicated that HBx function and the interaction among HBx protein, oncogene and tumor suppressor gene may play important roles in the development of HCC associated with HBV infection. Obviously, HBx mRNA transcription and its relationship with oncogene activation and tumor suppressor gene inactivation should be focused in further researches.

**Acknowledgments:** We would like to thank Professor Liu CB for providing us the recombinant plasmid pBR322-2HBV.

## REFERENCES

- 1 Kekule AS, Lauer U, Weiss L, Luber B, Hofschneider PT. Hepatitis B virus transactivator HBx uses a tumor promoter signalling pathway. *Nature*, 1993; 361(6414):742-745
- 2 Twu JS, Lai MY, Chen DS, Robinson WS. Activation of protooncogene C-jun by the x protein of hepatitis B virus. *Virology*, 1993;192(1):346-350
- 3 Cao YN, Sun WS, Song J, Wang DF, Zhang LN, Cao YL. The preparation of HBV-DNA probe labeled with digoxigenin. *Acta Acad Med Shandong*, 1993; 31(4):279-282
- 4 Wang SW. Gene diagnostic technique. Beijing: *Beijing Medical University and PUMC Press*, 1993:70-72
- 5 Sambrook J. Molecular cloning. 2nd ed. New York: *Cold Spring Harbor Laboratory Press*, 1992
- 6 Robinson WS. The role of hepatitis B virus in development of primary hepatocellular carcinoma: Part II. *J Gastro Hepat*, 1993;8(1):96-105
- 7 Unsal H, Yakicier C, Marçais C, Kew M, Volicmann M, Zentgraf H *et al*. Genetic heterogeneity of hepatocellular carcinoma. *Proc Natl Acad Sci USA*, 1994;91(2):822-826
- 8 Wang WL, London WT, and Feitelson MA. Hepatitis B x antigen in hepatitis B virus carrier patients with liver cancer. *Cancer Res*, 1991;51(18):4971-4977
- 9 Benn J, Schenider RJ. Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls. *Proc Natl Acad Sci USA*, 1995;92(24):11215-11219
- 10 Greenblatt MS, Bennett WP, Hallstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res*, 1994;54(18):4855-4878

# Expression and kinetic changes of alkaline phosphatase and its isoenzymes in experimental rat hepatoma

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**Subject headings** liver neoplasms/enzymology; alkaline phosphatase/analysis; alkaline phosphatase isoenzymes/analysis; disease models, animal; 2-fluoenylacetamide/analysis

## Abstract

**AIM** To explore the expression and changes of hepatoma specific alkaline phosphatase (ALP) in rats during canceration.

**METHODS** The ALPs and isoenzymes of rat livers and sera were investigated in SD hepatomas induced with 0.05% 2-fluoenylacetamide (2-FAA).

**RESULTS** By pathological examination and biochemical analysis. ALPs were overexpressed in rat livers during canceration and then were secreted into blood. Serum total ALP activities, liver ALP specific activities (U/g) including soluble and membrane-combined ALP activities of each group were all significantly higher ( $P < 0.01$ ) than those of control group. The average ratios of soluble ALP to membrane-combined ALP were increased significantly after 6 weeks. ALP isoenzymes of rat sera and livers showed 5 bands on PAGE: ALP-I and ALP-II were specific for normal liver and rat hepatoma tissues, the ALP-II appeared in rat liver after 6 weeks and in sera after 8 weeks.

**CONCLUSIONS** ALP with carcino-embryonic protein was overexpressed in hepatoma tissues; the abnormal ALP-II of ALP isoenzymes in sera and liver of rats can be used as a tumor marker for early diagnosis of rat hepatoma.

## INTRODUCTION

Alkaline phosphatase (ALP) (EC, 3.1. 3.1) may separate several isoenzymes bands on Polyacrylamide Gel Electrophoresis (PAGE) and Starch Gel Electrophoresis. The ALP-I of nearly anode (also called hepatoma specific ALP) can diagnose hepatoma with a specificity of 100% clinically. It is the characteristic of hepatoma of fibrolamellar type. ALP-I was not related to patient age, sex and total ALP activities, being positive when AFP was in low concentration, which has drawn great attention in clinical practice<sup>[1-3]</sup>. But the expression mechanism of the band is still unclear in tumorigenesis. We used a chemical carcinogen, 2 Fluoenylamide (2-FAA), to feed Wistar rats so as to examine the expression and kinetic changes of ALP and isoenzymes during carcinogenesis of rat hepatoma.

## MATERIALS AND METHODS

### *Animal model*

Forty-eight male Wistar rats weighing 140g-180g were obtained from Shanghai Experimental Animal Centre. The rats were divided into 8 groups at random. Each group had 6 animals. One of these groups was randomly chosen as the control group. Control group was fed with ordinary fodder, and experimental groups were fed with 0.05% 2-FAA. One group was killed every two weeks respectively after feeding. Peripheral blood was collected for biochemical assay and a part of the liver was used for histopathological examination.

### *Liver homogenate preparation*

Fresh rat liver was washed in 0.9% NaCl solution, dried with filter and cut into pieces. Two parts (A and B) of 1g wet tissue were weighed respectively. Five ml homogenate solution (pH 8.6, 0.1mol/L Tris-HCl buffer solution) was added to part A for purified soluble ALP; and 5ml homogenate solution with 0.5% Trito X-100 to part B for purified soluble and membrane-combined ALP. They were then put in ice bath, homogenate was produced on YQ 3 homogenizer at 12 000×g, stopped for 5 minutes every 30 seconds and repeated for five times, then centrifuged for 45 minutes at 15000×g, the

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**Project supported by National Education Ministry.**

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**Received** 1998-04-01

supernatant was kept at  $-20^{\circ}\text{C}$ .

### Enzymes activity and isoenzyme analysis

The ALP activities (U/L) of rat livers and sera were measured by the method of Disodium-phenyl-orthophosphate and the protein of liver homogenate was analyzed by Lorry's method, and homogenate ALP's ratio activities (U/g) were calculated. For the sake of convenience in analysis, soluble and membrane-combined ALP activities and their ratios were also calculated. ALP isoenzymes of the livers and sera were analysed with the improved Stage Polyacrylamide Gel Electrophoresis (4%, 7.8%, 11.8%) and were named ALP I-V band in turn from anode to cathode<sup>[4]</sup>.

## RESULTS

### Histopathological findings

Table 1 shows the results of histological examination of experimental rat livers with H.E. staining. From the second week, precancerous lesions occurred in all livers and from the fourth week 1/3 of the livers presented with precancerous lesions and 2/3 of the livers had canceration and from the 6th to 12th week, all the rat livers were found to have canceration. At the precancerous stage of the experimental group, most of the rat liver tissues demonstrated normal tissue structure, a few of hyperplastic oval cells and the tendency of nodule formation were seen. The cancerous tissues indicated that the structure of hepatic lobules was destroyed, and necrosis spread all over the rat livers and a large number of small oval cells and cancer nest lobules were observed. In the experimental rats fed with 2-FAA from the 6th to 12th week, all the rat livers were seen to have canceration and the morphology of hepatic cells showed highly differentiated hepatocellular carcinoma.

**Table 1 Results of histological examination of rat livers**

Groups	n	Precancerosis	Canceration
Control group	6	0	0
2nd week	6	6	0
4nd week	6	2	4
6nd week	6	0	6
8nd week	6	0	6
10nd week	6	0	6
12nd week	6	0	6

### Kinetic changes of rat serum and liver ALP

The kinetic changes of ALP activities in sera and livers during canceration are shown in Table 2. A lot of ALPs were expressed after 2-FAA was given, ALP activity of the experimental group was much

higher than that of control group after 2 weeks ( $P<0.01$ ), from the 4th to 10th week it kept rising, although decreased a little to the twelfth week, it still remained at a high level. The total ALPs, soluble and membrane-combined ALP activities of liver homogenate in the experimental groups were also higher than those of the control group. The ratios between the soluble ALP and membrane-combined ALP in each experimental group were much higher than that of the control group.

**Table 2 Kinetic changes of ALP of rat sera and livers**

Groups	n	Serum ALP (U/L)	ALP of homogenate (U/g)			
			B (Total ALP)	A (Soluble)	B-A (Membrane combined)	A/B-A
Control group	6	9.2±4.1	16.8±2.7	7.5±3.5	9.3±4.2	0.81
2nd week	6	22.0±6.3 <sup>a</sup>	57.8±8.2 <sup>a</sup>	24.9±7.2 <sup>a</sup>	32.9±6.5 <sup>a</sup>	0.76
4nd week	6	26.2±20.9 <sup>a</sup>	56.2±31.7 <sup>a</sup>	25.7±13.9 <sup>a</sup>	30.4±21.1 <sup>a</sup>	0.85
6nd week	6	25.0±8.0 <sup>a</sup>	127.5±43.9 <sup>a</sup>	72.1±32.4 <sup>a</sup>	55.4±22.9 <sup>a</sup>	1.30 <sup>a</sup>
8nd week	6	32.8±4.0 <sup>a</sup>	73.1±11.8 <sup>a</sup>	51.2±6.8 <sup>a</sup>	21.9±6.5 <sup>a</sup>	2.34 <sup>a</sup>
10nd week	6	33.3±3.1 <sup>a</sup>	77.6±20.3 <sup>a</sup>	52.1±7.1 <sup>a</sup>	25.5±14.5 <sup>a</sup>	2.04 <sup>a</sup>
12nd week	6	27.0±2.9 <sup>a</sup>	60.5±4.1 <sup>a</sup>	38.0±6.9 <sup>a</sup>	22.5±6.6 <sup>a</sup>	1.69 <sup>a</sup>

<sup>a</sup> $P<0.01$  compared with control group.

### Changes of ALP isoenzymes of sera and livers

The electrophoresis analysis of ALP isoenzymes of livers and sera during canceration is shown in Table 3. ALP isoenzymes showed 5 bands on PAGE, band V still stayed where samole was added, band IV was situated at the boundary of condensation gel and separate gel, in band I electrophoresis speed was the fastest, it was a bit nearer to anode than band II. Electrophoresis results are as follows: band I was not found in rat sera but found in liver homogenates of the control group and the experimental group in the 2nd and the 4th week, but in the 4th week only one sample of the experimental group was detected. Band II was seen in liver homogenates from the 6th week and in the sera from the 8th week, six samples were found to have deeply stained band II in the sera and the liver homogenates in the 10th and the 12th week, and bands III, IV and V all could be detected from the sera and the liver homogenates of both the control and the experimental groups, and the isoenzyme results were the same.

**Table 3 Changes of ALP's isoenzymes**

Groups	n	Serum (liver tissues) ALP isoenzyme				
		I	II	III	IV	V
Control group	6	0(6)	0(0)	6(6)	6(6)	6(6)
2nd week	6	0(6)	0(0)	6(6)	6(6)	6(6)
4th week	6	0(1)	0(0)	6(6)	6(6)	6(6)
6th week	6	0(0)	0(4)	6(6)	6(6)	6(6)
8th week	6	0(0)	4(6)	6(6)	6(6)	6(6)
10th week	6	0(0)	6(6)	6(6)	6(6)	6(6)
12th week	6	0(0)	6(6)	6(6)	6(6)	6(6)

## DISCUSSION

Various hepatoma-related proteins, polypeptides and isoenzymes can be synthesized and secreted, such as AFP, GGT-II, etc. in hepatoma tissues. When the genes which control ALP synthesis is expressed abnormally, it will produce and secrete a few- tumor-associated ALP such as ALP-I, Nagao, Regan, Kasahara, Warnock isoenzymes, etc. There is no ALP-I in healthy persons or patients with non-malignant hepatopathy, its specificity in diagnosis of hepatoma is 100%, but the sensitivity is low, probably due to the detection method. Its positive rate in diagnosis of hepatoma is 18% in Europe and American, 31% in Japan and about 10%<sup>[4,6]</sup> in China. To understand the expression and mechanism of hepatoma ALP, the expression of ALP and abnormal changes of isoenzyme were observed during carcinogenesis of rat hepatoma induced with 2-FAA.

In the early stage of the rat liver carcinogenesis induced by chemical carcinogens, oval cells were observed in the histopathological examination and non-differentiation was revealed in morphology. This may be relevant to the cytotoxic effects of liver cells in resisting carcinogen, and in the meantime, the ALP activities of liver tissues and sera of the experimental groups were obviously higher than the normal control group ( $P < 0.01$ ), indicating that the oval cells can express ALP and secrete into blood. ALP activities increased continuously and the highest in the experimental groups, and the livers of the experimental groups in the 6th week were seen to have canceration. In the following weeks, the liver tissues still synthesized a lot of ALP, but the activities became a bit lower than that in the 6th week, however, they still remained at a high level.

Tissue ALP has two types: soluble type and membrane-combined type. Soluble ALP could be purified only with homogenate solution without surface activity agent, if added surface activity agents (such as Triton X-100, deoxycholic acid, etc), membrane-combined ALP could be isolated, which constituted tissue total ALP with soluble ALP. Their average ratios (Table 2) reflected the tendency of simultaneous increase in the early stage of carcinogenesis (the 2nd and the 4th week). But it was proved by histology that liver tissues which began to have canceration from the 6th week synthesized a large quantity of soluble ALP. The ratios of the soluble and the membrane-combined ALP were obviously higher, probably due to the destroyed structure of hepatic lobules in the later stage of canceration and the large quantity of secretion of the enzyme to the blood after necrosis of liver cells.

According to the family system theory of ALP isoenzyme, hepato-type ALP is controlled by tissue

non-specific genes. Cancer induced by chemical carcinogens is a course with multiple stages, the primary reason for this is that the carcinogens induce the activation of the original-cancer genes and/or the inactivation of repressive-cancer genes. It can make the genes controlling ALP synthesis express abnormally, producing ALP which can be shown on electrophoresis<sup>[7,8]</sup>. ALP of rat liver is different from that of human liver. Only 5 bands were shown on electrophoresis: normal rat liver is bands I, III, IV and V, and the latter 3 bands in the serum. In the early stage of carcinogenesis, ALP-I of liver tissue began to disappear, in the 6th week band ALP-II appeared; in the 8th week ALP-II appeared in sera, after that, it appeared with liver tissues simultaneously. In the whole process of carcinogenesis, bands III, IV and V had no obvious changes except for increased enzyme activities. ALP-II produced hepatoma, and secreted to blood, then formed soluble ALP, which was only found in the liver tissue in early stage of carcinogenesis and detected in sera in late stage of carcinogenesis. From these, we can infer that clinically, liver biopsy during the early stage of canceration or in the sera of hepatoma produced may detect hepatoma-specific isoenzymes.

It suggested that the rat hepatoma tissues can express abnormal ALP isoenzyme bands in the animal model, and all of them were positive in the late stage of carcinogenesis. It still remains unknown whether ALP-II is synthesized by hepatoma tissues or degradation of ALP-I in normal liver tissues. Clinically although the specificity of hepatoma diagnosis is high, the positive rate of the appearance of ALP-I in human sera is not so desirable at present<sup>[4]</sup>, and much lower than the results of animal model, and the number of bands does not coincide with human being either. It is suggested that ALP varied among different races. In addition, the cause of human hepatoma is associated with many factors. ALP-I could become a valuable marker for diagnosis of hepatoma if the sensitivity of the detection method is improved.

## REFERENCES

- 1 Fishman WH. Alkaline phosphatase isoenzyme, recent progress. *Clin Biochem*, 1990;23(2):99
- 2 Yao DF, Men XY. Detection and clinical significance of tumour-relative ALP isoenzyme. *Tumour*, 1995;15(1):49
- 3 Fei SJ, Men XY, Ge ZL. Significance of ALP isoenzyme diagnosis in hepatobiliary diseases. *CJCH*, 1995;11(1):10
- 4 Yao DF, Yang ZH. Separation of ALP isoenzymes by a stage polyacrylamide gel electrophoresis. *J Nantong Med Coll*, 1983;3(1):136
- 5 Fishman WH. Clinical and biological significance of an isozyme tumor marker-PLAP. *Clin Biochem*, 1987;20(6):387
- 6 Smith A. Multiple marker screening in primary liver cancer (PLC). *J Hepatol*, 1986;3(suppl 1):19
- 7 Wan B, Ke Y. Chemical carcinogens and cancer-genes and repressive-cancer genes. *Tumour*, 1995;15(2):107
- 8 Koyama I, Miura M, Matsuzaki H, Sakagishi Y, Komoda T. Sugar chain heterogeneity of human alkaline phosphatase: differences between normal and tumour-associated isozymes. *J Chromat*, 1987;413:65

# Orientated thermotherapy of ferromagnetic thermoseed in hepatic tumors

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**Subject headings** liver neoplasms/therapy; eletromagnetic field; thermotherapy; liver neoplasms/pathology

## Abstract

**AIM** To study the thermotherapeutic effects of implanted ferromagnetic thermoseeds in high frequency electromagnetic field in hepatic tumors.

**METHODS** The ferromagnetic thermoseeds made of nickel-copper alloy, which has a lower Curie temperature, were implanted into hepatic tumors of mice. The high frequency electromagnetic field was then applied in vitro to make the ferromagnetic thermoseeds produce the hyperthermia. Before and after thermotherapy, the tumor size, pathologic alteration and animal survival period were assessed.

**RESULTS** The temperature at the central area of the tumor could be heated up to 50°C. Most of tumors in mice disappeared with a large amount of tumor necrosis. The survival period of mice was prolonged.

**CONCLUSION** This thermotherapy is beneficial to directional selection and temperature control for treatment of hepatic tumors.

## INTRODUCTION

The problem of selected hyperthermia for tumors has not been well solved so far. In this paper the orientated heating technique of implanted ferromagnetic thermoseed induced by high frequency electromagnetic field was evaluated in mice hepatic tumors.

## MATERIALS AND METHODS

Thirty-eight female Kunming mice (provided by Xuzhou Medical College) weighing 18 to 22 g were used. Hepatic tumor models were made by subcutaneous inoculation of H<sub>22</sub> cells (provided by Shanghai Institute of Materia Medica, Academia Sinica) to the back of the mice. Tumors were allowed to grow for 1 week. When the tumors were measured to be as large as 10 mm in diameter in 1 week, the animals were used for experiment. Part of them were used to observe the temperature change during the heating of tumors and the histopathological changes after the tumors were heated. The others were divided at random into four groups to observe the growth of tumors and the survival period of the animals.

Untreated control group (G<sub>1</sub>). Tumor-bearing animals received no treatment.

Thermoseed group (G<sub>2</sub>). The tumors of the mice were implanted with two ferromagnetic thermoseeds which were 1cm in length in parallel.

Electromagnetic group (G<sub>3</sub>). Tumor-bearing animals were exposed to high frequency electromagnetic field for 60 minutes.

Heat-treated group (G<sub>4</sub>). G<sub>4</sub>=G<sub>2</sub>+G<sub>3</sub>, tumor-bearing animals were kept in a position in which the thermoseed axes must be aligned with the direction of the magnetic field.

High frequency electromagnetic field was produced by the induction coil of our "heating apparatus". Megnetic field strength at the center of the coil was 5 mT and the frequency was 200 kHz. Water was supplied for cooling the coil during the induced heating.

Ferromagnetic thermoseed (provided by Beijing Science and Technology University) 1 mm in diameter was made of nickel-copper alloy. Induced heating of the seed by eddy current would generate when the seed was exposed to electromagnetic field. Then the seed of this alloy had its Curie point at 60°C. When the seed was heated up to the Curie

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**Supported in part by the Youth Medical Scientific Foundation of Chinese Ministry of Public Health, No.90207 and the Natural Scientific Foundation of Jiangsu Province, No.90058.**

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Received 1998-03-08

point in the magnetic field, the seed would lose its ferromagnetic properties, thereby providing an automatic temperature regulative effect.

A laboratory-built thermometer was used to measure the temperature during hyperthermia. The sensor of the thermometer was made of thermocouple with the error of +/- 0.5°C. And the sensor was shaped in needle, therefore it could be inserted into tissue directly for measuring the temperature.

The diameter of the tumors was measured 2-dimensionally using a sliding caliper. The average size of a tumor was expressed by the product of maximum diameter and minimum diameter. The statistical differences were assessed using Student's *t* test.

**RESULTS**

Thermoseed-induced heating at the centre of tumors occurred rapidly after the magnetic field was applied. To heat the tumor up to 42°C, the effective therapeutic temperature in tumor hyperthermia, only 5min was needed. The maximum temperature to 50°C was recorded in 30 min and was stabilized in subsequently continuous heating. The temperature of the boundary between tumor and normal tissues increased less rapidly and maintained at 39°C (Table 1).

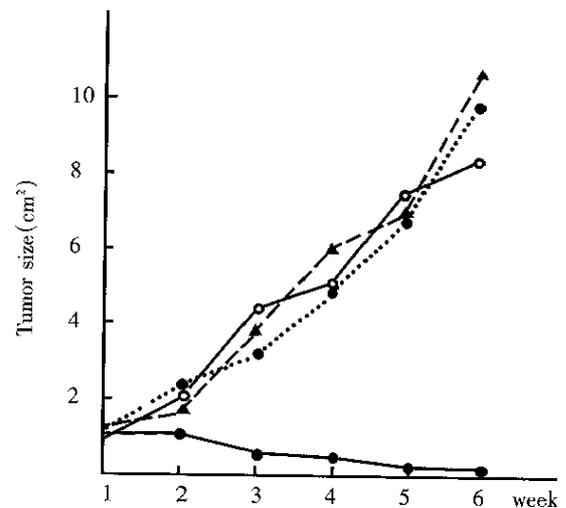
**Table 1 Temperature changes at the centre and boundary of the tumors ( $\bar{x}\pm s$ , °C)**

Heating time (min)	Centre of tumor	Boundary of tumor
5	42.93±1.10	34.91±0.81
10	46.90±1.14	35.41±1.00
20	47.38±1.52	35.94±1.03
30	50.02±2.46	38.13±1.42
40	49.67±2.68	38.92±2.33
50	49.74±3.12	39.22±2.94

Coagulation necrosis occurred in the tumors after thermal injury. Tumor tissues for the light microscopic examination were prepared in 1 week after the induced heating was performed. The sections showed that there were extensive necrosis and infiltration of inflammatory cells appeared in the peripheral region.

In all groups of animals, there was no significant difference in tumor size before thermotherapy. In the heat-treated group ( $G_4$ ,  $n = 7$ ) the tumor began to decrease in size in 2 weeks after the induced heating and disappeared in 5 of 7 animals 5 weeks later. And the tumor growth in the remaining two animals was significantly delayed. Meanwhile, the tumor in the other 3 groups ( $G_2$ ,  $G_3$ ,  $G_4$ ) grew progressively (Figure 1). By the time

of 5 weeks, the tumor size of  $G_1$ - $G_4$  was  $9.74\text{cm}^2\pm 4.72\text{cm}^2$  ( $n = 8$ ),  $10.71\text{cm}^2\pm 5.16\text{cm}^2$  ( $n = 8$ ),  $8.51\text{cm}^2\pm 3.42\text{cm}^2$  ( $n = 8$ ) and  $0.15\text{cm}^2\pm 0.17\text{cm}^2$  ( $n = 7$ ). The difference was significant in tumor size between heat-treated groups and the control groups ( $P<0.01$ ). The survival time in 4 groups is shown in Table 2. All of the animals in the 3 control groups died within 10 weeks. While the survival time of the heat-treated animals was markedly prolonged as compared with the control ( $P<0.01$ ). Five of seven animals of the heat-treated group were still alive at the end of experiment with no evidence of tumors.



**Figure 1** Changes of  $H_{22}$  tumor growth after thermotherapy. ---●---Untreated control group ( $G_1$ ) ---▲---Thermoseed groups ( $G_2$ ) ---○---Electromagnetic group ( $G_3$ ) —●— Heat-treated group ( $G_4$ )

**Table 2 Survival period of mice after thermotherapy ( $\bar{x}\pm s$ )**

Groups	<i>n</i>	Survival time (d)
Untreated control group	8	50.1±12.2
Thermoseed group	8	38.6±8.8
Electromagnete group	8	46.0±12.1
Heat-treated group	7	123.1±45.9 <sup>a</sup>

<sup>a</sup> $P<0.01$  vs untreated control group.

**DISCUSSION**

Traditional hyperthermia methods are limited in the treatment of hepatic tumors. Many problems, such as over-heating of surface tissue and reflection from tissue-air interface, are often encountered with external microwave or ultrasound heating techniques. Interstitial technique, on the other hand, produces heat by means of directly implanting devices into the target area. Although invasive, interstitial source usually can localize heating in a tumor and minimize thermal damage to the surrounding normal tissues. Implanted electrodes

and microwave antennas are used most frequently in interstitial techniques. However, the need for connecting the implanted devices to a power source and the invasive temperature monitoring limit the practice of these methods.

In recent years, thermoseed method for the brain tumors was reported in literatures<sup>[1-6]</sup>. The clinical trials described in tumor-bearing animals and patients have shown the thermoseed technique to be a promising method in tumor hyperthermia. In an effort to obtain orientated hyperthermia for hepatic tumors, we investigated the effects of thermoseed heating on subcutaneous hepatic tumors (H<sub>22</sub>) as the first step of our studies. This preliminary report suggests that the method is beneficial to heating selection and temperature control for treatment of hepatic tumor. When thermoseed is implanted into tumors, it can absorb energy from an externally applied electromagnetic induction field and act as a "seed in heating" within the tumors, thus make the localized heating of deep-seated hepatic tumor possible. Automatic temperature regulation is another advantage in this method. Because of its lower Curie temperature, thermoseed has a function of self-regulating in the electromagnetic induction heating so as not to

damage the surrounding normal tissues.

However, it is necessary to investigate how to warm relatively a large volume of tumor tissues before clinical applications. In our another experiment on rabbit (to be published), we found that when the renal artery was ligated during thermoseed hyperthermia, the temperature of the kidney increased by 10°C. Obviously, the cooling effect by blood flow can not be ignored. Therefore, the combination therapy of hyperthermia and ligation or embolization of tumor supply artery may be beneficial to the treatment of large, deep-seated tumors.

## REFERENCES

- 1 Kobayashi T, Kida Y, Matsui M, Amemiya A. Interstitial hyperthermia of malignant brain tumors using implant heating system (IHS). *No Shimkei Geka*, 1990;18(3):247-252
- 2 Mark CF, Stea B, Kittelson JM, Shimm DS, Sneed PK, Phillips TL *et al*. Interstitial thermoradiotherapy with ferromagnetic implants for locally advanced and recurrent neoplasms. *Int J Radiat Oncol Biol Phys*, 1993;27(1):109-115
- 3 Brezovich IA, Atkinson WJ, Chakraborty DP. Temperature distributions in tumor models heated by self-regulating nickel-copper alloy thermoseeds. *Med Phys*, 1984;11(2):145-152
- 4 Paulus JA, Molloy JA, Ritter RC, Broaddus WC, Grady MS, Quate EG *et al*. Evaluation of inductively heated ferromagnetic alloy implants for therapeutic interstitial hyperthermia. *IEEE Trans Biomed Eng*, 1996;43(4):406-413
- 5 Indik JH, Indik RA, Cetas TC. Fast and efficient computer modeling of ferromagnetic seed arrays of arbitrary orientation for hyperthermia treatment planning. *Int J Radiat Oncol Biol Phys*, 1995;30(3):653-662
- 6 Chin RB, Stauffer PR. Treatment planning for ferromagnetic seed heating. *Int J Radiat Oncol Biol Phys*, 1991;21(2):431-439

# Effect of endotoxin on fibronectin synthesis of rat primary cultured hepatocytes

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**Subject headings** liver cirrhosis; endotoxins; fibronectin/analysis; liver/cytology; cells, cultured; lipopolysaccharide

## Abstract

**AIM** To investigate the effect of endotoxin on liver fibrosis and further define the role of hepatocytes in production of fibronectin in primary liver cell culture by endotoxin.

**METHODS** After isolation and seeding of hepatocytes, the obtained cells were added to various doses (0, 5, 10, 15 and 20 mg/L) of LPS treated culture media. The cells were collected and counted at various periods (0, 12, 24, 48, 72, 96, 120 h). The concentrations of fibronectin were tested by electrophoresis.

**RESULTS** The fibronectin levels tended to increase with prolongation of culture time. There was a sharp increase after 72 h in 10 or 15 LPS treated group. The peak level of fibronectin was above 20 mg/L. However, cell proliferation was inhibited during the course. Cell number of untreated control group ( $4.6 \pm 0.1 \times 10^6$ ) was about three fold that of 20 LPS treated group ( $1.6 \pm 0.2 \times 10^6$ ) at 120 h.

**CONCLUSION** Hepatocytes have a potent ability to produce fibronectin stimulated by endotoxin, suggesting that hepatocytes might participate in the process of liver fibrosis.

## INTRODUCTION

Endotoxin may contribute to the pathogenesis of several disease processes, including thermal injuries, bowel diseases, pancreatitis and septic syndrome<sup>[1]</sup>. Previous experiments proved that gut-derived endotoxemia was a hallmark of hepatic failure<sup>[2]</sup>. As an essential presupposition of chronic hepatic failure, liver fibrosis was also directly associated with gut-derived endotoxemia<sup>[3]</sup>. Besides Ito cells, Kupffer cells, endothelial cells and biliary epithelial cells, some experiments in vivo or in vitro indicated that liver parenchymal cells participated in the process of liver fibrosis<sup>[4]</sup>. Fibronectin, a component of extracellular matrix, arises at the early stage of liver fibrosis. In this study, we observed the alteration of cell population and fibronectin secreted by primarily cultured rat hepatocytes in response to lipopolysaccharide (LPS).

## MATERIALS AND METHODS

### *Isolation and culture of hepatocytes*

Hepatocytes were isolated from Wistar rats (250g-300g in weight, fasted overnight) by the collagenase method of Gressner<sup>[5]</sup>. The liver was preperfused with D-Hank's solution containing 0.2% glucose for 10min at a flow rate of 10 ml/min to wash out the blood. Then it was perfused with D-Hank's solution containing 0.05% collagenase (Collagenase type I, Sigma) for another 10min. After removal, the liver was cut into tiny pieces. Passing through a stainless steel mesh (mesh size: 100  $\mu$ m), hepatocytes were purified by centrifugation ( $50 \times g$ , 1min $\times$ 2, 4 $^{\circ}$ C). The viability of the final parenchymal cell suspension, checked by trypan blue exclusion, was between 90% and 95%. Cell recovery was about  $2 \times 10^8$  cells/liver and contamination with non-parenchymal cells was less than 1%. Cells were seeded at a density of  $30 \times 10^6$  cells/10cm culture dish (Corning, N.Y.) and cultured at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in 10ml RPMI-1640 (Gibco) containing 10% fetal bovine serum, 0.02U/ml insulin, penicillin (100 IU/ml) and streptomycin (100  $\mu$ g/ml). The medium was changed 3 h after plating, during which non-adherent and non-vital cells were removed. Lipopolysaccharide

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**Supported by the Science and Technology Development Foundation of Shanxi Provincial Education Commission (No. 9725) and the Scientific Foundation of Ministry of Public Health, No.2179412.**

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Received 1997-08-26

(*Escherichia coli*, 0111B4, Sigma) was added to hepatocyte culture media at various concentrations of 0, 5, 10, 15 and 20 mg/L. Finally, the number of cells per dish were counted at 0, 12, 24, 48, 72, 96 and 120 h. The morphology of hepatocytes was observed under microscope.

#### Determination of cellular fibronectin

Rocket electrophoresis was employed to determine the fibronectin concentration in the culture medium of hepatocytes. Gelatin was made from 0.05M pH 8.2 sodium barbiturate-HCl buffer containing 2% agarose and 1% fibronectin antibody (Shanghai Biologic Products Research Institute). Time of electrophoresis was limited within 6 h. Voltage and current intensity were controlled respectively at 80 v and 100 mA. The final numerical value was determined by linear regression according to peak-shift length of fibronectin standard ladder concentrations.

#### Statistical analysis

The results were expressed as mean $\pm$ SD. Variance analysis and *q* test were used to determine the statistical significance by SPSS/PC + 4.0 general software. When the *P* value was less than 0.05, the result was considered statistically significant.

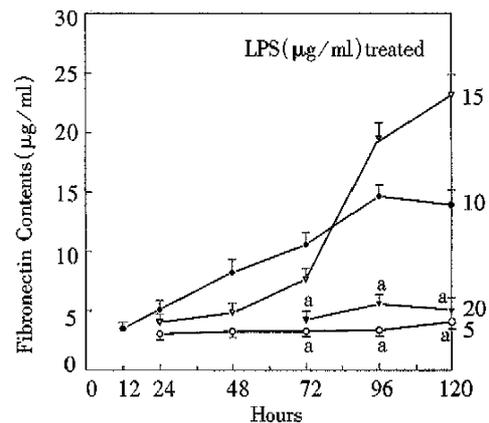
## RESULTS

#### Alteration of cellular fibronectin

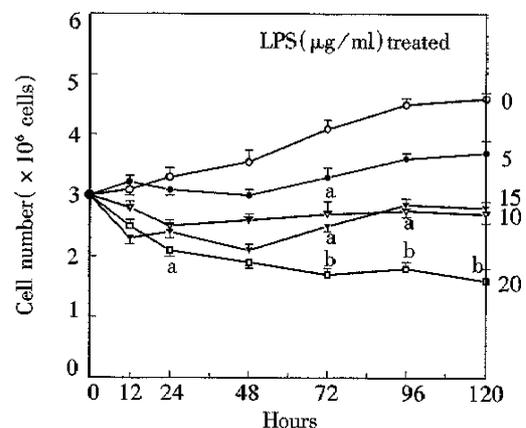
The present experiment demonstrated the effect of LPS on cellular fibronectin. As shown in Figure 1, in 10 and 15 mg/L LPS treated groups, fibronectin tended to increase progressively, especially after 72 h. However, the fibronectin concentrations of 5 and 20 mg/L LPS treated groups varied slightly, but were significantly different from those of 10 and 15 mg/L groups (*P*<0.05). It should be noted that fibronectin concentration of 0 mg/L LPS treated group could not be tested by the present means. So did it in 5 and 10 mg/L LPS treated group before 24 h, 15 mg/L group before 12 h, and 20 mg/L group before 72 h.

#### Changes of hepatocytes population

As shown in Figure 2, proliferation of cultured hepatocytes was remarkably inhibited in all LPS treated groups, especially in 20 mg/L group. Hepatocyte population of untreated control group increased progressively. After 72 h, multiplication rate in all groups was accelerated. Hepatocyte population of 10, 15, or 20 mg/L was significantly less than that of control group. Population of control group ( $4.6\pm 0.1\times 10^6$ ) was approximately three-fold that of 20 mg/L LPS treated group at 120 h.



**Figure 1** Effect of various doses of LPS on cellular fibronectin concentrations in primary hepatocytes culture. Mean  $\pm$  SD of six groups are given. <sup>a</sup>*P*<0.05 compared with 10 or 15  $\mu$ g/ml LPS treated group.



**Figure 2** Effect of various doses of LPS on cell population in primary hepatocytes culture. Mean  $\pm$  SD of six groups is given. <sup>a</sup>*P*<0.05 compared with 0 or 5 mg/L LPS treated group. <sup>b</sup>*P*<0.05 compared with 10 or 15 mg/L LPS treated group.

#### Morphological observation in cultured hepatocytes

In morphological study, appearance of hepatocytes exhibited a slight difference if they were cultured at various concentrations of LPS. Almost in every cultured dish, binuclear and even trinuclear cells could be observed, which indicated active proliferation in the culture. The higher the LPS levels were, the more hepatocyte lesions exhibited. These pathologic changes included cloudy swelling, hydropic degeneration, and pyknosis or karyolysis. After 72 h, there were cell debris and new products (without defined characteristics) around vital cells.

## DISCUSSION

Fibronectin is a high molecular weight glycoprotein and increases firstly among extracellular matrix proteins during liver fibrosis. The increased serum fibronectin level is now considered as an important

and susceptible marker of fibrosis and necrosis in chronically injured liver<sup>[6]</sup>. Normally, it is very difficult to detect fibronectin because of its low concentration. Thus enhanced fibronectin may serve as an indicator of pathophysiological process. Fibronectin in the liver mainly exists in the portal, septal and perisinusoidal matrix. Most of fibronectin is secreted from mesenchymal cells including Kupffer cell, Ito cell and endothelial cells. In some pathological processes, hepatocytes may secrete fibronectin<sup>[7]</sup>. Until now, no study has shown the direct relation between endotoxin and hepatocytes in the liver fibrosis process. In this study, hepatocytes culture in vitro was used to observe this relationship. In 10 and 15 mg/L LPS treated groups, fibronectin concentrations tended to increase progressively during the whole culture process, especially after 72 h. It may indicate that it is endotoxin that stimulates fibronectin synthesis of hepatocytes. In 5 and 20 mg/L LPS treated group, however, synthesis was not stimulated. Therefore, there may be response limits in which endotoxin acts as a stimulator of fibronectin synthesis. As for control group, hepatocytes did not secrete fibronectin because there was no stimulator.

This study demonstrated that the proliferation of hepatocytes in primary culture was inhibited by endotoxin. There was no positive correlation between fibronectin secretion and hepatocyte proliferation in the first 72 h. Then, the increase of hepatocyte population coincided with that of fibronectin concentration, indicating that there may be refractory period for hepatocytes to secrete fibronectin or proliferate. In terms of morphology,

it may be relevant to degeneration, production of uncertain new protein and nuclear mitoses. Previous studies confirmed that biological behavior of hepatocytes was influenced by some mediators secreted by Kupffer cells, such as tumor necrosis factor, transforming growth factor, etc. In our study, it is presumed that there may be a mechanism to modulate the function of liver cells by some factors secreted from hepatocytes (still unknown in this study) if they are stimulated by endotoxin. According to Roeb's opinion<sup>[8]</sup>, besides playing a controvertible role as initiator and regulator of fibrogenesis, fibronectin acts as a scaffold for other matrix proteins. Enhanced fibronectin secretion of hepatocytes in the culture media containing LPS suggests that endotoxin plays a crucial role in the liver fibrosis. Therefore, hepatocytes may take part in the liver fibrosis which is stimulated by endotoxin

## REFERENCES

- 1 Manthous CA, Hall JB, Samsel BW. Endotoxin in human diseases. *Chest*, 1993;104(5):1572-1581
- 2 Han DW. Advance on hepatic failure mechanism research. *Chin J Hepatol*, 1995;3(3):134-137
- 3 Jia JB, Han DW, Xu RL, Chen XM, Zhao YC, Yan JP *et al.* Role of gut-derived endotoxemia in the pathogenesis of hepatic fibrosis. *Chin J Pathophysiol*, 1998; in press
- 4 Friedman SL. Cellular sources of collagen and regulation of collagen production in liver. *Seminars in Liver Disease*, 1990;10(1):20-29
- 5 Gressner AM, Pfeiffer T. Preventive effects of acute inflammation on liver cell necrosis and inhibition of heparan sulfate synthesis in hepatocytes. *J Clin Chem Clin Biochem*, 1986;24(11):821-829
- 6 Soresi M, Di Martino D, Montalto G, Carroccio A, Ruggeri MI, Bascone F *et al.* Plasma fibronectin in chronic liver diseases. *Recent Prog Med (Italy)*, 1993; 84(9):602-607
- 7 Koukoulis GK, Shen J, Virtanen I, Gould VE. Immunolocalization of cellular fibronectins in the normal liver, cirrhosis, and hepatocellular carcinoma. *Ultrastruct Pathol*, 1995;19(1):37-43
- 8 Roeb E. Fibronectin - a key substance in pathogenesis of liver cirrhosis? *Leber Magen Darm (Germany)*, 1993;23(6):239-242

# Comparative studies on epithelial lesions at gastric cardia and pyloric antrum in subjects from a high incidence area for esophageal cancer in Henan, China \*

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**Subject headings** esophageal neoplasms; stomach neoplasms; gastric cardia/pathology; pyloric antrum/pathology; precancerous lesions; mass survey

## Abstract

**AIM** To investigate the pathogenesis of gastric cardia and the distal part of stomach cancer and to further characterize the histopathogenesis model for gastric cardia cancer from the high-risk population for esophageal cancer.

**METHODS** Mass survey with endoscopic mucosa biopsy and histopathological examination were carried out on 226 subjects aged above 30 years. Three biopsies were collected one each from the middle part of the esophagus, the gastric cardia and the pyloric antrum. The biopsy tissue was fixed with 85% alcohol and paraffin-embedded.

**RESULTS** The incidence of intestinal metaplasia and dysplasia at gastric cardia epithelium was higher than that at the pyloric antrum from the subjects in the same area. And there were high incidences of both esophageal and gastric cardia cancer, but a low incidence of gastric cancer at the distal part of the stomach.

**CONCLUSION** There might be different etiology and pathogenesis of gastric cardia and pyloric cancer at the distal part of the stomach.

## INTRODUCTION

Gastric cardia cancer is one of the most common malignancies in the population at high-incidence areas for esophageal cancer in Henan, China. In spite of the mechanism for this coincidence of high incidence of esophageal and gastric cardia cancer in this special population, the evidence indicated that the pathogenesis and the risk factors of gastric cardia carcinogenesis are quite different from those of distal part of gastric adenocarcinoma. Another characteristic for gastric cardia cancer in this special population is the discordant higher incidence of gastric cardia cancer and much lower incidence of distal part stomach cancer. It appears very important to compare the pathogenesis in gastric cardia and distal part of the stomach cancers, which will facilitate the understanding of the factors and mechanisms of carcinogenesis of esophagus and gastric cardia in this area. The specific aim of this study is to further characterize the pathogenesis model of gastric cardia cancer by comparing the lesions at gastric cardia and pyloric antrum in the symptom-free subjects from the high incidence area for both esophageal and gastric cardia cancer.

## MATERIALS AND METHODS

### Subjects

All the subjects were from Loucun Village, Henan Province, a high incidence area for esophageal cancer, subjects ( $n = 226$ ) aged above 30 years without gastrointestinal symptoms were selected randomly. The biopsied tissues were fixed in 85% alcohol, embedded in paraffin and cut in 5  $\mu\text{m}$  for HE staining.

### Endoscopic biopsy examination

Three biopsies were obtained one each from the middle part of the esophagus, the gastric cardia and the pyloric antrum.

### Histopathological analysis

Histopathological diagnosis for gastric cardia and pyloric antrum epithelia were made according to the previously established criteria. Based on the cell

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Received 1998-07-05

morphology and tissue structure, the epithelia were divided into normal, the epithelial cells differentiated well, without obvious inflammatory cell infiltration; chronic superficial gastritis, an inflammation manifested by mild lymphocyte and plasma cell infiltration; chronic atrophic gastritis, mucosal glandular morphology eradicated partially or completely by connective tissues with

interglandular space infiltrated mainly by plasma cells and lymphocytes; dysplasia, characterized by nuclear atypia with or without architectural abnormalities in the gastric epithelium, but without invasion; and intestinal metaplasia, columnar absorptive cells and mucous goblet cells of intestinal phenotype partially replace portions of the gastric mucosa.

**Table 1** Histological results of gastric cardia and pyloric mucosa biopsy

Sites	Cases	Normal(%)	Chronic superficial gastritis (%)	Chronic atrophic gastritis (%)	Intestinal metaplasia (%)	Dysplasia (%)
Gastric cardia	226	117(51)	54(23)	36(16)	12(7)	8(3)
Gastric pyloria	226	128(57)	43(19)	46(20)	7(3)	2(1)

## RESULTS AND DISCUSSION

The histopathological results of gastric cardia and pyloric mucosa biopsy of 226 subjects from the high incidence area for esophageal cancer are listed in Table 1. The results show that the incidence of intestinal metaplasia and dysplasia in gastric cardia was significantly higher than that in pyloria in the subjects from the same area, although the incidence of chronic superficial gastritis and chronic atrophic gastritis in gastric cardia was similar to that in gastric pyloria. This observation suggested that different factors and mechanism might be involved in the carcinogenesis of gastric cardia carcinoma and pyloric carcinoma in the area, which was consistent with the previous results that there was a high incidence of gastric cardia cancer, but low incidence of distal part of stomach cancer in the high incidence area for esophageal cancer.

Gastric adenocarcinoma is one of the most common malignant diseases in China and was previously considered as the cancer occurring at the distal parts of the stomach and the adenocarcinoma occurring at gastric cardia was usually included, and classified into the type of esophageal cancer. However, the pattern of gastric cancer has changed over the past decades. In contrast to the decrease in cancer incidence from the distal part of the stomach, the adenocarcinoma incidence from the gastric cardia and the esophageal and gastric cardia junction increased dramatically, especially in western countries<sup>[1-4]</sup>. This phenomenon was particularly obvious in the high-incidence area for esophageal cancer in northern China. Retrospective

analysis for esophageal and gastric cardia cancer occurrence in Linzhou City (originally known as Linxian) from 1987-1995 indicated that gastric cardia cancer accounted for 45% of all the cancers from digestive tract, and esophageal cancer 55%<sup>[5]</sup>.

It is worth emphasizing that both the incidences of esophageal and gastric cardia carcinomas were higher among the subjects from the high-incidence area for esophageal cancer, while the incidence of distal gastric carcinoma was very low. It suggested that different factors and mechanism of carcinogenesis were involved in these two kinds of tumors. Recent reports showed that the infection of *Helicobacter pylori* was different between the gastric cardia and pylori. Therefore, further studies of the molecular basis for cardia and pyloria epithelial pathogenesis will be of great importance in the understanding of molecular mechanism and the etiological factors of carcinogenesis in the gastric cardia cancer.

## REFERENCES

- 1 Pera M, Cameron AJ, Trastek VF, Carperter HA, Zinsmeister AR. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. *Gastroenterology*, 1993;104(2):510-513
- 2 MacDonald WG, MacDonald JB. Adenocarcinoma of the esophagus and/or gastric cardia. *Cancer*, 1987;60(7):1094-1098
- 3 Cranen ME, Dekker E, Blok P, Ferwerda J, Tytgat G. Time trends in gastric carcinoma: changing pattern of type and location. *Am J Gastroenterol*, 1992; 87(5):572-579
- 4 Wang LD, Stephanie TS, Zhou Q, Goldstein S, Hong JY, Shao P *et al.* Changes in P53 and cyclinD1 protein levels and cell proliferation in different stages of human esophageal and gastric cardia carcinogenesis. *Int J Cancer*, 1994; 59(4):514-519
- 5 Wang LD, Gao WJ, Yang WC, Li XF, Li J *et al.* Statistics about the 9-year admissions of patients with esophageal cardiac tumors in People's Hospital of Linzhou City. *J Henan Med University*, 1997;32(1):9-11

# Two novel gastric cancer-associated genes identified by differential display

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**Subject headings** stomach neoplasms; gene clone; nucleotides sequence analysis; in situ hybridization; polymerase chain reaction; RNA, messenger

## Abstract

**AIM** To clone novel gastric cancer-associated genes and investigate their roles in gastric cancer occurrence.

**METHODS** A method called differential display was used which allows the identification of differentially expressed genes by using PAGE to display PCR-amplified cDNA fragments between gastric cancer cells and normal gastric mucosa cells. These fragments were cloned into plasmid vector pUC18. Homology analysis was made after sequencing these fragments.

**RESULTS** Two novel genes were identified compared with sequences from GenBank. One was registered with the AD number AF 051783. In situ hybridization showed that these two novel genes expressed specifically in gastric cancer tissues.

**CONCLUSION** The two novel genes obtained by differential display were confirmed to be gastric cancer-associated genes using in situ hybridization.

## INTRODUCTION

Cancer is a disease state caused by multiple genetic alterations, which lead to uncontrolled cell proliferation. The process often involves the activation of cellular protooncogenes and inactivation of tumor-suppressor genes. We used a method called differential display<sup>[1]</sup> to search for novel gastric cancer associated genes. mRNA from normal gastric mucosa cell line GES and gastric cancer cell line 7901 were compared. The identification and characterization of the two cDNA fragments designated GCC1 and GCC2 expressed exclusively in the gastric cancer cell line 7901 are reported below. These sequences have been included into the database in GenBank.

## MATERIALS AND METHODS

### Cells

Gastric cancer cell line SGC7901 and normal gastric mucosa cell line GES were routinely grown at 37 °C with 10% CO<sub>2</sub> in RPMI 1640 medium (Gibco) supplemented with 4mM glutamine and 10% fetal bovine serum.

### RNA isolation

Total cellular RNA from 1×10<sup>7</sup> SGC7901 and GES cells were extracted by the single-step method for RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction<sup>[2]</sup>. Poly(A)+RNA was isolated using mRNA Isolation Kit (Promega).

### Primer design

According to the primer design principle of differential display<sup>[3]</sup>, 3'-end anchored primers: T12A, T12G and T12C; 5'-end random primers: P1:GCCACCATGC; p2:GCCACCATGA were designed by us and synthesized by Sangon Company.

### Differential display<sup>[4-7]</sup>

Reverse transcription of 0.2 µg mRNA, 10 µmol/L T12M (M=G, C, A), 1 mmol/L dNTP was carried out using the Reverse Transcription Kit (Promega) according to the manufacturer's instruction in 20 µl total volume. The mixture was kept warm at 42 °C for 2 hours, added with 180 µl TE and boiled for 5 minutes. PCR was conducted using the RT products as templates. The total volume of 20 µl contained 2 µl RT products, 2 µl dNTP (100 µmol/L), 5 µl H<sub>2</sub>O, 2 µl 3'-end primer, 2 µl 5'-end primer, 2 µl 10×Buffer, 4 µl Taq enzyme (0.5×10<sup>6</sup>U/L) and 1 µl

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Received 1998-06-06

[ $\alpha$ - $^{32}$ P] labeled dATP ( $3.7 \times 10^8$  Bq/L). Reacting condition: 95°C, 30sec; 42°C, 3min; 72°C, 30sec; 40 cycles, and finally extended at 72°C for 10min. Four  $\mu$ l PCR products were analyzed on a 6% polyacrylamide DNA sequencing gel. Recovery and reamplification of positive fragments from the differential display gel was as described above.

#### Purification and subcloning of PCR products

Recovered PCR products from 2% agarose gel and purified by PEG8000 (Sigma), and subcloned into pUC18 which was previously digested by *Hinc* II. Identification of recombinants were carried out by restriction enzyme digestion. DNA sequencing reaction was performed by a model 373A automated sequencer (Applied Biosystem).

#### Database searches and homology analysis

Database searches and sequence alignment were done using the FASTA and BLAST search servers at the NCBI.

#### In situ hybridization

mRNA probes were labeled by digoxigenin (DIG) using DIG-labeling and detection Kit (Boehringer Mannheim Inc). *In situ* hybridization was done according to the manufacturer's instructions. Frozen sections were fixed in 4% paraformaldehyde/PBS and then rinsed with PBS, 0.1N HCl and Triton-100PBS in turn. Hybridization buffer contained 120  $\mu$ g DIG-mRNA probes, 5 $\times$ SSPE, 50% formamide, 5 $\times$ Denhardt's solution, 0.1% SDS and 100mg/L ssDNA. Prehybridized for 2 hours at 42°C and then added labeled probes into prehybridized solution to hybridize in humid chamber overnight at 42°C.

## RESULTS

#### Identification and cloning of differential cDNA fragments by display

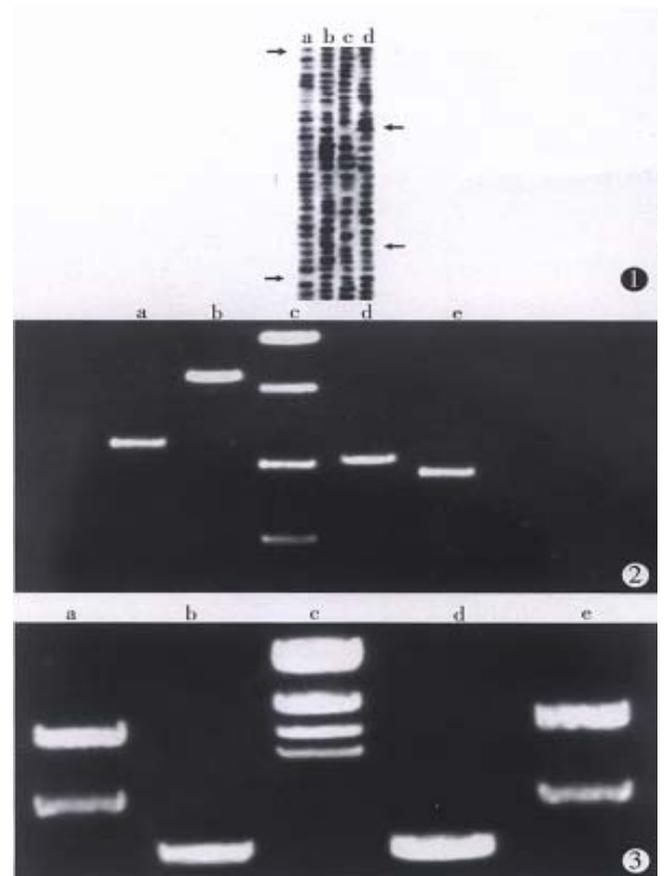
Total RNA of 100  $\mu$ g was isolated from 107 cells. The ratio of A260/A280 was above 2.0, which indicated good quality of RNA. Four cDNA fragments that appeared to be differentially expressed in SGC7901 cells were identified (Figure 1). These fragments were not detected in normal gastric mucosa cells. Positive results were observed after reamplification of these cDNA fragments (Figure 2). Identification was conducted by restriction enzyme digestion after subcloning these fragments into pUC18 vector. *Pst*-I and *Kpn* I were used to identify recombinants. Two fragments, 500bp (GCC1) and 800 bp (GCC2), were obtained (Figure 3). Sequence analysis of these two fragments revealed that GCC1 had 80% homology to protein tyrosine kinase encoding gene, while GCC2 had 30% homology to leukemia virus

encoding gene. Nucleotides were partly shown as follows:

```
GCC1:ATCCTGCTTTGCACATGCAGCGCCCGATG
GGCACCAGCCACTCGCCGTCCTCCGTTACAGTAGAGCT
TGATGGGTACATCCACCTCTTCCGCATTGGCGATGCAG
CTGCAGCTGC;GCC2:TAGTCAGCCATGGGGCGG
AGAATGGGCGGAAGTGGGCGGAGTTACGCGGGGG
GGGGATGGGCGGAGTTAGGGGCGGGACTTAATGGT
TTGCTGGACTTAATTTTGGAGATGGCAT(GenBank:
AF051783).
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#### In situ hybridization

Various normal tissues from dead infants were used, including: heart, liver, lung, brain, kidney, bladder, spleen, esophagus, stomach, intestine and colon. Malignant gastric cancer tissues served as control. Positive hybridization signals were mainly distributed in the cytoplasm of gastric cancer tissues. No positive signals were detected in normal tissues mentioned above, indicating the expression specificities of these two genes.



**Figure 1** 6% polyacrylamide DNA sequencing gel with DDPCR. a, c. GES cells; b, d. 7901 cells.

**Figure 2** Electrophoresis results of reamplification of differential display cDNA fragments.

a. fragment 1, b. fragment 2, c. PCR marker, d. fragment 3, e. fragment 4.

**Figure 3** Electrophoresis result for GCC1 and GCC2.

a. pUC GCC2 cDNA; b. pUC GCC2/*Kpn* I + *Pst* I; c.  $\lambda$  DNA Marker/*Hind* III; d. pUC GCC1/*Kpn* I + *Pst* I; e. pUC GCC1 cDNA.

## DISCUSSION

One approach to learn about the mechanisms underlying functional or structural changes in cells or tissues is to determine the changes in their pattern or rate of gene expression. Techniques aiming at making an inventory of differentially expressed gene products in two populations of cells being compared often employ (PCR) amplification of all gene products to obtain detectable levels, normally as cDNA fragments. The subsequent identification of these differentially expressed and amplified gene products is generally achieved by one of the two following methods. The differential display method relies on amplification of the entire mRNA populations, followed by a direct comparative display of the resulting populations of cDNA on a sequencing gel to visualize differences in prevalence. Gastric cancer still remains a leading cause of death among all malignant tumors in China. So various mechanisms are involved in cancer. Genetic alterations could be considered as one of the most important one. Multiple genetic alterations can be subgrouped into three groups: ① the deletion, inactivation or mutation of tumor suppressor genes. For example, *p53*, the hottest focus in tumor suppressor populations, protects malignant subtype transformation of mutated cells by initiating programmed cell death. Mutated *p53* gene expression was up to 49%-61.5% in gastric cancer tissues by immunohistology<sup>[8]</sup>. Deletions of other tumor suppressors were often found in gastric cancer, including APC and DCC. ② The overexpression of oncogenes which plays an important role in tumor occurrence, development and metastasis by encoding growth factors and their related receptors which could promote proliferation of cells. *EGF*, *EGF-R*, *TGF- $\alpha$* , *c-erbB-2*, etc, secreted by gastric mucosa cells, were proved to be involved in gastric cancer occurrence. ③ *nm23*,

which is thought to be related to metastasis in gastric cancer<sup>[9]</sup>. All these genes contribute to the diagnosis and prognosis of gastric cancer, only to some extent. Most of them could be detected at the latest period of malignant gastric cancer. And results often vary among different detecting methods. People can not expect to use them for early diagnosis and treatment. Clone novel gastric cancer-associated genes may provide a clue to clinical treatment. We cloned the novel gastric cancer-associated genes using differential display. Homology analysis indicated that GCC1 was 80% homologue to Trk gene, while GCC2 was 30% homologue to leukemia virus gene. It is well known that Trk plays a significant role in signal transduction, so does leukemia virus in leukemia occurrence. Do these two novel fragments belong to the same families of Trk and leukemia virus? What mechanisms of these two fragments are involved in gastric cancer? All these questions still need further investigations.

## REFERENCES

- 1 Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, 1992;257(5072):967-671
- 2 Bauer D, Muller H, Reich J, Riedel H, Ahrenkiel V, Warthoe P, Strauss M. Identification of differentially expressed mRNA species by an improved display technique (DD-PCR). *Nucleic Acid Res*, 1993 Sep 11;21(18):4272-4278
- 3 Chromczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 1987;162(2):156-159
- 4 Guimaraes MJ, Lee F, Zlotnic A, McClanahan T. Differential display by PCR: novel findings and applications. *Nucleic Acid Res*, 1995;23(10):1832-1833
- 5 Shoham NG, Arad T, Rosin-Abersfeld R. Differential display assay and analysis. *Bio Tech*, 1996;20(2):182-184
- 6 Mou L, Miller H, Li J, Wang E, Chalifour L. Improvements to the differential display method for gene analysis. *Biochem Biophys Res Commun*, 1994;199(2):564-569
- 7 Callard D, Lescure B, Mazzolini L. A method for the elimination of false positive generated by the mRNA differential display technique. *Bio Tech*, 1994;16(6):1096-1097
- 8 Ichiyoshi Y, Qiwa-H, Tomisaki S, Sakaguchi Y, Ohno S, Maehara Y, Sugimachi K. Overexpression of *p53* is associated with growth pattern and prognosis in advanced gastric cancer. *Hepatogastroenterology*, 1997;44(14):546-553
- 9 Ura H, Denno R, Hirata K. Correlation between *nm23* protein and several cell adhesion molecules in human gastric carcinoma. *Jpn J Cancer Res*, 1996; 87(5):512-517

# Changes of lipid metabolism in plasma, liver and bile during cholesterol gallstone formation in rabbit model

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**Subject headings** cholelithiasis; lipids/metabolism; cholesterol/metabolism; apolipoproteins/ metabolism; triglycerides/metabolism; rabbits; diseases models, animal

## Abstract

**AIM** To find out the relationship between the disturbances of lipid metabolism and the formation of cholesterol gallstones by studying the changes of lipid metabolism in plasma, liver tissue and the bile.

**METHODS** Male and female white Japanese rabbits were divided randomly into a control group (Con) and four experimental groups of 10 rabbits each fed with a diet containing 1.2% cholesterol for one, two, three and four weeks (1 wk, 2 wk, 3 wk and 4 wk group). The measurement of plasma triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and its subfractions (HDL2-C, HDL3-C), very low and low density lipoprotein cholesterol (VLDL-C, LDL-C) was taken with standard enzymatic techniques. Apolipoprotein (apo) concentrations in plasma were measured by radial immunodiffusion assay for apoA1, apoB100, apoC<sub>II</sub> and apoC<sub>III</sub>. Total cholesterol of liver was measured by the enzymatic procedure for each animal. Bile acids, mainly glycocholate (GCA) and glycodeoxycholate (GDCA) were detected by dual wavelength thin layer scanner.

**RESULTS** In all the experimental groups fed with dietary cholesterol, cholesterol crystal was found in the gallbladder in 2/10 cases of the 1 wk group, 4/10 of the 2 wk group, 6/10 of the 3 wk group and 7/10 of the 4 wk group respectively. The concentration of plasma total cholesterol (TC), triglyceride (TG), phospholipid (pl), VLDL-C, LDL-C, apoB100, apoC<sub>II</sub>, apoC<sub>III</sub> gradually increased ( $P < 0.05$ ) with the prolonged feeding time of

dietary cholesterol. High density lipoprotein cholesterol and its subfractions (HDL-C, HDL2-C, HDL3-C) showed a tendency to decrease, but without statistical significance ( $P > 0.05$ ). ApoA1 was reduced with increased feeding time of dietary cholesterol ( $P < 0.05$ ). The hepatic and biliary cholesterol increased 1-1.5 times as compared with the control group ( $t = 5.221$  and  $3.445$ ,  $P < 0.05$ ). The GCA gradually decreased beginning from the control group to the 4 wk group ( $P < 0.05$ ).

**CONCLUSION** Owing to the high cholesterol diet, the increased concentrations of plasma TC, TG, VLDL-C, LDL-C, hepatic TC and TG, apoB100, apoC<sub>II</sub> and apoC<sub>III</sub> possibly enhanced the secretion of biliary cholesterol into bile; the decreased plasma apoA1 level might reduce the secretion of antinucleating factor into bile. All those factors mentioned above probably contribute to the formation of cholesterol gallstones.

## INTRODUCTION

Cholesterol cholelithiasis is one of the common diseases in China. The mechanism of gallstone formation is quite complicated and it has been supposed to be synthesized from a variety of related factors. Among them oversaturation of cholesterol and decrease of bile acids, as well as lecithin in bile might be the fundamental causative factors. Furthermore, these changes are closely related to the disorders of lipoprotein metabolism in liver. However, during the formation of cholesterol gallstones, different links in the disturbance of lipoprotein cholesterol metabolism and their effects in lithogenesis still has much controversies. Besides, the mechanism and regulating elements are still unclear. This study is aimed to investigate the changes of lipid metabolism in plasma, liver tissue and bile in order to find out the relationship between the disturbance of lipid metabolism and the formation of cholesterol gallstones.

## MATERIALS AND METHODS

### *Animals and diets*

Male and female white Japanese rabbits were obtained from the Experimental Animal Center of West China University of Medical Sciences. They

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Project supported by the Youth Scientific Research Fund Ministry of Health P.R.C (942170) and in part by China Medical Board of New York (Y9411).

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Received 1998--

were divided randomly into a control group (Con) and four experimental groups of ten rabbits each fed with 1.2% cholesterol diet for one week (1 wk), two weeks (2 wk), three weeks (3 wk) and four weeks (4 wk). All animals were maintained in separate cages with free access to water.

### Gallstone evaluation

At the end of experiment following an overnight fasting, animals were exsanguinated under anesthesia, blood samples were collected for the measurement of plasma lipoprotein cholesterol and apolipoproteins, and portions of liver were removed for the analysis of cholesterol. Bile specimen was aspirated from gallbladder and kept for subsequent analysis. The gallbladder was cut open under the microscope, and the gallstones were evaluated according to Juniper's method. Cholesterol stones proved by infra-red spectrometry were collected.

### Plasma lipid and lipoprotein analysis

The measurement of plasma triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and its subfractions (HDL2-C, HDL3-C), very low and low density lipoprotein cholesterol (vLDL-C, LDL-C) was taken with standard enzymatic techniques. Apolipoproteins (apo) concentration in plasma were measured by radial immunodiffusion assay for apoA1, apoB100, apoC II and apoC III.

### Analysis of hepatic cholesterol

Following lipid extraction of liver samples with chloroform and methanol (2:1, v/v) total cholesterol of liver was estimated by the enzymatic method for each animal.

### Analysis of bile cholesterol and bile acids

Gallbladder bile was extracted with chloroform and methanol (2:1, v/v), and cholesterol was analyzed in the chloroform phase. Bile acids, mainly glycocholate (GCA) and glycodeoxy-cholate (GDCA), were detected by dual wavelength thin

layer scanner.

### Statistical analysis

Results were shown as  $\bar{x} \pm s$  (means  $\pm$  SD) and a paired Student's *t* test was used for quantitative information,  $P < 0.05$  is considered statistically significant. Statistical analysis of the results was carried out using SPSS software in computer.

## RESULTS

### Gallstones evaluation

Cholesterol crystal in gallbladder was not found in any animal of the control group fed with routine diet. However, in all the experimental groups fed with dietary cholesterol, cholesterol crystal in gallbladder was found in 2/10 of 1 wk group, 4/10 of 2 wk group, 6/10 of 3 wk group and 7/10 of 4wk group, suggesting the formation of cholesterol gallstones. Plasma lipids and apolipoproteins (Table 1) Table 1 shows that the concentrations of plasma total cholesterol (TC), triglyceride (TG), phospholipid (p1), VLDL-C, LDL-C, apoB100, apoC II, apoC III gradually increased with the prolonged feeding time of dietary cholesterol ( $P < 0.05$ ). High density lipoprotein cholesterol and its subfractions (HDL-C, HDL2-C, HDL3-C) showed a decreasing tendency, without statistical significance ( $P > 0.05$ ). ApoA1 was reduced with increasing feeding time of dietary cholesterol in rabbits ( $P < 0.05$ ).

### Hepatic and biliary cholesterol and bile acids (Table 2)

Table 2 shows that the hepatic and biliary cholesterol significantly increased by more than 1-1.5 times as compared with the control group. The GCA gradually decreased from the control group to the 4 wk group, and GDCA showed an increase at the beginning of dietary cholesterol feeding in 1 week group, then decreased gradually without statistical significance in the other experimental groups.

**Table 1** The results of plasma lipids (mmol/L) and apolipoproteins (mg/L) for each group

	Control	Experimental groups			
		1 wk	2 wk	3 wk	4 wk
TC	0.79 $\pm$ 0.03	4.50 $\pm$ 1.86 <sup>a</sup>	7.23 $\pm$ 3.87 <sup>a</sup>	16.35 $\pm$ 3.49 <sup>a,e,c</sup>	17.21 $\pm$ 4.78 <sup>a,e,c</sup>
TG	0.83 $\pm$ 0.21	1.10 $\pm$ 0.45	1.45 $\pm$ 0.51 <sup>a</sup>	1.52 $\pm$ 0.41 <sup>a</sup>	2.87 $\pm$ 0.81 <sup>a,e,c</sup>
PL	0.94 $\pm$ 0.29	2.37 $\pm$ 0.68 <sup>a</sup>	3.10 $\pm$ 1.32 <sup>a</sup>	6.24 $\pm$ 2.43 <sup>a,e,c</sup>	6.71 $\pm$ 2.80 <sup>a,e,c</sup>
HDL-C	0.70 $\pm$ 0.21	0.72 $\pm$ 0.30	0.52 $\pm$ 0.23	0.55 $\pm$ 0.21	0.58 $\pm$ 0.24
HDL2-C	0.32 $\pm$ 0.10	0.35 $\pm$ 0.11	0.25 $\pm$ 0.13	0.27 $\pm$ 0.11	0.31 $\pm$ 0.16
HDL3-C	0.31 $\pm$ 0.11	0.37 $\pm$ 0.17	0.26 $\pm$ 0.12	0.29 $\pm$ 0.17	0.28 $\pm$ 0.15
LDL-C	0.05 $\pm$ 0.02	0.90 $\pm$ 0.13 <sup>a</sup>	1.88 $\pm$ 0.97 <sup>a,c</sup>	4.86 $\pm$ 0.98 <sup>a,e,c</sup>	5.56 $\pm$ 1.07 <sup>a,e,c</sup>
VLDL-C	0.06 $\pm$ 0.01	0.45 $\pm$ 0.18	1.22 $\pm$ 0.91 <sup>a,c</sup>	3.43 $\pm$ 0.87 <sup>a,e,c</sup>	3.76 $\pm$ 1.01 <sup>a,e,c</sup>
apoB100	0.15 $\pm$ 0.01	0.54 $\pm$ 0.06 <sup>a</sup>	0.54 $\pm$ 0.10 <sup>a</sup>	5.36 $\pm$ 0.11 <sup>a,c</sup>	6.45 $\pm$ 0.54 <sup>a,e,c</sup>
apoA I	20.82 $\pm$ 1.57	18.10 $\pm$ 2.23	17.87 $\pm$ 2.07	18.16 $\pm$ 0.74	15.96 $\pm$ 1.13 <sup>g</sup>
apoC II	0.05 $\pm$ 0.01	0.15 $\pm$ 0.02 <sup>a</sup>	0.30 $\pm$ 0.04 <sup>a,c</sup>	0.25 $\pm$ 0.01 <sup>a,c</sup>	0.69 $\pm$ 0.046 <sup>a,e,c</sup>
apoC III	0.17 $\pm$ 0.02	0.45 $\pm$ 0.06 <sup>a</sup>	1.22 $\pm$ 0.05 <sup>a,c</sup>	1.62 $\pm$ 0.06 <sup>a,e,c</sup>	2.91 $\pm$ 0.07 <sup>a,e,c,g</sup>

<sup>a</sup> $P < 0.05$ , vs Con; <sup>c</sup> $P < 0.05$ , vs 1 wk; <sup>e</sup> $P < 0.05$ , vs 2 wk; <sup>g</sup> $P < 0.05$ , vs 3 wk.

**Table 2** The results of hepatic and biliary cholesterol and bile acids

	Control	Experimental groups			
		1 wk	2 wk	3 wk	4 wk
TCd	25.02±6.21	33.63±16.86 <sup>a</sup>	36.23±8.31 <sup>a</sup>	65.75±33.41 <sup>a,b</sup>	83.87±35.10 <sup>a,b,c</sup>
TGd	158.72±58.15	145.37±21.34	142.27±30.24	148.22±38.16	151.52±35.52
PLd	29.21±7.04	34.65±7.56	30.23±10.16	29.02±10.89	27.81±9.53
GDCAe	275.35±101.46	532.83±258.71	413.73±132.37	393.54±108.71	348.14±132.71
GCAe	97.81±59.28	87.56±59.52	73.26±49.54	58.61±9.89 <sup>a</sup>	43.79±38.74 <sup>a</sup>
CHf	2.51±1.36	3.58±1.98	3.85±1.41	4.32±1.23 <sup>a</sup>	5.02±1.86 <sup>a</sup>

<sup>a</sup>*P*<0.05, vs Con; <sup>b</sup>*P*<0.05, vs 1, 2 wk; <sup>c</sup>*P*<0.05, vs 3 wk; <sup>d</sup>Hepatic TC, TG and PL (mg/g); <sup>e</sup>Bile GDCA and GCA (mg/L); <sup>f</sup>Bile cholesterol (mol/L).

## DISCUSSION

In this study the changes of plasma lipoproteins, hepatic lipids and bile lipids have been observed in the processes of cholesterol gallstone formation, which was induced by the cholesterol diet in animal model.

In epidemiological studies<sup>[1,2]</sup>, the concentrations of plasma lipoprotein cholesterol were various in cholesterol gallstone patients. In normal people, the plasma LDL-C concentration appeared to be related to the biliary cholesterol concentration<sup>[3]</sup>, but the pathway of its metabolism is still unknown. In type IV hyperlipidemia patients with elevated VLDL-C and LDL-C, the incidence of gallstone was high<sup>[4]</sup>. Recent investigations showed<sup>[5]</sup> that in cholesterol-fed hamsters with the VLDL-C/HDL-C ratio greater than 1.0, cholesterol gallstone formation occurred easily. Hayes *et al.*<sup>[4]</sup> found an expanded pool of VLDL and LDL cholesterol and a reduced pool of HDL<sub>2</sub> predominated in hamsters with cholesterol gallstone. Likewise, increased VLDL and decreased HDL, primarily HDL<sub>2</sub>, were common in obese persons with gallstones, therefore, elevation of VLDL-C and LDL-C is closely related to excessive dietary cholesterol<sup>[5]</sup>. The decreased HDL, as recently found<sup>[6]</sup>, bears a close relationship to the elevated activity of plasma CETP (cholesteryl ester transfer protein) which promotes the lipoprotein cholesterol of HDL to be transferred to other lipoproteins such as VLDL and LDL in patients with cholesterol gallstones. Our study also showed elevated plasma TC, TG, VLDL-C, LDL-C and a slightly lowered HDL-C and its subfractions (DHL2-C, HDL3-C). Kern Jr<sup>[7]</sup> reported that the diet-induced cholesterol increase was found in biliary cholesterol secretion in the gallstone subjects, but not in the controls, indicating that dietary cholesterol might be important in the pathogenesis of cholesterol gallstones, and also supported the hypothesis that hepatic metabolism of cholesterol in gallstone patients differs from those without stones. Although the factors that regulate biliary cholesterol secretion are not certain, a number of researches have suggested that most

biliary cholesterol were derived from the existed rather than newly synthesized cholesterols. This study shows that dietary, hepatic and biliary cholesterols increased concomitantly, which is consistent with the above conclusion. Robins *et al.*<sup>[8]</sup> suggested that the precursor cholesterol secreted by the gallbladder was transported directly from plasma through the plasma membrane of hepatocytes to the biliary canaliculi without entering the interior of the cell.

The concentration of apolipoproteins in bile is about 10% of that in plasma. Although apolipoproteins are potential antinucleating protein in bile, their functional role in vivo as a factor in the solubilization of biliary cholesterol is relatively unexplored. In vitro apoA1 in low concentrations can delay the shift from micelles to vesicles, thereby enhancing the cholesterol-solubilizing capacity of bile acids. Another finding<sup>[9]</sup> is that apoA1 stabilizes phospholipid lamellae and thus prolongs nucleation time in model bile systems. This study demonstrates that the concentration of plasma apoA1 gradually decreased following dietary cholesterol which might result in a reduced concentration of apoA1 and cholesterol crystal formation in bile.

## REFERENCES

- Hayes KC, Livinston A, Trautwein EA. Dietary impact on biliary lipids and gallstones. *Annu Rev Nutr*, 1992;12:299-326
- Busch N, Matern S. Current concepts in cholesterol gallstone pathogenesis. *Eur J Clin Invest*, 1991;21(1):453-768
- Lee DWT, Gilmore CJ, Bonorris G, Cohen H, Marks JM, Cho-sue M *et al.* Effect of dietary cholesterol on biliary lipids in patients with gallstones and normal subjects. *Am J Clin Nutr*, 1985;42(1):414-420
- Hayes KC, Khosla P, Pronczuk A. Diet-induced type like hyperlipidemia and increased body weight are associated with cholesterol gallstones in hamsters. *Lipids*, 1991;26(2):729-735
- Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J Lipid Res*, 1993;34(10):1637-1659
- Juvonen T, Savolainen MJ, Kairaluoma MI, Lajunen LHI, Humphries SE, Kesaniemi YA *et al.* Polymorphisms at the apoB, apoA I and cholesterol ester transfer protein (CETP) gene loci in patients with gallbladder disease. *J Lipid Res*. 1995;36(4):804-812
- Kern F Jr. Effects of dietary cholesterol on cholesterol and bile acid homeostasis in patients with cholesterol gallstones. *J Clin Invest*, 1994;93(3):1186-1194
- Robins SJ, Brunengraber H. Origin of biliary cholesterol and lecithin in the rate contribution of new synthesis and preformed hepatic stones. *J Lipid Res*, 1982;23(4):604-608
- Everson GT, McKinley C, Kern F Jr. Mechanisms of gallstone formation in women: effect of exogenous estrogen and dietary cholesterol on hepatic lipid metabolism. *J Clin Invest*, 1991;87(1):237-246

# Copper-chelating therapeutic effect in Wilson disease with different clinical phenotypes and polymorphisms of ATP7B gene

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**Subject headings** Wilson disease/therapy; copper chelating agents; ATP7B gene; mutations

## Abstract

**AIM** To investigate the copper-chelating therapeutic effect in Wilson disease (WD) with different clinical phenotypes and polymorphisms of ATP7B gene.

**METHODS** One hundred and twenty-two WD patients with different clinical phenotypes were given DMPS intravenously and Gandou copper-chelating tablet orally for one month. The therapeutic effect was judged by modified Goldstein method. Exon 18 of ATP7B gene extracted from the DNA of patients and 20 healthy volunteers was amplified with PCR mutation and polymorphism were screened with SSCP technique.

**RESULTS** Four kinds of abnormal migration bands in PCR-SSCP were observed in 37 WD patients, mutation frequencies of three different disease phenotypes, and curative effect between mutation group and non-mutation group showed no statistically significant difference ( $P > 0.05$ ), but the total effectiveness rates in patients with Wilson type or pseudosclerosis type were significantly higher than those of patients with hepatic type ( $\chi^2 = 6.17$ ,  $P < 0.05$ ).

**CONCLUSION** Most WD patients are compound heterozygotes, the patients with different clinical phenotypes have different response to copper-chelating therapy. Specific mutation, at least in part, plays a role in influencing the disease phenotypes and therapeutic effect.

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Supported by the National Natural Science Foundation of Anhui Province, No.97412001.

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Received 1998-03-01

## INTRODUCTION

Wilson disease (WD) occurs most frequently in adolescence and characterized by failure to incorporate copper into ceruloplasmin in the liver, and failure to excrete copper from the liver into bile<sup>[1]</sup>. This results in toxic accumulation of copper in the liver, and also in kidney, brain and cornea. The induced liver cirrhosis and/or progressive neurological damage are fatal if not treated with copper chelating agents promptly. WD is divided into different clinical phenotypes according to the age at onset and clinical presentations. The therapeutic effect and its prognosis depend on the clinical phenotypes. With the cloning of WD gene (ATP7B) and elucidation of its molecule structure, it is possible to explore the relationships between clinical therapeutic effect and phenotypes or mutations. In this report, we observed the effect of copper-chelating therapy in WD with different phenotypes and polymorphisms of ATP7B gene.

## MATERIAL AND METHODS

### Subjects

From March 1995 to July 1996, 122 inpatients with WD were chosen for this study. There were 74 men and 48 women with a mean age of  $19 \pm 6.5$  years and an average disease course of  $2.5 \pm 1.5$  years. They had clinically definite WD by criteria that there are typical extrapyramidal system and/or liver symptoms; the concentration of ceruloplasmin  $< 200 \text{ mg/L}$  and urinary copper excretion  $> 100 \mu\text{g}/24 \text{ hours}$ <sup>[2]</sup>. According to clinical symptoms, we divided all WD patients into Wilson type (74 cases), pseudosclerosis type (36 cases) and hepatic type (12 cases)<sup>[3]</sup>. The severity degree of sickness were graded by modified Goldstein method<sup>[4]</sup>. They included 21 patients with grade I, 45 grade II, 41 grade III and 15 grade IV. Twenty healthy volunteers consisting of 11 men and 9 women with a mean age of  $20 \pm 4.5$  years were chosen as control.

### Method of treatment

All WD patients were given DMPS (unithiol) intravenously, at a dose of 20 mg/kg daily. Meanwhile, Gandou copper-chelating tablets were given orally, 10 tablets, three times daily, with one month as a course of treatment. They had low-

copper diet throughout the study. Before and after treatment, typing, grading and therapeutic effect judgment were accomplished independently by two experienced neurologists in our institute.

#### PCR-SSCP analysis and silver staining

White blood cells were first separated from 10mL peripheral blood anti-coagulated with 3 mL ACD, then genomic DNA was extracted as described by salting-out method<sup>[5]</sup>. The primer sets for exon 18 mutation analysis were: 5' - ACCTGTTGCCAACACTAGCAT-3' and 5' - TCCCAGCACCCACAGCC-3'<sup>[6]</sup>. Genomic DNA of 800ng was dissolved in 25  $\mu$ l PCR buffer solution containing 20  $\mu$ mol/L TrisCl (pH 8.5), 50 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>, 200  $\mu$ mol/L dATP, dGTP, dCTP, dTTP, 0.5  $\mu$ mol/L each primer and 1 unit Taq DNA polymerase (Promega Corporation). The cDNA was amplified in a DNA thermal cycler (MJ research, U.S.A.). The cycle of denaturation at 93 °C for 45 seconds, renaturation at 58 °C for 45 seconds and extension at 70 °C for 90 seconds was repeated for 35 times, with the last step extended to 10 minutes. Nondenatured 8% polyacrylamide gel (acrylamide/bis-acrylamide cross linker with a ratio of 49:1, and glycerol content 5%) was poured between two glass plates of DYY-III vertical electrophoresis gel tank apparatus with cooling compartment, placed at least 2 hours in room temperature for the gel to set, the wells were added with 1 $\times$ TBE buffer using a Pasteur pipette or pipette tip. After electrophoresis for 30-60 minutes in advance, 5  $\mu$ l-8  $\mu$ l PCR product was added with same amount of SSCP gel loading buffer (containing 96% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). All samples were denatured at 98 °C for 5 minutes, and then quickly chilled on ice for electrophoresis. Fragments were resolved by electrophoresis (30W, 15 °C-20 °C) for 6-8 hours. Silver staining was performed and photograph was taken for further analysis according to the method reported by Yu Long<sup>[7]</sup>.

## RESULTS

#### Therapeutic effect criterion

Marked effectiveness. After one month of copper-chelating treatment, conditions were improved remarkably by two grades.

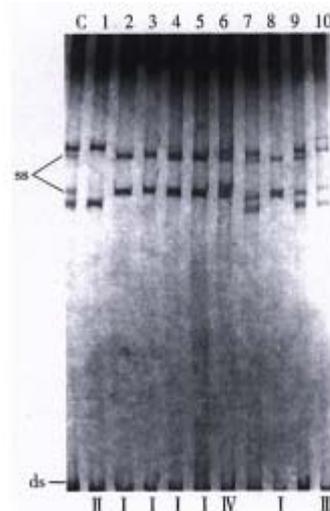
Improvement. There were some improvement in clinical condition, conditions were one grade better than before treatment.

Inefficiency or exacerbation. Conditions had no obvious changes or became worse.

#### Polymorphism of exon 18 and clinical phenotypes

The exon 18 of 122 WD patients was analyzed. Four

kinds of abnormal migration bands in PCR-SSCP were observed in 37 WD patients, which were named by us as type I, II, III and IV temporarily (Figure 1). It was found that type I, II and III had both abnormal band shifts and similar band shift as control group, which indicated that most WD patients were compound heterozygotes, i.e., they carry two different mutations. According to the relationship of ssDNA conformation with molecular electrophoresis shift, mutation frequencies of Wilson type, pseudosclerosis type and hepatic type WD were 29.7%, 30.6% and 33.3%, respectively. No statistically significant differences were found by Chi-square test ( $P>0.05$ , Table 1).



**Figure 1** Abnormal SSCP band shift (exon 18) C:Control, 1-10: WD patients, I-IV: Abnormal band shift type.

**Table 1** Clinical phenotype and polymorphisms of exon 18

Phenotype	Cases	Length of PCR product (bp)	Abnormal SSCP				Total	Mutation frequency (%)
			I	II	III	IV		
Wilson	74	204	17	4	1	0	22	29.7
Pseudosclerosis	36	204	9	2	0	0	11	30.6
Hepatic	12	204	3	0	0	1	4	33.3

#### Relationship between therapeutic effect and phenotypes or mutations

No statistically significant difference was found by Ridit analysis in notable effectiveness between three different phenotypes ( $P>0.05$ ), while the total effectiveness of Wilson type or pseudosclerosis type was much better than that of hepatic type WD ( $\chi^2=6.17$ ,  $P<0.05$ ). Comparison of total and notable effectiveness between mutation group and non-mutation group showed no significant difference ( $P>0.05$ ).

**Table 2 Therapeutic effect and phenotypes or mutations**

	Cases	Marked effectiveness	Improved	Ineffective/exacerbated	Total effectiveness
Wilson	74	20(27)	45(61)	9(12)	65(88) <sup>a</sup>
Pseudosclerosis	36	8(22)	23(64)	5(14)	31(86) <sup>a</sup>
Hepatic	12	1(8.0)	5(42)	6(50)	6(50)
Mutation	37	8(22)	22(59)	7(19)	30(81)
Non-mutation	85	21(25)	51(60)	13(15)	72(85)

<sup>a</sup> $P < 0.05$ , vs WD with hepatic type. The No in parenthesis indicates the percentage.

## DISCUSSION

WD is certainly preventable and to some extent is curable, if it is diagnosed and treated in early stage. Recently, we have achieved satisfactory therapeutic effects in treating WD patients with copperchelating agents DMPS, DMSA and Gandou copperchelating tablet<sup>[8-10]</sup>. The gene for WD, predicted to encode a coppertransporting membrane protein, has now been cloned, making it possible to explore the relationship between therapeutic effect and different phenotypes or mutations. It has been proved that mutation of WD gene could disrupt ATPase function so that copper was detained in tissues and cells, leading to the occurrence of different clinical phenotypes. Up to now, Thomas et al has identified 23 disease-specific mutations including 59 monosomic mutations in 58 WD patients, which explained the diversity of the disease phenotypes, but he did not sum up the relationship between mutation and clinical phenotypes due to the limited number of cases<sup>[11]</sup>. These mutation sites involved largely functional regions of ATPase (exon 13-18), but occasionally detected in copperbinding regions. It was reported that the frequencies of missense mutation detected in exon 14 and 18 were 28% and 10%, so that they were usually chosen as important sites of genetic screening<sup>[12]</sup>. PCR-SSCP technique is of great value in direct mutation identification and polymorphism analysis, and SSCP analysis of PCR products was made by denaturing ds DNA and fractionating the strands on a non-denaturing polyacrylamide gel. Under the appropriate condition, the electrophoretic mobility of the DNA was dependent not only on its length and molecular weight, but also on its conformation. This secondary structure was determined by the balance between destabilizing thermal forces and weak stabilizing forces such as

intra-strand base pairings and stacking, which were in turn dependent on the primary structure of the DNA strand, not only did the complementary strands migrate as separate bands on the gel, but small differences in sequence also altered the mobility of these strands. Aberrant bands were then sequenced to confirm the presence of mutation or polymorphism<sup>[13]</sup>. In this study, 37 WD patients had aberrant migration bands in PCR-SSCP, indicating strongly that the exon 18 of ATP7B gene may be one of the regions with a higher mutation frequency, although the exact mutation sites and its trait could be identified by further sequential analysis. At same time, comparative results of the therapeutic effect showed that there were no statistically significant differences between the three kinds of clinical phenotypes as well as mutation group and non-mutation group ( $P > 0.05$ ). In view of the fact that most WD patients who carry two different mutations are compound heterozygotes and total effectiveness of Wilson type or pseudosclerosis type WD are much better than that of hepatic type WD, we consider that specific mutation, at least in part, plays a role in influencing the disease phenotype and curative effect.

## REFERENCES

- 1 Ren MS, Fan YX, Yang RM, Han YZ, Wu GJ, Xin YR *et al*. A comparative study of biliary elements and clinical phenotypes in Wilson disease. *China Natl J New Gastroenterol*, 1997;3(4):260-262
- 2 Shi YQ. Practical neurology. 2th ed. Shanghai: Shanghai Science and Technology Press, 1995:771-772
- 3 Walshe JM. Wilson's disease. In: Vinken PJ, Bruyn GW, Klawans HL, eds. Handbook of clinical neurology, extrapyramidal disorder. Amsterdam: Elsevier, 1986:223-224
- 4 Yang RM. Hepatolenticular degeneration. 1st ed. Hefei: Anhui Science and Technology Press, 1995:204-205
- 5 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res*, 1988;16(6):1215-1216
- 6 Bull PC, Thomas GR, Forbes J, Rommens JM, Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes disease gene. *Nature Genet*, 1993;5(12):327-337
- 7 Yu L, Gong LQ, Deng Y, Shi SL, Zhao SY. A simple and easy preparation method of silver staining PAG glue film for PCR-SSCP and STR analysis. *Chin J Med Genet*, 1996;13(3):177
- 8 Ren MS, Yang RM, Zhang B, Xu SH. Comparison of therapeutic effects between unithiol, succimer and penicillamine on hepatolenticular degeneration. *Chin J New Drugs Clin Remedies*, 1998;17(1):23-25
- 9 Ren MS, Yang RM. Clinical curative effects of dimercaptosuccinic acid (DMSA) on hepatolenticular degeneration and the impact of DMSA on biliary trace elements. *Chin Med J*, 1997;110(9):694-697
- 10 Ren MS, Zhang B, Yang RM, Han YZ, Wang X. Clinical study of integrated traditional and western medicine therapy on hepatolenticular degeneration. *Chin J Integrated Traditional Western Med*, 1997;17(3):136-138
- 11 Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW. The Wilson disease gene: spectrum of mutations and their consequences. *Nature Genet*, 1995;9(2):210-217
- 12 Fan YX, Yang RM, Yu L, Wu MC, Shi SL, Ren MS *et al*. Identification of a novel missense mutation in Wilson disease gene. *Chin Med J*, 1997;110(11):887-890
- 13 Griffin HG, Griffin AM. PCR technology: current innovation. Florida: CRC Press, 1995:165-166

# Role and pitfalls of hepatic helical multi-phase CT scanning in differential diagnosis of small hemangioma and small hepatocellular carcinoma

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**Subject headings** liver neoplasms/radiography; carcinoma, hepatocellular/radiography; hemangioma/radiography; diagnosis, differential; tomography, X-ray computed

## Abstract

**AIM** To compare and analyze the contrast enhancement appearance of small hemangioma (SHHE) and small hepatocellular carcinoma (SHCC) with helical multi-phase CT scanning so as to determine their roles and pitfalls in the differential diagnosis of SHHE and SHCC.

**METHODS** The pre and postcontrast CT scanning of the liver in 73 cases (38 SHHE, 35 SHCC) were carried out. The first phase scan of the entire liver began at 30s after the injection of contrast medium, the second and third phases began at 70s, and 4 min respectively. The contrast enhancement patterns and characteristics of all lesions were observed and compared.

**RESULTS** In SHHE, 64.29% (27/42) had typical manifestations in two-phase dynamic scanning, such as peripheral dramatic high-density enhancement of the lesions with progressive opacification from the periphery toward the center, 30.95% (13/42) were hyperdense in both phases and 4.76% (2/42) were hypodense in both phases. In the third phase scanning, 96.67% (28/30) of SHHE were hyperdense and isodense. In SHCC 59.52% (25/42) presented typical appearances, such as hyperdense in the first phase and hypodense in the second phase, 23.81% (10/42) were hyperdense in the first phase and isodense in the second phase with 4.76% (2/42) of hypodense in both phases. In the third phase scanning, 85.71% (24/28) of SHCC were hypodense.

**CONCLUSION** According to the contrast enhancement patterns of SHHE and SHCC in the two-phase or multi-phase scanning by helical CT, diagnosis can be established in the majority of lesions, while some atypical cases needed MRI for further investigation.

## INTRODUCTION

Hemangioma is the most common benign tumor of the liver. It is found incidentally during the abdominal imaging examination, which is important in the differential diagnosis from hepatocellular carcinoma (HCC)<sup>[1]</sup>. In most cases, the differential diagnosis is easy, but in some cases, it is difficult. With the application of helical CT, the scan speed is greatly improved, the entire liver can be scanned during a single breath-hold and it can be done during dual-phases or multi-phases of hepatic enhancement for optimal visualization of small hemangioma (SHHE) and small hepatocellular carcinoma (SHCC)<sup>[2]</sup>. To improve the capability of differential diagnosis between SHHE and SHCC, it is necessary to renew their enhancement patterns on helical CT scanning. We analyzed the appearance of 38 SHHE (42 lesions) and 35 SHCC (42 lesions) on dual-phase and multiphase helical CT scanning, and evaluate their roles and pitfalls in the differential diagnosis between the two.

## MATERIALS AND METHODS

Seventy-three patients were reviewed, 52 men, 21 women, aged 32-76 years, averaging 53 years. Thirty-five cases of SHCC were confirmed by surgical pathology or biopsy and 10 of 38 cases of SHHE were proved by surgery, and the others confirmed with the combination of other examinations (US, MRI) and the follow-up CT scan at least for one year. All lesions were  $\leq$  3cm in diameter. The CT imaging was performed with a GE-Hispeed Advantage Scanner, scanning time 1s, 120 kV-140 kV, 230 mA-280 mA, an image matrix of 512 $\times$ 512, section thickness and interscan gap of 5 mm or 10 mm. The postcontrast scanning was started after the plain scan. The contrast

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**Project supported by the National Natural Science Foundation of China, No.39282229.**

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**Received** 1998-01-31

medium (CM, Ultravest) was injected at a rate of 3 or 4 ml/s with a total amount of 80 ml-120 ml (1.5 ml/kg body weight). The first (arterial) phase scan of the whole liver began at 30s after initial injection of CM into the antecubital vein, and the second (portal venous) phase scan started at 70s. In addition, 28 SHCC patients and 30 SHHE patients underwent the third (delayed) phase scan with 4 min.

All cases received US examination (HITANICA-EUB-40B, Japan), and 25 of the patients underwent MR imaging (GE-1.5T Sigma Superconducting Magnet). The SE technique was used and T<sub>1</sub>-weighted and T<sub>2</sub>-weighted (2000/30-50) imaging of the entire liver was carried out. The dynamic contrast enhancement MR imaging was performed with the fast multiplanner spoiled gradient recalled sequence.

## RESULTS

Fourty-two lesions were detected in 38 SHHE patients with a size of 1 cm-3 cm, averaging 2.2 cm, and 42 lesions were detected in 35 SHCC patients, with a size of 0.8 cm-3.0 cm, averaging 1.8 cm.

### *Plain scanning*

Twenty-eight lesions of SHHE were detected, 25 of them had low attenuation with the edge clear or unclear, 3 of them presented high attenuation because of the existence of fatty liver. Twenty-six SHCC were detected on the plain scan, 24 of them had low attenuation and 2 had high attenuation with fatty liver background.

### *Contrast enhancement*

On the first phase, 30.95% (13/42) of the SHHE revealed homogeneous high attenuation, 50% (21/42) of them with unhomogeneous high attenuation, and 19.05% (8/42) homogeneous low attenuation. On the second phase, 61.90% (26/42) presented with homogeneous high attenuation, 28.57% (12/42) with unhomogeneous high attenuation, 2.38% (2/42) isoattenuation and low attenuation, respectively.

On the first phase scan, SHCC presented the following enhancement findings: homogeneous high attenuation in 71.43% (30/42), unhomogeneous high attenuation in 16.67% (7/42), isoattenuation in 7.14% (3/42) and low attenuation for 4.76% (2/42). On the second phase, 71.43% (30/42) of them became low attenuation (homogeneous or unhomogeneous), 23.81% (10/42) isoattenuation, 4.76% (2/42) had high attenuation associated with fatty liver.

On the delayed phase scan, the appearance of SHHE varied, but the majority (96.67%) were

hyperdense (homogeneous or unhomogeneous) and isodense, and the other 2 of the 30 were homogeneously hypodense, one of them was hypodense, the other associated with fatty liver was hyperdense on both plain scan and the first phase scan, and became hypodense compared with the liver parenchyma enhanced markedly on the second phase. Those two cases were misdiagnosed as hepatocellular carcinoma, but on the dynamic MR contrast imaging, they began to be opacified fully at the 10 min. For SHCC, 85.71% (24/28) were hypodense on the delayed phase scan, 4 were isodense (with fatty liver), 2 hyperdense on the first and the second phase, and the other 2 were hyperdense on the first phase and isodense on the second phase, resulting in misdiagnosis of SHHE preoperatively.

## DISCUSSION

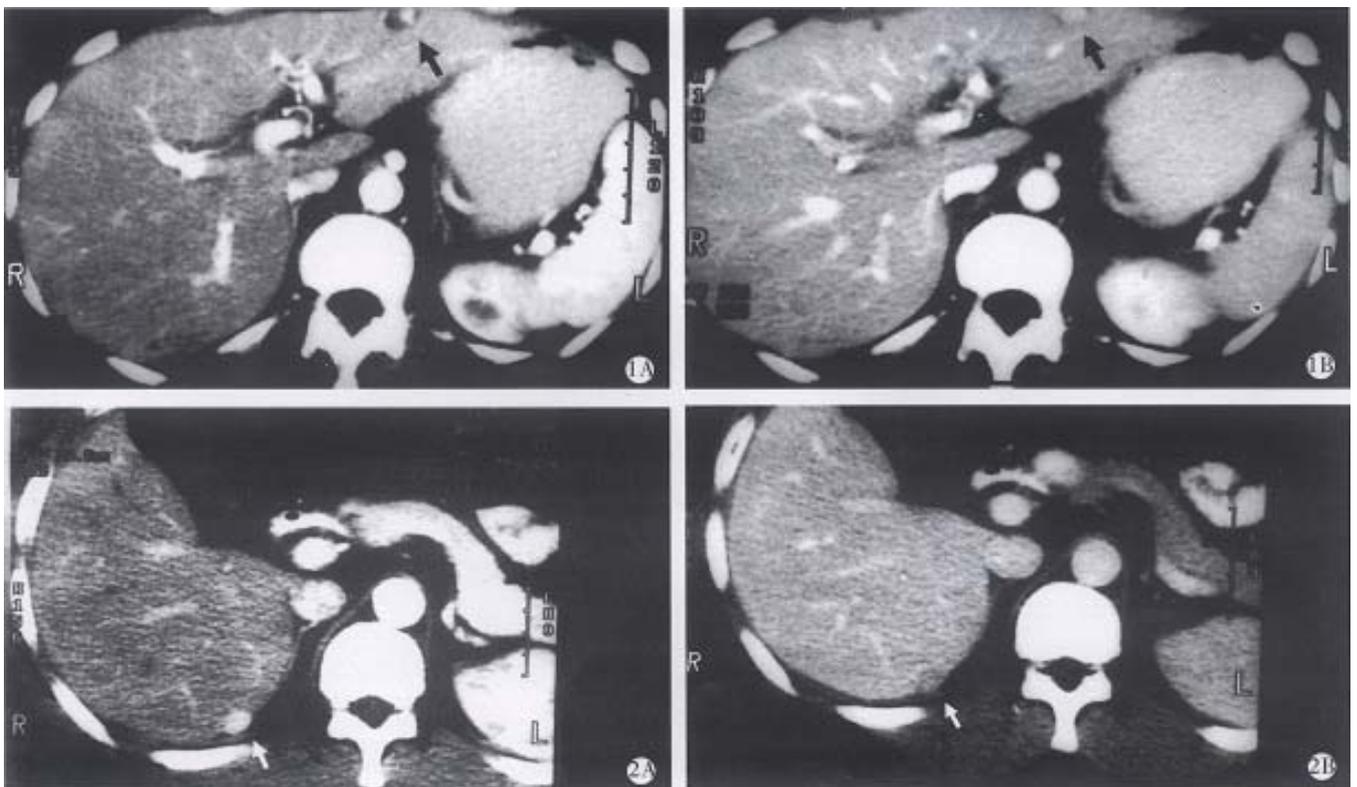
The diagnostic standards of HHE suggested by Freeny<sup>[3]</sup> were: ① early contrast enhancement in the peripheral areas; ② progressive opacification from the periphery to the center of the lesion; ③ full opacification in the delayed scan, but only 55% HHE were typical due to the different characteristics of each lesion and pitfalls of the scan protocol. The single-level dynamic CT can remedy the shortcomings of conventional contrast CT, but the scan phase can not be controlled accurately to pass through the center of small lesions. The helical CT is able to overcome the disadvantages of the conventional dynamic CT. The scanning of entire liver can be finished during a single breath-hold without respiration motion. On the other hand, the major advantage of helical CT is that the entire liver can be scanned at the different periods of hepatic enhancement, facilitating lesion's characterization in general<sup>[4]</sup>. The arterial phase scan usually started at 25s-35s after initial injection of contrast medium, and the portal phase at 65s-70s, sometimes an additional delayed scan at 3 min-5 min was needed in patients suspected of having HHE in order to distinguish from HCC.

Our results showed that, 27 of 42 SHHE (64.28%) had obvious globular enhancement peripherally (Figure 1 A, B), and it was not seen in the other 15 lesions. Thirteen of 15 lesions were homogeneous hyperdense on the early phase, which was difficult to differ from hypervascular SHCC, but in the second phase, the lesions remained hyperdense, which was helpful in making definite diagnosis of SHHE. The time of opacification needed in SHHE was usually 1 min-4 min or more, but the smaller the lesion, the faster the spreading of contrast medium, becoming homogeneously hyperdense rapidly, with a longer peak period of about 1 min-2 min<sup>[1,5]</sup>, therefore, remaining

hyperdense on the second phase scan. Thirty-seven of 42 of SHCC (88.09%) cases were homogeneous or unhomogeneous hyperdense on the first phase, but 71.43% of them became hypodense on the second phase (Figure 2 A, B), and 23.81% (10/42) of them were isodense. The result showed that the peak period of early enhancement of SHCC lasted short and waked out rapidly, so the dual-phase (arterial phase and portal venous phase) scans can reflect sufficiently the specific feature of blood supply of SHHE and SHCC. Findings during the portal venous phase scan was especially important for the differential diagnosis. Early homogeneous hyperdense findings in some SHHE have been reported to be atypical<sup>[1-7]</sup>, but the prolonged hyperdense on the second phase can make a positive diagnosis (Figure 3 A-D). In addition, Hanafusa<sup>[5]</sup> *et al* reported a group of SHHE, 7 of them were considered atypical as hyperdense on the first phase and isodense on the second phase. Ten lesions in our group had the same appearance, all were performed with the delayed phase scan, 8 of them being hypodense and diagnosed as SHCC (Figure 4 A-D). The other two lesions of isodense were misdiagnosed as SHHE preoperatively, but later were confirmed as SHCC by the surgical pathology. The misdiagnosis might be inferred that

a few SHCC received the blood supply from portal vein rendering them more hypervascular and prolonged enhancement into the portal venous phase<sup>[8]</sup>. On the other hand, in the patients with fatty liver, due to the lower parenchymal attenuation, the difference between the lesion and the liver parenchyma was minimized. The delayed phase scan was necessary in these patients, so as to lower the misinterpretation. The demonstration of capsule of the lesions was also important in the differential diagnosis, but rarely seen on CT, and MR imaging may be helpful under this condition.

Thirty SHHE and 28 SHCC had the third phase scanning, the result showed that 96.67% (20/30) of SHHE were hyperdense or isodense, and 85.71% (24/28) of SHCC were hypodense. These reflected further changes of the enhancement patterns of SHHE and SHCC. The early enhancement patterns of SHHE were various, but the majority of lesions were opacified gradually or rapidly and lasting longer while those of SHCC had the enhancement pattern of both ascending and descending rapidly. In some atypical cases, especially in the patients associated with fatty liver, either dual-phase or multi-phase scan showed the limitation in the differential diagnosis, and MR imaging would be the best choice.



**Figure 1** SHHE confirmed by surgery.

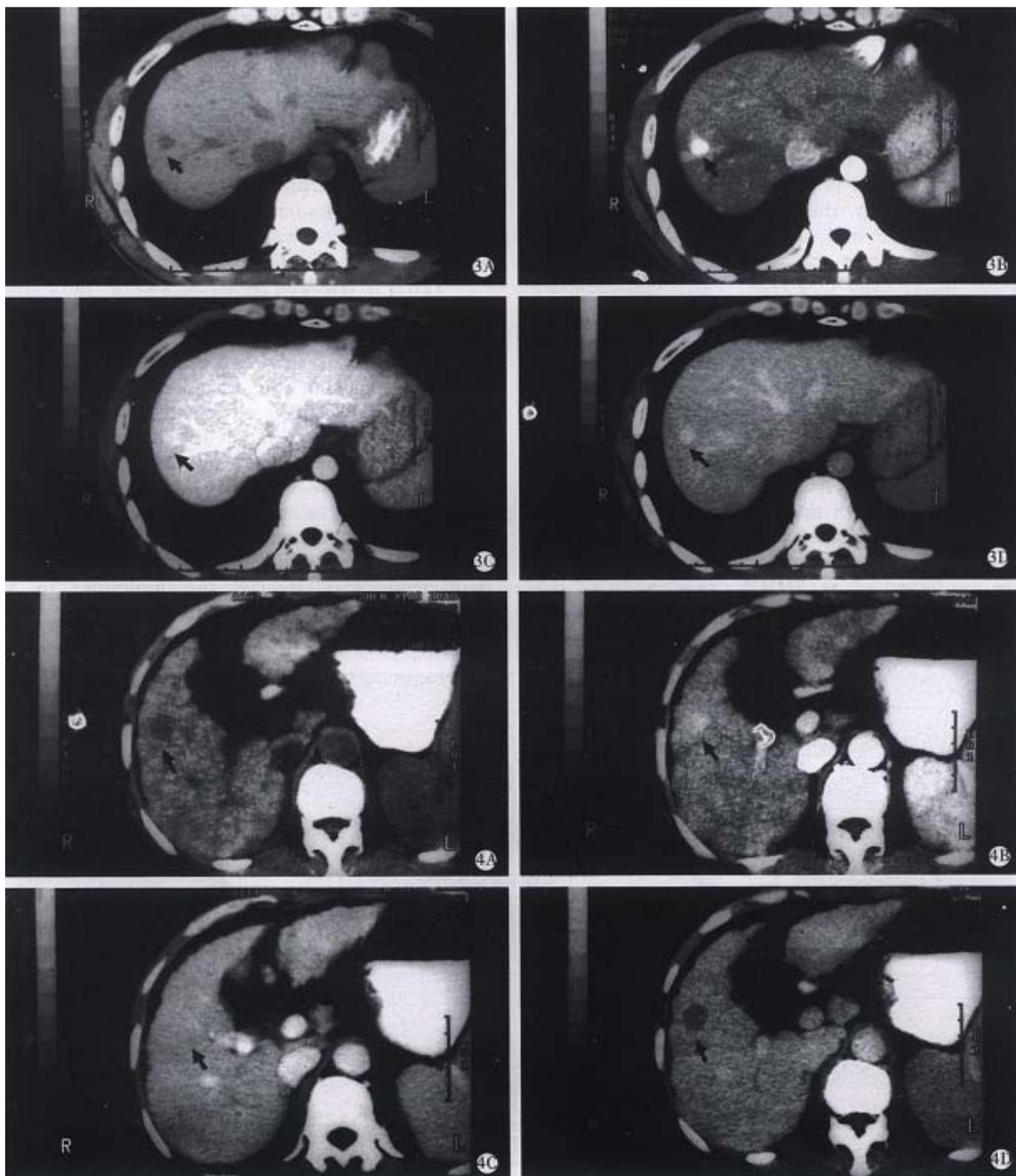
A. Arterial phase CT scan demonstrated the peripheral nodular enhancement of the lesion in the left lobe (arrow).

B. Portal venous phase CT scan demonstrated the lesion was opacified fully, its dense was higher than normal liver parenchyma (arrow).

**Figure 2** SHCC confirmed by surgery.

A. Arterial phase CT scan demonstrates the lesion enhanced homogeneously and became hyperdense (arrow).

B. Portal venous phase scan demonstrated the lesion was hypodense homogeneously (arrow).



**Figure 3** SHHE confirmed by surgery.

- A. Plain scan demonstrated a hypodense lesion in the right lobe, the edge was unclear (arrow).
- B. Arterial phase CT scan demonstrates the lesion enhanced homogenously and became hyperdense (arrow).
- C. Portal venous phase CT scan demonstrated the lesion remained hyperdense (arrow).
- D. Delay phase scan demonstrated the same finding as portal venous phase scan (arrow).

**Figure 4** SHCC confirmed by surgery.

- A. Plain scan demonstrated a small hypodense lesion in the right lobe, the edge was clear (arrow).
- B. Arterial phase CT scan demonstrates the lesion enhanced homogenously and became hyperdense (arrow).
- C. Portal venous phase CT scan demonstrated the lesion was isodense and could not be found (arrow).
- D. Delay phase scan demonstrated the lesion was hypodense with clear boundary (arrow).

Depending on the enhancement patterns of SHHE and SHCC, the following findings were helpful in the diagnosis of SHHE: ① peripheral globular enhancement on the first phase; ② homogeneous hyperdensity on the first phase and maintaining to the second phase or/and the third phase; ③ homogeneous hyperdense on the first phase and isodense on the second phase and the third phase (except the cases associated with fatty liver). Forty of 42 SHHE had the above-mentioned findings (95.24%), the other two was difficult to diagnose.

MRI was considered to be the most accurate method in the diagnosis of HHE among the imaging modalities with an accuracy of 95%<sup>[9]</sup>, helical CT could also reflect sufficiently the blood supply of SHHE, thus helping with a definite diagnosis. The result of our study showed that the accuracy of diagnosis with helical CT was similar to that of MRI, and significantly superior to that of conventional CT especially in the portal venous phase scan for the differential diagnosis. Two phase

scan should be routinely used, the addition of the third phase scan or multiphase scan would be the choice for further confirmation, and if the diagnosis is still unclear, MRI ought to be the preference.

## REFERENCES

- 1 Zhong KR. CT of the abdomen. 1st Ed. Shanghai: *Shanghai Medical University Publisher*, 1993:47
- 2 Berland LL. Slip-ring and conventional dynamic hepatic CT: contrast material and timing considerations. *Radiology*, 1995;195(2):1-8
- 3 Freeny PC, Marks WM. Hepatic hemangioma: dynamic bolus CT. *AJR*, 1986; 147(4):711-719
- 4 Heiken JP, Brink JA, Vannier MW. Spiral CT. *Radiology*, 1993;189(3): 647-656
- 5 Hanafusa K, Ohashi I, Yoshiro H, Suzuki S, Shibuya H. Hepatic hemangioma: findings with two-phase CT. *Radiology*, 1995;196(4):465-469
- 6 Gaa J, Saini S, Ferrucci JT. Perfusion characteristics of hepatic cavernous hemangioma using intravenous CT angiography (IVCTA). *Eur J Radiol*, 1991; 12(2):228-233
- 7 Itai Y, Ohtomo K, Kokubo H, Yoshida M, Minami M, Yashiro N. Well defined, dense and continuously spreading enhancement on single level dynamic CT of the liver: a characteristic sign of hepatic cavernous hemangioma. *Fortschr Roentgenstr*, 1989;151(4):697-701
- 8 Matsui O, Kadoya M, Kameyama T, Yoshikawa J, Takashima T, Nakanuma Y *et al.* Benign and malignant nodules in cirrhotic livers: distinction based on blood supply. *Radiology*, 1991;178(2):493-497
- 9 Whitney WS, Herfkens RJ, Jeffery RB, McDonnell CH, Li KCP, Van Dalsem WJ *et al.* Dynamic breath-hold multi-planner spoiled gradient-recalled MR imaging with gadolinium enhancement for differentiating hepatic hemangiomas from malignancies at 1.5T. *Radiology*, 1993;189(3):863-870

# Effects of Fuzheng Huayu 319 recipe on liver fibrosis in chronic hepatitis B \*

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**Subject headings** liver cirrhosis/therapy; hepatitis B; Fuzheng Huayu; procollagen; hyaluronic acid; metalloproteinases; hydroxyproline

## Abstract

**AIM** To investigate clinic effects of Fuzheng Huayu 319 recipe (319 recipe) on liver fibrosis in chronic hepatitis B.

**METHODS** Ninety-five patients with chronic hepatitis B were divide into the treated (63 cases) and control (32 cases) group, and orally administrated with 0.5g 319 capsule or 0.5g Dahuang Zhachong pill tid for 3 months, respectively. The liver functions and serological fibrotic markers were observed before and after treatment, 12 cases in the treated group were examined with liver biopsy.

**RESULTS** Three hundreds nineteen recipe could remarkably decreased serum ALT level and total bilirubin and significantly improve serum albumin and A/G ratio. Its effects were better than Dahuang Zhachong pill. Before treatment, patientsiäserum monamine oxidase activities, tissue inhibitor of metalloproteinase (TIMP)-1, procollagen type III and laminin were all higher than those of health peoples. These levels decreased remarkably after treatment, and urine hydroxyproline level increased significantly ( $P < 0.001-0.05$ ). Compared with the control, the improvement in treated group was better than that in the control except TIMP-1. According to the scoring system for staging of chronic hepatitis, the fibrotic extents of 7 cases among 12 cases examined by liver biopsy decreased remarkably (1 case decreased by 3 scores, 5 by 2 scores, 1 by 1 score).

**CONCLUSION** Fuzheng Huayu 319 recipe had good therapeutic effects on chronic hepatitis B, it could reverse the development of liver fibross to some extent. In general its effects were better than that of Dahuang Zhachong pill.

## INTRODUCTION

Our previous study showed that Fuzheng Huayu 319 recipe had good effect on the patients with post-hepatic cirrhosis<sup>[1]</sup>. In order to study this recipe actions on chronic liver diseases and liver fibrosis, 95 patients with chronic hepatitis B (CHB) were treated and observed. Among them 63 cases were treated with 319 recipe, 32 with Dahuang Zhachong pill as controls. Patients' liver functions and serum fibrotic markers were observed and the liver histological changes in 12 cases were examined by biopsy before and after treatment.

## OBJECTS AND METHODS

### Patients

Ninety-five in-patients were included, 11 female and 84 male, aged from 20 to 65 years, averaging 37.6. They were clinically diagnosed as CHB according to the revised standard of the sixty Shanghai national virus hepatitis symposium in 1990. Each case had at least one positive serum HBV marker. Among them, there were 63 cases with HBeAg positive, 33 with jaundice, 29 with vascular spider or liver palm, 37 with splenomegaly. 26 cases were examined randomly by laparoscopy or biopsy and the histopathological assessment was in accord with clinic diagnosis.

### Groups and therapy

Ninety-five patients were randomly divided into the treated group (63 cases) and the control group (32 cases). The patients' ratio of sex, range of age, course of disease and liver functions were paralleled between two groups.

The treated group orally took 1.5g 319 recipe, tid, composed of Semen Persicase, Radix Salviae, Miltiorrhiae, Cordyceps Sinnensis (Berk) Sace, Pollen Pini, etc. and was purified and made into capsules (0.3g per capsule, equivalent to 8.17g raw herbs) by Shanghai Zhonghua Pharmaceutical

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\* Project supported by the National 8-5 Scientific Program, No. 859190402

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Received 1997-12-18

Factory. The control group orally took 1.5g Dahuang Zhachong pill, tid, which was one of the traditional typical recipes for supporting healthy energy and eliminating blood stasis effectively, produced by Shanghai First Herb company (lot No.91221A1) and smashed to powder and made into capsules (0.3g per capsule). Both groups were treated for 3 months, accompanying with the same expectant treatment and observed by double blind method and continuously observed for half a year after the treatment.

#### **Observed items and methods**

After thoroughly medical history inquiring, physical examination and the course of treatment, the blood samples were collected from patients with empty stomach in the morning before and after treatment and the following items were detected such as HBV markers, liver functions, monamine oxidase (MAO) activity and fibrotic markers etc. Also 24 h urine amount was recorded and measured for fibrotic markers. The normal serum samples were collected from healthy donors, whose sex ratio and age range were paralleled to the patients.

**MAO activity** colorimetric assay, the kit was from Wenzhou Yilikang Biology Technique Company.

**Tissue inhibitor of Metalloproteinases-1 (TIMP-1) content** ELISA, the kit was from Japan Fuji Medical Industrial Company.

**Type-III-procollagen-N-peptide (P-III-P) content** ELISA, the kit was from Jingshi Medical Technique Development Institute.

**Type IV Collagen (IV-C) content** ELISA, the kit was from Japan First Chemical Medicine Company.

**Hyaluronic Acid (HA) content** radioimmunoassay, the kit was from Shanghai Navy Medical Institute.

**Laminin (LM) content** Radioimmunoassay, the kit was from Shanghai Navy Medical Institute.

**Hydroxyproline (Hyp) content (Jamall's method)** <sup>[2]</sup>. **Laparoscopy and biopsy** Twelve cases in the treated group were examined with liver biopsy before and after treatment, among them 6 were also examined with laparoscopy. The patients' liver, spleen, peritoneum, mesentery, etc. were observed under STORZ fiber laparoscope and photographed. Also under laparoscopy, liver tissues were quickly taken in the point on the diaphragmatic surface of liver left lobe 2.5 cm away from falciform ligament and liver edge. The needle liver biopsy was punctured with 1 second paracentesis.

**Liver histology** The liver tissue samples were fixed

in 10% neutral formalin buffer, and embedded in paraffin regularly, continuously cut to 5  $\mu$ m slice and stained with HE. The pathologic examination standard was observed according to the grading and staging of the draft "The project of preventing and treating virus hepatitis" in 1995<sup>[3]</sup>. The inflammation activities were classified into 5 grades (G0-4), fibrosis degrees into 5 stages (S0-4).

Rabbit anti-bovine type I collagen (I-C) serum, rabbit anti-human type III collagen (III-C) anti-serum, and rabbit anti-human IV-C serum were from the pathology department, Shanghai Medical University and rabbit anti-human LM serum from Sigma Company.

The paraffin sections of liver samples were deparaffined, gradient dehydrated with alcoholic solution, incubated at 37°C for 1h with H<sub>2</sub>O<sub>2</sub> methyl alcohol so the endogenous peroxidase was removed, and digested with 0.2% peptic solution at 37°C for 1 h, stained with regular PAP method. The negative and blank control were made.

**Statistical analysis**  $\chi^2$ , *t* test Ridit and *u* test methods were used.

## **RESULTS**

### **The liver function and clinic manifestation**

**ALT activities** There were 52 cases were with increased ALT activities in the treated group before treatment, 48 (recovery rate 92.3%) recovered in 14 d-80 d after treatment, the average recovery time was 33.8 d. 24 had higher ALT activities in the control before treatment, 19 (79.2%) recovered after treatment in 14 d-90 d, the average recovery time was 37.0 d.

**Serum total bilirubin** In the treated group, 30 cases were higher before treatment, 23 (76.7%) became normal after treatment, 6 decreased but 1 increased. In the control, 12 cases were higher before treatment, 8 (66.7%) became normal after treatment, 2 decreased and 2 increased.

**Serum albumin** In the treated group, the serum albumin (Alb) content was 40.04 $\pm$ 5.2 before treatment, and 45.3 g/L $\pm$ 5.1 g/L ( $P$ <0.001) after treatment. Among them 13 cases were less than 35 g/L before treatment, and 11 were normal after treatment. In the control, that was 39.0 $\pm$ 4.9 before treatment and 39.7 g/L $\pm$ 5.3 g/L after treatment. Six cases were less than 35 g/L before treatment, 2 were normal after treatment. The Alb differences between two groups before and after treatment were 4.9 g/L $\pm$ 4.9 g/L, 0.7 g/L $\pm$ 2.5 g/L respectively. Significant difference ( $P$ <0.001) was found.

**Serum-A/G ratio** In the treated group, it was 1.46 $\pm$ 0.43, and 1.72 $\pm$ 0.48 respectively before and

after treatment ( $P<0.001$ ). Twenty-one cases were less than 1.3 before treatment, 13 were normal after treatment. In the control, it was  $1.32\pm 0.34$  and  $1.31\pm 0.34$  before and after treatment, 8 cases were less than 1.3, 1 case normal. A/G difference between two groups before and after treatment were  $0.26\pm 0.34$ ,  $-0.01\pm 0.21$  respectively and significant difference was found ( $P<0.001$ ).

The main symptoms and signs in both groups were improved obviously, such as fatigue, abdominal flatulence, nausea, yellow in eyes and hypochondriac pains etc., but no significant difference was found (Table 1).

**HBV markers** Before treatment there were 52 cases with positive HBsAg and HBeAg. 36 cases in the treated group, 2 or 16 of them had HBsAg or HBeAg negative conversion (5.6% or 41.0%) after treatment respectively. For 16 in the control, 1 was with HBsAg and 6 with HBeAg negative conversion (6.3% or 37.5%). No case had HBsAb negative conversion in both groups. There were 8 cases in the treated group (20.5%) and 1 in the control (6.3%) with positive HBeAb conversion, but no significant difference.

**Follow-up observation** Half a year after the treatment, ALP of 5 among the 48 recovered cases relapsed in the treated group, and 7 of 19 cases in the control relapsed. The HBsAg and HBeAg negative conversion rates were 2.8% and 38.5% in the treated group, 0 and 25% in the control respectively. For the therapeutic evaluation, 54 cases were effective, 9 not effective, and the effective rate was 85.7% in the treated group. 21 cases were effective, 11 not effective, and the effective rate was 65.6% in the control (Tables 2, 3).

#### Serum fibrotic markers

MAO activities Compared with normal people, patients' serum MAO activities increased obviously before treatment, 22 cases (59.5%) among 37 patients were more than 54U (normal peoples'  $\bar{x}+2SD$  the same as following).

**TIMP-1** Serum TIMP content increased obviously before treatment, in 16 cases (38.1%) with more than 204 ng/ml ( $-379$  ng/ml) among 42 cases.

**Table 1 The main symptoms and signs before and after treatment (cases)**

Group	n		Fatigue	Anorexia	Abdominal flatulence	Nausea	Yellow urine	Yellow eye	Hypochondriac pain
Treated	63	Before	34	20	21	13	16	21	21
		After	1	1	1	1	3	3	4
Control	32	Before	24	10	9	12	6	9	10
		After	1	1	1	0	1	2	1

**Table 2 Serum fibrotic markers in patients with Chronic hepatitis B**

Group	MAO(u)	TIMP	P-III-P	IV-C	HA	LM(ng/ml)
Normal people	$28\pm 13$ (20)	$164\pm 20$ (20)	$0.31\pm 0.12$ (15)	$99\pm 23$ (60)	$38\pm 36$ (60)	$234\pm 69$ (60)
Chronic hepatitis B	$63\pm 29^d$ (37)	$192\pm 39^d$ (42)	$1.41\pm 2.19^b$ (34)	$494\pm 300^d$ (46)	$362\pm 235^d$ (61)	$358\pm 64^d$ (61)

<sup>b</sup> $P<0.01$ , <sup>d</sup> $P<0.001$ , vs control. ( ) inside number was patient cases.

**Table 3 The serum fibrotic markers and urinary hydroxyproline ( $\bar{x}\pm s$ )**

Group		MAO(U)	TIMP	P-III-P	IV-C	HA	LN(ng/ml)	Urine Hyp
Treated	Cases	30	24	20	29	44	42	29
	Before	$64\pm 30$	$184\pm 58$	$1.81\pm 3.9$	$547\pm 345$	$377\pm 293$	$369\pm 73$	$21.6\pm 8.9$
	After	$28\pm 16^d$	$153\pm 54^b$	$0.80\pm 1.69^a$	$386\pm 212^b$	$210\pm 241^d$	$208\pm 85^d$	$24.5\pm 7.8^a$
	Difference	$36\pm 27^c$	$31\pm 44$	$1.01\pm 2.08$	$161\pm 262$	$167\pm 199$	$161\pm 116$	$3.2\pm 7.5$
Control	Cases	7	18	14	17	17	17	17
	Before	$60\pm 30$	$204\pm 59$	$1.57\pm 1.46$	$404\pm 179$	$356\pm 313$	$382\pm 68$	$20.7\pm 7.3$
	After	$47\pm 33$	$139\pm 39^d$	$1.71\pm 3.14$	$333\pm 150^a$	$220\pm 218^a$	$282\pm 103^d$	$22.6\pm 5.6$
	Difference	$13\pm 14$	$65\pm 47$	$0.14\pm 2.51$	$72\pm 121$	$136\pm 251$	$99\pm 92$	$2.0\pm 6.5$

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , <sup>d</sup> $P<0.001$ , vs before treatment. <sup>c</sup> $P<0.05$  vs control.

**P-III-P and IV-C** Serum P-III-P and IV-C increased obviously before treatment, in 17 cases (50%) with more than 0.55 ng/ml (-24.22 ng/ml) P-III-P in 34 cases, in 45 cases (97.8%) with more than 145 ng/ml (-1511 ng/ml) in 46 cases.

**HA** Patients' serum HA content increased obviously before treatment, in 44 cases (72.1%) with more than 110 ng/ml. In the treated group, there were 31 cases with HA levels more than 110 mg/ml before treatment, after treatment 14 cases recovered, 15 decreased, 1 had no change and 1 increased. In the control there were 13 cases with HA levels more than 110 mg/ml before treatment; 5 recovered, 6 decreased, 1 had no change and 1 increased after treatment.

**LM** The serum LM contents of patients increased significantly compared with the normal ( $P < 0.001$ ). There are 31 cases among 61 patients with LM contents more than 372 ng/ml (-540 ng/ml). All 21 cases in the treated group with LM more than 372 ng/ml recovered. For 10 cases in the control with more than 372 ng/ml LM, 6 recovered, 3 decreased and 1 increased. After treatment both groups had less MAO, TIMP-1, P-III-P, IV-C, HA and LM contents than before treatment, but the treated group decreased more significantly than the control ( $P < 0.05$ ).

**Urinary Hyp** In the treated group urinary Hyp increased significantly after treatment compared with that before treatment ( $P < 0.05$ ). It slightly increased in the control, but there was no significant difference.

#### **The histological changes of 12 cases in the treated group**

**Laparoscopic examination** One case was diagnosed as chronic persistent hepatitis (CPH) according to the old classification, the liver right lobe enlarged slightly; the edge was a little bit blunt, the small vessels on the ligamentum teres hepatis proliferated slightly and the spleen swelled mildly before treatment. After treatment the vessel proliferation attenuated. The livers of 4 patients with chronic active hepatitis (CAH) swelled and enlarged to varying degrees before treatment. The liver edges were slightly blunt, the color was pink or red, the most of the surface capsule thickened, the stripe was not clear enough. Among these 4 cases, 1 showed wave wrinkle, 2 had mass ( $\pm 4$  mm diameter), 1 osmosised fibrin, and liver surface of 1 case covered by the greater omentum. After treatment, in 1 case the liver surface was separated from omentum (Figure 1). In another case with early liver cirrhosis, the right and left lobe of front liver swelled, accompanied with blunt edge, dark red color, thick capsule in the liver surface, vague

stripe, 4 mm-6 mm diameter mass surrounded by the fibrous bundle, edemaed ligamentum teres hepatis, swollen spleen, and slightly varicose veins in the great omentum and mesentery etc. After treatment the swelling of the liver lobe attenuated, fibrous bundles on liver surface reduced and the varicose vein on the omentum and mesentery recovered.

#### **Pathological observation**

After treatment the liver inflammatory grades of 4 cases had decreased (1 decreased by 3 scores, 1 by 2 scores, 2 by 1 score), 6 cases had no obvious changes, and 2 cases increased by 1 score. Therefore, the improvement of liver inflammation was not significant on the whole ( $P > 0.05$ ). But the improvement of the liver fibrotic stages was significant ( $P < 0.005$ ). 7 cases improved (1 decreased by 3 scores, 5 by 2 scores, 1 by 1 score), 4 cases had no marked change, only 1 case increased by 1 score (Table 4).

**Table 4 The liver inflammatory grades and fibrotic stages**

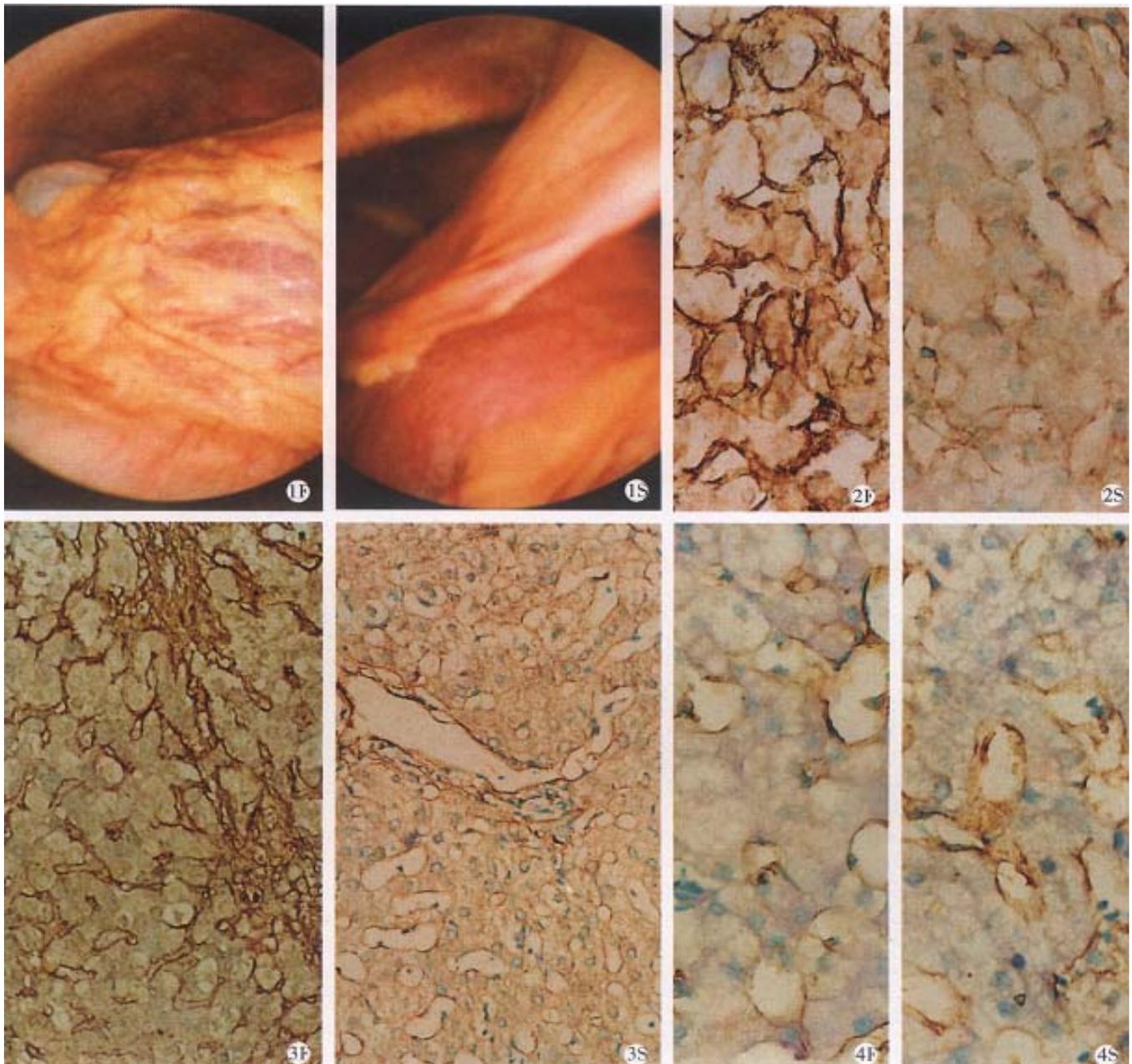
	Inflammatory grades					Fibrotic stages				
	G0	G1	G2	G3	G4	S0	S1	S2	S3	S4
Before treatment	0	1	7	3	1	1	1	3	5	2
After treatment	1	2	7	1	1	3	5	1	2	1 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , vs after before treatment.

#### **Immunohistochemical observation**

**Type I collagen** Before treatment, I-C was positively stained along the most liver sinusoid with a ground circular shape. The portal areas became wider, surrounded by hepatocyte necrosis and formed fibrous septum stretched from the portal areas to hepatic lobules. After treatment, these collagen became smaller, thinner or discontinuous. Stained areas decreased in 1/3 of cases, unchanged in 1/2 of cases, increased in only 1 case. The portal areas decreased in 1/2 of cases, the others unchanged. Although positive stained in hepatocellular necrotic areas, 6 of 8 cases positive stained in fibrous septum before treatment showed either negative stain or decreased areas, none increased after treatment (Figure 2).

**Type III collagen** Before treatment, most III-C with ground circular shape expressed around the sinusoids as well as I-C, in widen portal areas, in hepatocyte necrotic areas along limit plate and in fibrous septum. After treatment, the positive stains did not change obviously around sinusoid, but decreased, unchanged or increased respectively for 1/3 of cases in the portal areas. Of the 9 cases positive stained before treatment in fibrous septum, 6 cases became negative or decreased, 1 unchanged and 2 increased slightly (Figure 3).



**Figure 1** Liver surface of patients' with CBH under laparoscopy before (F) and after (S) 319 recipe treatment.

**Figure 2** Type III collagen expression in the liver of patients' with CBH under laparoscopy before (F) and after (S) 319 recipe treatment ( $\times 200$ ).

**Figure 3** Type I collagen expression in the liver of patients' with CBH under laparoscopy before (F) and after (S) 319 recipe treatment ( $\times 200$ ).

**Figure 4** Type IV collagen expression in the liver of patients' with CBH under laparoscopy before (F) and after (S) 319 recipe treatment ( $\times 200$ ).

**Type IV collagen** Before treatment, IV-C around sinusoids expressed as thicker or thinner circles, some of them were thin and continuous. In the portal areas, hepatocellular necrosis areas and fibrous septum, IV-C expressed as I-C and III-C. After treatment, IV-C expression around sinusoid in half of the cases increased, or became grosser or become continuous from discontinuous, none decreased. 10 cases were stained positive for IV-C in fibrous septum before treatment, and after treatment, 6 became negative or decreased, 2 unchanged, 2

slightly increased (Figure 4).

**Laminin** Before treatment, LM was stained slightly positive, scattered around sinusoids and in fibrous septum like thin circle or discontinuous string. But in sinusoids with hepatocyte space meal necrosis and in the portal areas, LM expressed strong positive and thick circles. After treatment, among 10 cases with LM positive stain around sinusoids, 2 decreased, 6 unchanged, 2 slightly increased. The changes in the portal areas were

similar to those around sinusoids. Among 9 cases with positive staining in fibrous septum, 4 decreased or became negative, 4 unchanged, 1 increased.

## DISCUSSION

The histological staging for fibrotic degrees was emphasized on the new classification of chronic hepatitis<sup>[4]</sup>. WANG reported that although most inflammation activities decreased after treatment, fibrosis still existed even developed in the patients observation of twice liver biopsy<sup>[5]</sup>. It is suggested that it is necessary to treat fibrosis during chronic hepatitis.

319 recipe had good therapeutic effect on post-hepatic cirrhosis. In this study it had been found that 319 recipe could decrease serum ALT activities and serum bilirubin, improve serum Alb and A/G ratio. The effects on CHB were better than Dahuang Zhachong pill.

The fibrotic degrees could demonstrate pathological development of chronic hepatitis. Because the difficulty and limit of liver biopsy, serum fibrotic markers important for diagnosis and evaluation of drug effects<sup>[6]</sup>. Serum MAO was first used in clinic<sup>[7]</sup>, later serum P-III-P, LM, IV-C, HA and TIMP-1 were applied<sup>[8-12]</sup>. But these markers could be influenced by body connective tissue metabolism, and single marker lacked specificity, so recently it is maintained that multi-markers should be combined for practice<sup>[6]</sup>. In this paper, serum MAO, TIMP, P-III-P, IV-C, HA and LM in patients all increased remarkably compared with normal people. If " $\bar{x} \pm 2SD$ " was set as abnormal value, their abnormal ratios were IV-C (97.8%), HA (72.1%), MAO (59.4%), LM (50.8%), P-III-P (50.0%), TIMP (38.1%), similar to other reports<sup>[10-12]</sup>. After the treatment, however, these 6 markers were improved to some extents. Except TIMP, the other 5 markers improvement in the 319 recipe treated group were more obvious than the control, especially the serum MAO and LM. Urinary Hyp excretion increased also after the treatment. It is shown that 319 recipe not only

improved patients' liver functions, but also decreased serum fibrotic markers which pathologically increased in CHB. Its comprehensive effects are better than Dahuang Zhachong pill.

Among 11 cases with fibrotic stage 1-4 in the treated group, 7 cases had obviously decreased fibrotic septum, the enlarged portal areas reduced, and hepatic lobule structure nearly recovered. In 5 cases, fibrotic stages decreased by 2 scores, 1 case by 3 stages and 1 case by 1 stage. Type I, III and IV collagen expressions in portal areas and fibrotic septum in most samples decreased or became negative, but in some cases the changes were not obvious. LM expressions in these places also decreased and were paralleled to pathological staging. All these indicated that 319 recipe could inhibit CBH fibrotic development, improve degradation of proliferated fibrous tissues in liver, and had a significant application on inhibition of liver cirrhosis.

## REFERENCES

- 1 Liu P, Liu C, Hu YY, Zhu JL, Xue HM, Xu LM. The clinical investigation of Fuzheng Huayu 319 recipe effects on post hepatic cirrhosis. *Chin J Inter Med*, 1996;16(8):459-462
- 2 Jamal IS, Finelli VN, QUE HEE SS. A simple method to determine nanogram level of 4 hydroxyproline in biological tissues. *Anal Biochemistry*, 1981;112:70-75
- 3 Zai WR, Wang TL, Zhou XJ, Zhang TH. Diagnosis, grading and staging for chronic hepatitis. *Chin J Dis*, 1996;16(5):277-281
- 4 Desmet VJ, Gerber M, Hoffnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*, 1994;19:1513-1520
- 5 Wang TL, Liu X, Zhao JB, Dong J, Chen FX, Zhang M *et al.* Investigation of classification for chronic hepatitis: Pathological analysis about liver biopsy in 316 cases. *J Diagn Pathol*, 1994;1(1):19
- 6 Liang KH, Li SB. *Hepatology*, 1st edition, Beijing: Peoples' Health Press, 1995:570-830
- 7 Itokenyiqi. Diagnostic meaning of monamine oxidation for liver fibrosis. *Clin Pathol*, 1973;21:143
- 8 Rohde H, Vergas L, Hahn E, Timpl R. Radioimmunoassay for type III collagen peptide and its application to human liver: collagen type IV, laminin and fibronectin. *Gut*, 1980;21(1):63-70
- 9 Niemela O, Risteli L, Sotaniemi EA, Risteli J. Type IV collagen and laminin-related antigens in human serum in alcoholic liver disease. *Eur J Clin Invest*, 1985;15(3):132-137
- 10 Engstrom A, Laurent UBG, Laurent TC, Lavrent TC. Concentration of sodium hyaluronate in serum. *Scan J Clin Lab Invest*, 1985;45(6):497-504
- 11 Murawaki Y, Yamamoto H, Kawasaki H, Shinia H. Serum tissue inhibitor of metalloproteinase in patients with chronic liver disease and with hepatocellular carcinoma. *Clin Chim Acta*, 1993;218(1):47
- 12 Hu PF. Significance of serum hyaluronic acid for chronic hepatitis and liver cirrhosis. *Liver*, 1993;34:859-867

# Effects of Dangshen on isolated gastric muscle strips in rats \*

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**Subject headings** Dangshen; stomach; muscle, smooth; muscle contraction; receptors, cholinergic; rats

## Abstract

**AIM** To study the effects of Dangshen [dried root of *Codonopsis Pilosula* (Franch) Nannf.] on contractile activity of isolated gastric muscle strips in rats and its possible mechanism involved.

**METHODS** Each isolated gastric muscle strip was put in a tissue chamber containing 5 ml Krebs solution, constantly warmed by water jacket at 37°C and supplied with a mixed gas of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After incubating for 1h with 1g tension, Dangshen of varied concentration was added cumulatively in the tissue chamber at intervals of 2 minutes. The isometrical response was measured on ink-writing recorders.

**RESULTS** Dangshen dose dependence increased the resting tension of longitudinal muscle (LM) of fundus ( $r = 0.96, P < 0.01$ ), the mean contractile amplitude of circular muscle (CM) of the stomach body ( $r = 0.87, P < 0.05$ ) and CM of antrum ( $r = 0.98, P < 0.01$ ), and the motility index CM of pylorus ( $r = 0.87, P < 0.05$ ). Atropine ( $5 \times 10^{-8}$  mol/L) or Hexamethonium ( $10^{-5}$  mol/L) or Indomethacin ( $5 \times 10^{-7}$  mol/L) was given 2 minutes before the administration of Dangshen, it did not abolish its dose related manner. Atropine apparently reduced the increasing action of 10% and 30% Dangshen on the resting tension of LM of fundus ( $P < 0.05$ ), 30%, 100% and 200% Dangshen on bodied strips ( $P < 0.05$ ), 100% and 200% Dangshen on antral strips ( $P < 0.05$ ). Hexamethonium reduced the increasing action of 10% and 30% Dangshen on the resting tension of LM of fundus ( $P < 0.05$  and  $P < 0.05$ ), 30%, 100% and 200% Dangshen on

bodied strips ( $P < 0.05$ ), and 100% and 200% Dangshen on pyloric strips ( $P < 0.05$ ). Indomethacin inhibited the effect of 10% Dangshen on the resting tension of LM of fundus ( $P < 0.05$ ), but did not affect the exciting action of Dangshen on strips of body, antrum and pylorus.

**CONCLUSION** The results showed that Dangshen possessed exciting action on the isolated gastric smooth muscle strips of the rat. The exciting action of Dangshen was partially mediated via cholinergic M and N receptors.

## INTRODUCTION

Recently, close attention has been paid to the Chinese drugs that affect the alimentary tract and are used to treat gastrointestinal motility disorders. Dangshen [*Codonopsis Pilosula* (Franch) Nannf.] is a main Chinese drug to treat spleen and stomach disorders. Previous studies had shown that Dangshen regulated gastric basic electrical rhythm disorder on stress condition and inhibited gastric motility on acute gastric ulcer in rats<sup>[1]</sup>. The effect of Dangshen on isolated gastric muscle strips in rats has not been published, so we have studied the action of Dangshen on gastric smooth muscle in rats and the possible mechanism involved.

## MATERIALS AND METHODS

Dangshen was ground into coarse powder, boiled in distilled water, filtered and made 100% extract solution. The 100% Dangshen extract solution was dispensed in the concentration of 1%, 2%, 7%, 20%, 70% and 100% respectively (The drug was made and identified by Gansu Institute for Drug Control).

Wistar rats, weighing 250 g-300 g were fasted with free access to water for 24 hours. Then they were hit to lose consciousness and the whole stomach was removed. The stomach was opened along greater curvature and four strips (8mm×2mm) were cut, parallel to either the longitudinal or circular fibers and named longitudinal muscle (LM) of fundus, circular muscle (CM) of body, antrum and pylorus<sup>[2]</sup>. Each strip with the mucosa removed was suspended in a tissue chamber containing 5 ml Krebs solution, constantly warmed by circulating water jacketed at 37°C and supplied with a mixed gas of 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

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**Project Supported by the Natural Scientific Foundation of Gansu Province, No.ZR-94-085.**

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**Received** 1998-03-08

One end of the strip was fixed to a hook on the bottom of the chamber while the other end was connected by a thread to an external isometric force transducer (JZ-BK, BK) at the top. Preparation were subjected to 1g load tension and washed with 5 ml Krebs solution every 20 minutes. The contractions of gastric strips in 4 tissue chambers were simultaneously recorded on ink-writing recorders (LMS-ZB, Cheng-Du)<sup>[3]</sup>. After incubating for 1 h, Dangshen (1%, 2%, 7%, 20%, 70% and 100%) were added in a 5 ml bath continuously at intervals of 2min, so that the cumulative concentration reached 1%, 3%, 10%, 30%, 100% and 200%. Atropine ( $5 \times 10^{-8}$  mol/L) or Hexamethonium ( $10^{-5}$  mol/L) or Indomethacin ( $5 \times 10^{-7}$  mol/L) was given 2 minutes before administration of Dangshen.

We measured the resting tension of LM of fundus, the mean contractile amplitude of CM of body and antrum and the motility index [MI=  $\Sigma$  (amplitude $\times$ duration)] of CM of pylorus. Frequency of contraction was calculated by counting the contraction waves. The results were demonstrated by the increasing percentage (%) of the control spontaneous contraction ( $\bar{x} \pm s_{\bar{x}}$ ). The data were analyzed with the Student's *t* test. The correlation coefficients were calculated, and value of  $P < 0.05$  was considered to be significant.

**RESULTS**

**Effect of Dangshen on spontaneous contraction of fundic strips**

Dangshen dose dependence increased the resting tension of LM of fundus ( $r = 0.96, P < 0.01$ ). Atropine ( $5 \times 10^{-8}$  mol/L) or Hexamethonium ( $10^{-5}$  mol/L) or Indomethacin ( $5 \times 10^{-7}$  mol/L) did not affect its action in dose related manner ( $r = 0.99, P < 0.01$ ). Atropine or hexamethonium reduced the effect of 10% and 30% Dangshen ( $P < 0.05$ ), while Indomethacin inhibited the effect of 10% Dangshen on the resting tension of LM of fundus significantly ( $P < 0.05$ , Table 1).

**Effect of Dangshen on the spontaneous contraction of bodied strips**

Dangshen dose dependence increased the mean

contractile amplitude of CM of body ( $1.7\% \pm 3.9\%$ ,  $4.2\% \pm 3.3\%$ ,  $7.5\% \pm 6.8\%$ ,  $15.7\% \pm 5.4\%$ ,  $16.6\% \pm 4.9\%$  and  $32.2\% \pm 11.8\%$ , respectively,  $r = 0.87, P < 0.05$ ). Atropine ( $5 \times 10^{-8}$  mol/L) or hexamethonium ( $10^{-5}$  mol/L) apparently reduced the increasing action of 30%, 100% and 200% Dangshen ( $-1.5\% \pm 4.3\%$ ,  $-1.5\% \pm 5.3\%$ ,  $-2.3\% \pm 3.9\%$ , and  $-1.9\% \pm 3.8\%$ ,  $-2.4\% \pm 3.1\%$ ,  $-0.7\% \pm 5.8\%$  respectively,  $P < 0.05$ ), while Indomethacin ( $5 \times 10^{-7}$  mol/L) did not apparently affect the increasing action of Dangshen (Figure 1). All drugs did not affect the contractile frequency of bodied strips significantly.

**Effect of Dangshen on the spontaneous contraction of antral strips**

Dangshen dose dependence increased the mean contractile amplitude of CM of antrum ( $5.4\% \pm 5.5\%$ ,  $5.6\% \pm 5.1\%$ ,  $6.3\% \pm 6.1\%$ ,  $8.6\% \pm 6.2\%$ ,  $23.9\% \pm 7.5\%$  and  $43.8\% \pm 7.5\%$ , respectively,  $r = 0.98, P < 0.01$ ). Atropine ( $5 \times 10^{-8}$  mol/L) significantly reduced the increasing action of 100% and 200% Dangshen ( $-12.5\% \pm 8.6\%$  and  $-2.0\% \pm 11.4\%$ ,  $P < 0.05$ ), while Hexamethonium ( $10^{-5}$  mol/L) or Indomethacin ( $5 \times 10^{-7}$  mol/L) didn't affect the increasing action of Dangshen (Figure 2). All drugs did not significantly affect the contractile frequency of antral strips.

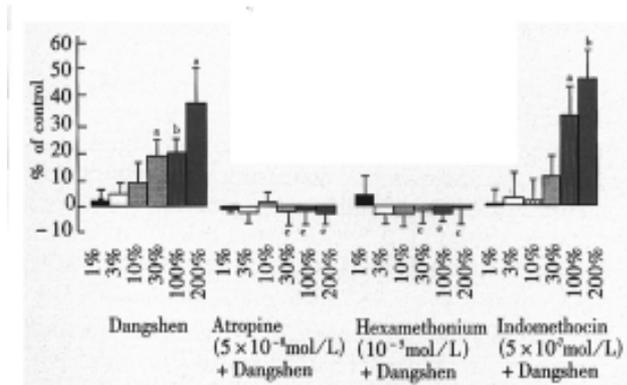
**Effect of Dangshen on the spontaneous contraction of pyloric strips**

Dangshen dose dependence increased the motility index of CM of pylorus ( $5.1\% \pm 9.7\%$ ,  $7.4\% \pm 5.0\%$ ,  $8.5\% \pm 7.1\%$ ,  $14.4\% \pm 6.5\%$ ,  $28.8\% \pm 4.1\%$  and  $38.8\% \pm 10.6\%$ , respectively,  $r = 0.87, P < 0.05$ ). Atropine ( $5 \times 10^{-8}$  mol/L) or hexamethonium ( $10^{-5}$  mol/L) significantly reduced the increasing action 100% and 200% Dangshen ( $5.3\% \pm 9.3\%$  and  $5.7\% \pm 7.7\%$ ;  $7.0\% \pm 6.9\%$  and  $3.4\% \pm 7.9\%$ , respectively,  $P < 0.05$ ). Indomethacin ( $5 \times 10^{-7}$  mol/L) didn't affect the increasing action of Dangshen (Figure 3). All drugs did not affect the contractile frequency of pyloric strips significantly.

**Table 1 Effect of Dangshen and antagonists plus Dangshen on the resting tension of the fundus in rats**

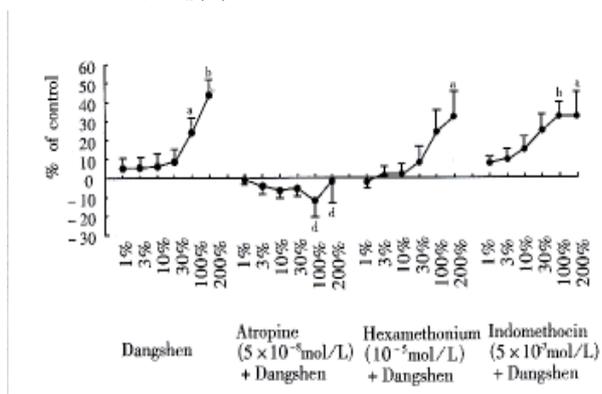
	1%	3%	10%	30%	100%	200%
Dangshen	0 (n = 12)	5.4±3.3 (n = 12)	38.8±6.9 <sup>b</sup> (n = 12)	75.8±11.9 <sup>b</sup> (n = 12)	153.8±17.9 <sup>b</sup> (n = 12)	163.3±20.0 <sup>b</sup> (n = 12)
Atropine ( $5 \times 10^{-8}$ mol/L)+Dangshen	1.0±0.7 (n = 15)	4.3±2.2 (n = 15)	18.0±4.8 <sup>b,c</sup> (n = 15)	38.0±10.5 <sup>b,c</sup> (n = 15)	120.3±21.9 <sup>b</sup> (n = 15)	132.3±23.0 <sup>b</sup> (n = 15)
Hexamethonium( $10^{-5}$ mol)+Dangshen	-1.8±1.3 (n = 17)	-1.5±1.8 (n = 17)	14.1±5.7 <sup>a,d</sup> (n = 17)	42.6±9.3 <sup>b,c</sup> (n = 17)	143.0±21.7 <sup>b</sup> (n = 17)	179.4±28.8 <sup>b</sup> (n = 17)
Indomethacin( $5 \times 10^{-7}$ mol/L)+Dangshen	0 (n = 14)	0 (n = 14)	14.3±5.4 <sup>a,c</sup> (n = 14)	42.1±14.9 <sup>a</sup> (n = 14)	116.4±24.1 <sup>b</sup> (n = 14)	157.8±29.9 <sup>b</sup> (n = 14)

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs Dangshen on the same concentration; 0, no effect;  $\bar{x} \pm s_{\bar{x}}$  (%) increase.



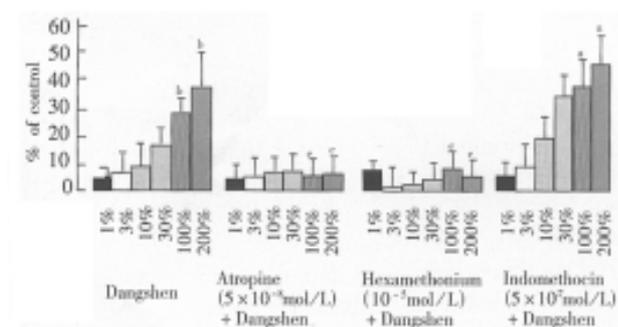
**Figure 1** Effect of Dangshen and antagonists plus Dangshen on the mean contractile amplitude of bodied strips in rats.

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control; <sup>c</sup> $P < 0.05$  vs Dangshen in the same concentration;  $\bar{x} \pm s_x$  (%) increase.



**Figure 2** Effect of Dangshen and antagonists plus Dangshen on the mean contractile amplitude of antral strips in rats.

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control; <sup>d</sup> $P < 0.01$  vs Dangshen on same concentration;  $\bar{x} \pm s_x$  (%) increase.



**Figure 3** Effect of Dangshen and antagonists plus Dangshen on the mean contractile amplitude of pyloric strips in rats.

<sup>a</sup> $P < 0.05$ , <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control; <sup>d</sup> $P < 0.01$  vs Dangshen on same concentration;  $\bar{x} \pm s_x$  (%) increase.

## DISCUSSION

Pi-Wei (spleen-stomach) is a functional conception that means alimentary-system involving the other system in traditional Chinese medicine. Dangshen is the main Chinese drug to maintain Pi-Wei and posses the effect in “Bu-Zhong-Yi-Qi” and “He-Wei-Sheng-Jing”.

It was reported that Dangshen might react experiment gastric ulcer and lesion of stomach mucosa and inhibit gastric acid secretion in rats. Dangshen had the regulating action on gastrointestinal motility, influenced gastric basic electrical rhythm disorder, and partly reduced the increasing action of gastric motility and emptying on stress condition of the rats<sup>[4]</sup>. It also had the inhibiting and exciting effects on isolated ileum segment in guinea pigs<sup>[5]</sup>. Our previous experiments have shown that BU-QI had the exciting action on isolated gastric strips<sup>[6]</sup>. Our results indicated that Dangshen dose dependence increased the resting tension of LM of fundus, the mean contractile amplitude of CM of body and antrum, and the motility index of CM of pylorus. This may support that on clinic Dangshen has the gastroprokinetic effect of treating “Pi-Wei-Xu-Han and Shi-Yu-Bu-Zhen”.

All smooth muscle is involuntary and the nerves in the gastrointestinal tract are controlled both by extrinsic autonomic nerves and by intrinsic neural plexus. These plexus extensively interact with each other and are independently extrinsic neural control. Many of the local neurogenic reflexes in the gut occur in these plexuses, and play an important role in regulating the gastrointestinal motility. Cholinergic N-receptors exist on the membrane of the nerve ganglion cell of cholinergic M-receptors on the membrane of gastric smooth muscles. Our experiment showed that Atropine partly blocked the increased action of Dangshen on the isolated strips of fundus, body, antrum and pylorus in rats. Hexamethonium had the same effect on fundus, body of the stomach and pylorus. This is an evidence that effect of Dangshen was partly mediated via cholinergic M and N receptors. Prostaglandin protected gastric mucosal cells and regulated gastric motility<sup>[7]</sup>. Indomethocin inhibited endogenous prostaglandin synthesis. In our experiment, Indomethocin did not influence the action of Dangshen on the rat stomach strips except 10% Dangshen on fundic strips.

It is very interesting to study traditional Chinese medicine and to select different drugs for treating the gastrokinetic disorder in patients.

## REFERENCES

- Zhou R, Zhang L. Search for a pharmacological role of Dangshen in alimentary system. *J Beijing Tradit Chin Med*, 1991;14(5):47-48<sup>a</sup>
- Qu SY, Song CW, Lee KY, Chey WY. Action of secretion and/or cholecystokinin on gastric smooth muscle in rats. *Gastroenterology*, 1992;104(2-4):A567
- Qu SY, Zheng TZ, Li W. Comparative study of ranitidine and cimetidine on contractile activity of isolated gastric muscle strips in rats. *China Natl J New Gastroenterol*, 1997;5(2):75-76
- Hou JY, Jiang ZW, He ZZ. Effect of Dangshen on gastric electricity, motility and emptying on rats of stress gastric ulcer. *Chin J of Integrated Tradit and Western Med*, 1989;9(1):31-32
- Wang YZ, Wang M, Tian SF. Influence of radix codonopsis pilosulae on isolated ileac movement of guinea pig. *Jiangsu J Tradit Chin Med*, 1983;4(3):58-59
- Zheng TZ, Li W, Qu SY. Action Tradit Chin Med BUQI on contractile of isolated gastric muscle strips in rats. *Caps News Communication*, 1996;15 (supp. 4):20-21
- Sanders KM. Role of prostaglandins in regulating gastric motility. *Am J Physiol*, 1984;247(1):117-126

# Traditional Chinese medicine "Qing Yi Tang" alleviates oxygen free radical injury in acute necrotizing pancreatitis

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**Subject headings** pancreatitis/therapy; Qing Yi Tang; free radicals; superoxide dismutase/analysis; malonyldiadehyde/analysis

## Abstract

**AIM** To observe the changes in oxygen free radical (OFR) and the curative effect of traditional Chinese medicine "Qing Yi Tang" in acute necrotizing pancreatitis (ANP).

**METHODS** After induction of ANP by injection of sodium taurocholate into pancreatic duct, 16 dogs were randomly divided into control group and Chinese medicine group. Serum amylase, SOD and MDA were determined on postoperative day 1, 2, 4 and 7. The animals were sacrificed on day 7. SOD and MDA in organs were determined, and pathological changes in pancreas were observed.

**RESULTS** As compared with control group, the serum level of amylase (734 U/L vs 2783 U/L) and MDA (7.8 nmol/ml vs 14.8 nmol/ml) in Chinese medicine group were decreased on day 7 ( $P < 0.05$ ), while SOD increased significantly (281 nU/ml vs 55 nU/ml,  $P < 0.01$ ), and similar changes occurred in MDA and SOD in organs, especially in the pancreas; the pathological changes in the pancreas were alleviated as well.

**CONCLUSION** "Qing Yi Tang" is effective in clearing OFRs and alleviating pathological changes in ANP.

## INTRODUCTION

It has been shown that OFR plays an important role in the mechanism of ANP<sup>[1]</sup>, and it mediates the earliest and most fundamental pathophysiological changes, leading to injury of tissues and organs, even to multiple organ dysfunction syndrome (MODS). Zhu N<sup>[2]</sup> reported that some Chinese medicines had inhibiting effects in peroxidation in acute pancreatitis. In this study, a formula of Chinese medicine "Qin Yi Tang" was used and its curative effect in ANP dog model was observed.

## MATERIALS AND METHODS

### Animal model

Adult mongrel dogs weighing 15 kg  $\pm$  2 kg were acclimatized in the laboratory for a week before the experiment was started. Laparotomy was performed under general anesthesia with sodium thiopental, 30 mg/kg intravenously. The duodenum was exposed and turned over, and the main pancreatic duct was identified at the mesenteric margin of the duodenum. Acute pancreatitis was induced by injection of 0.5 ml/kg of 5% sodium taurocholate with 3 000U/kg trypsin into the pancreatic duct under a pressure of 7.84kPa (80cmH<sub>2</sub>O). During the first 2-3 days after operation, 5% glucose-saline was infused as needed and anti-shock measures were taken when indicated. After induction of ANP, 16 dogs were equally divided in-to two groups at random. Group 1 (ANP,  $n = 8$ ) received no treatment for ANP, group 2 (CM,  $n = 8$ ) was fed with "Qing Yi Tang", 20 ml/kg  $\cdot$  d, by gavage everyday. The ingredients of "Qing Yi Tang" include: rhubarb root, bupleurum root, white peony root, 24 g each; scutellaria root, picrorhiza rhizome, corydalis tuber, aucklandia root, sodium sulphate, 18 g each. All animals were sacrificed on the 7th postoperative day.

### Parameters examined

**Serum and organ superoxide dismutase (SOD) and malonyldialdehyde (MDA)** Blood samples were obtained on d 1, d 2, d 4 and d 7 postoperatively. Tissues of the liver, pancreas, kidney, and ileum were harvested on d 7, weighed and homogenized with phosphate buffered solution. SOD activities were determined by xanthine oxydase method and expressed as nU/ml or mg protein. The levels of

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Received 1998-03-08

MDA were quantified according to the reference<sup>[3]</sup> and expressed as nmol/ml or mg protein.

**Serum GPT and BUN** Monarch biochemical analyser (USA) was used for determination.

**Serum amylase (Amy)** Amy was measured by iodoamylum method and expressed as U/L.

**Morphological studies** Tissue samples of pancreas were harvested and observed under light microscope and transmission electron microscope.

**Statistical analysis** All results were expressed as  $\bar{x} \pm s$ . Data were analyzed by *t* test. *P*- values <0.05 were considered statistically significant.

## RESULTS

### Morphological changes of pancreas

**Macroscopy** In ANP group, the pancreas was enlarged, swollen and tough, with grey to dark foci, while in CM group, only uniform edema of pancreas was noticed.

**Light microscopy** In ANP group, there were multiple areas of hemorrhage and necrosis on a background of inflammatory infiltration. In CM group, the pancreatic acini were basically intact. There was slight interstitial congestion and edema, with mild infiltration of inflammatory cells.

**Transmission electron microscopy** A significant dilatation of the rough-surfaced endoplasmic reticulum (RER) was revealed in ANP group, and the mitochondria were swollen markedly as well. In CM group, there was mild dilatation of RER, without significant swelling of the mitochondria, and the zymogen granules could be easily seen.

### Serum and organ SOD and MDA

In CM group, as compared with ANP group, the serum SOD activities were increased significantly ( $P < 0.05$ ) from second postoperative day, while the serum levels of MDA were decreased significantly ( $P < 0.05$ , Table 1). At the same time, SOD in organs were increased markedly ( $P < 0.05$ ), and MDA reduced significantly ( $P < 0.05$ ), especially in the pancreas ( $P < 0.01$ ).

### Serum GPT and BUN

In CM group, as compared with ANP group, serum levels of GPT and BUN were significantly lower (Table 3).

### Serum amylase

Serum amylase levels were markedly elevated after induction of ANP in both groups. But in CM group, amylase levels were significantly lower than those in ANP group on d 4, and returned to normal on d 7 (Table 4).

**Table 1 Changes in serum SOD (nU/ml) and MDA (nmol/ml) levels**

Group	Parameter	d 1	d 2	d 4	d 7
ANP	SOD	89.87±18.43	78.19±13.39	63.89±11.23	55.87±28.54
	MDA	21.48±3.62	26.20±5.37	20.49±4.53	14.83±2.03
CM	SOD	90.85±15.27	94.72±11.54 <sup>a</sup>	234.83±45.96 <sup>b</sup>	281.59±29.79 <sup>b</sup>
	MDA	20.29±3.44	16.62±2.67 <sup>a</sup>	11.85±2.36 <sup>a</sup>	7.84±1.69 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs ANP.

**Table 2 Levels of SOD (nU/mg) and MDA (nmol/mg) in organs**

Group	Parameter	Liver	Pancreas	Kidney	Ileum
ANP	SOD	149.53±24.05	146.94±31.48	115.65±18.29	126.25±14.54
	MDA	29.56±8.42	10.41±1.38	25.80±4.49	6.06±2.19
CM	SOD	279.46±38.66 <sup>a</sup>	295.92±53.38 <sup>a</sup>	200.87±31.37 <sup>a</sup>	298.88±22.26 <sup>a</sup>
	MDA	15.83±3.21 <sup>a</sup>	4.52±1.09 <sup>b</sup>	13.37±1.02 <sup>a</sup>	3.24±0.64 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs ANP.

**Table 3 Changes in GPT (U/L) and BUN (mmol/L) levels**

Group	Parameter	Before operation	d 1	d 2	d 4	d 7
ANP	GPT	22.25±8.25	72.10±12.61	54.88±10.67	41.38±6.75	25.85±8.41
	BUN	3.11±1.13	5.49±2.20	4.26±1.08	3.83±0.99	2.84±0.79
CM	GPT	18.96±4.32	68.56±12.68	34.71±9.46 <sup>a</sup>	28.92±5.28 <sup>a</sup>	24.35±6.44
	BUN	3.13±0.84	5.14±1.89	3.48±0.87 <sup>a</sup>	2.97±0.64 <sup>a</sup>	3.12±0.56

<sup>a</sup> $P < 0.05$  vs ANP.

**Table 4** Changes in serum amylase levels (U/L)

Group	Before operation	d 1	d 2	d 4	d 7
ANP	825.50±82.94	7363.25±1383.26	7060.75±1135.65	4590.25±1312.44	2783.75±893.42
CM	816.58±74.85	7158.60±1258.82	5673.43±1173.45	1567.43±863.55 <sup>b</sup>	743.68±101.44 <sup>b</sup>

<sup>b</sup>P<0.01 vs ANP.

## DISCUSSION

The tissues of intestine and pancreas are rich in xanthine oxidase (XOD). In healthy tissues, XOD exists as a dehydrogenase which is inactive or minimally active. During ischemia and hypoxia of the intestinal tissue and subsequent reperfusion, a large amount of xanthine dehydrogenase was converted rapidly to active XOD, promoting the oxidation of hypoxanthine which was accumulated in hypoxic tissue. Hence a burst of oxygen free radical (OFR) generation occurred, including O<sub>2</sub><sup>-</sup>, O<sup>-</sup>, OH<sup>-</sup>, ·O and H<sub>2</sub>O<sub>2</sub>. OFRs were highly reactive, with a half-life time in μs. Usually MDA, a product of lipid peroxidation, was quantified to reflect the OFR levels<sup>[4]</sup>. Dabrowski<sup>[5]</sup> showed a decrease of SOD in pancreatic tissue and blood in experimental acute pancreatitis and referred it to the enhanced lipid peroxidation caused by OFR. OFR can react on almost all components of cells such as phospholipids, proteins and DNA, exerting influences on cell metabolism and function, leading to destruction of tissue structure, producing a series of pathophysiologic changes. There has been evidence that OFR can cause dysfunction of acinar cell microtubule of the pancreas, releasing a large amount of abnormal secretion-zymogen granules directly into pancreatic interstitium and bloodstream. OFR-induced reduction of membrane stability may lead to release of acinar cell lysosome and activation of various pancreatic enzymes. OFR can also activate phospholipase A<sub>2</sub>, decompose cell membrane lecithin of the pancreas, bringing about further damage to pancreatic tissue. The long half-life lipid peroxides may reach via blood stream to the remote organs, causing extrapancreatic damage<sup>[6]</sup>. It is believed that some severe complications of ANP, especially ARDS and MSOF, are related to OFR injury which may play a

role as a “trigger”. The data of this study demonstrated that in Chinese medicine group, in contrast to the control group, the SOD levels in serum and organs were higher significantly, while the MDA levels decreased markedly, especially in the pancreatic tissues, and the functions of the liver and kidneys were improved remarkably. These results suggested that Chinese medicine “Qing Yi Tang” could reduce OFR generation significantly, thus attenuating the lipid peroxidation injury in ANP. The mechanism of alleviating OFR injury by Chinese medicine in ANP is not clear. The effects of “Qing Yi Tang” might include: ① inhibiting the XOD activity in tissue of the pancreas, attenuating the production of OFR; ② improving the blood perfusion of gastrointestinal mucosa, alleviating its ischemic and hypoxic state, thus inhibiting the OFR generation; ③ reducing the organ damage caused by OFR and their chain reactions; ④ decreasing pancreatic enzyme release; and ⑤ playing a part in regulating the immunologic function of the organism. A large number of clinical reports has indicated that “Qing Yi Tang” has satisfactory therapeutic effects in acute edematous pancreatitis. Our study suggested that “Qing Yi Tang” could also alleviate the pathophysiologic changes in ANP, and might be beneficial to improving the prognosis.

## REFERENCES

- 1 Chen HL, Wu XZ, Zheng XL. Oxygen free radicals and acute pancreatitis. *Basic Clin General Surg*, 1994;1(1):51-54
- 2 Zhu N, Du JH. The effect of the anti-oxidation of *Salvia Miltiorrhiza* on rats with acute pancreatitis. *Chin J Pathophysiol*, 1991;7(5):499-502
- 3 Hachimoku K. Peroxid lipid assay. *J Med Technol*, 1979;23(2):115-120
- 4 Parks DA, Bulkley GB, Granbger N. Role of oxygen derived free radicals in digestive disease. *Surgery*, 1983;94(3):415-422
- 5 Dabrowski A, Gabryelewicz A, Wereszeczynska U, Wereszczynskasmiatkowski, Chyczewski L. Oxygen derived free radicals in cerulein induced acute pancreatitis. *Scan J Gastroenterol*, 1988;23(10):1245-1250
- 6 Furukawa M, Kimura T, Yamaguchi H, Kinjoh M, Nawata H. Role of oxygen-derived free radicals in hemorrhagic pancreatitis induced by stress and cerulein in rats. *Pancreas*, 1994;9(1):67-72

# Traditional Chinese medicine for primary liver cancer

LI Zuo-Qing

**Subject headings** Liver cancer, primary; TCM; complication; pain; ascites; fever

Further progress has been made in the traditional Chinese medicine for primary liver cancer over the past few years, especially in the research of traditional Chinese medicine (TCM) treatment principle, improvement of therapeutic results and prolonging the survival.

## Research of TCM therapy principles

Strengthening the Spleen and replenishing Qi. According to Yu Er-Xin's<sup>[1]</sup> principle, medication to strengthen Spleen and replenish Qi is believed to be effective in hindering the growth of tumors, even decreasing the size of tumors, improving patients' health conditions and prolonging their lives. It is also believed to strengthen the function of T lymphocytes, adjust the NK cells and help protect the liver function. Song Ming-Zhi's<sup>[2]</sup> examination of cyclic nucleoside in the blood of cancer patients showed that cAMP in the patients with weakness and slackness of the Spleen decreased significantly, while in the type of damp and heat and Yin deficiency, cGMP tended to increase greatly. The medication to strengthen Spleen and replenish Qi can increase cAMP but decrease GMP.

Activating the blood flow and phosphating stasis (ABFPS). Modern medicine has proven that cancer patients usually have trouble with microcirculation. The therapy of ABFPS can improve microcirculation and enhance immunity, and some medications can directly kill cancer cells. Of 24 cases of 3 primary liver carcinoma treated with Wang Xiao's<sup>[3]</sup> ABFPS, 60% showed improvement, 26% had the size of carcinoma decreased, the medium survival was five months and the >1 year survival rate was 25%, which indicates that such medication can improve microcirculation, increase blood flow and decrease platelet

aggregation. The research by Ying Duo-Rong<sup>[4]</sup> in mechanism of this medication showed that it is effective in fighting against carcinoma, normalizing proteinogen of plasma and increasing the activation of lysozyme. Such a medication can also affect the synthesis of cellular toxin of S 180 RNA in mice and adjust the immunity. Some components of the medication such as *Rhizoma Sparganii*, *Rhizoma Zedoariae*, *Radix Angelica Sineasis*, *Flos Carthami* *Radix Paeoniae*, *Squama Manitis*, *Olibanum*, *Myrrha* and *Eupolyphaga Seu Steleophaga* had inhibitory effect in carcinoma of animal models. They also help prevent radioactive fibrosis and regulate nervous system and internal secretion. A pathological and ultrastructural study by Liu Jin-Fang<sup>[5]</sup> revealed that this medication can directly affect the structure of liver cancer cell membrane by breaking it down, expanding rough surfaced endoplasmic reticulum (RER) and swelling of mitochondrion so as to destroy the liver cancer cells.

Cleaning the heat and toxins. This medication of TCM is one of the main therapies for cancers. It was reported in Japan that treatment with herbs like -Herba Solani Lyrati, Herba Agrimoniae-and-Radix Sophorae Tonkinensis-proved effective for various kinds of carcinomas with an effective rate of 84.6%-100%. Lu Gui-Zhi's<sup>[6]</sup> compound -Herba Solani Nigri- injection (composed of Herba Solani, snake venom- and-Herba Solani Lyrati) administered to mice with ascitic carcinoma had an inhibiting effect of 87.35%. According to Pan Ming Ji's<sup>[7]</sup> theory, the medication of cleaning heat and toxins can remove the stasis of cancer toxin from human body, therefore, strengthen Yin exhausted by the long period of sickness and balance the body function.

Strengthening body resistance to eliminate pathogenic factors. Qiu Jia-Xin<sup>[8]</sup> in the principle of strengthening the Spleen and replenishing Qi, cleaning heat and eliminating toxin and softening hard mass and eliminating phlegm, used *Radix Pseudostellariae*, *Rhizoma Panacis Majoris*, *Rhizoma Atractyodis*, *Poria*, *Radix Salviae Miltiorrhizae*, *Flos Lonicerae*, *Radix Serobilanthes*, *Concha Ostreae*, *Spica Preunellae*, *Carapax Trionycis*, *Flow Rosae Rugosae*, *Prumus Mume*

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Received 1997-08-01 Revised 1998-02-15

(*sieb*), *Lumbricus*, *Fructose Akebiae* and *Rhizoma Arisaematis* to treat 123 cases of advanced cases of liver cancer with a 1-year survival rate of 32.5%. It was proved that the formula of cleaning heat and eliminating toxin was most effective in killing liver cancer cells, and some components of the medication such as *Rhizoma Atractylodis*, *Concha Ostreae*, *Rhizoma Arisaematis*, *Flos Mume Albus* can inhibit the cancer growth and transmission of Lewis cancer. Chen Kai's<sup>[9]</sup> medication was composed of roots of *Radix Quinguefolium*, *Radix Acanthopanax*, *Senticos*, *Radix Asparagus*, *Venenum Bufonis* and *Bulbus Bolbostemmae*, which were made into liquid and experimented on mice with transmitted cancer. The experiment revealed that the liquid can promote the activation of these mice's NK cells and IL2, and help enhance the transformation of T cells and the function of macrophages, with a 50% inhibitory rate of the development of carcinoma.

#### **Dialectical treatment**

In recent years, obvious progress has been made in clinical treatment of cancer and prolonging the survival of cancer patients. Wang Ze-Guang<sup>[10]</sup> treated 110 liver cancer patients with self-made anti-cancer prescriptions No.1.4 at one dose daily for 30 days as a therapeutic course. Liver cancer characterized by stagnation of Liver Qi was treated with No.1 formula composed of *Radix Bupleuri*, *Radix Paeonia Alba*, *Radix Curcumae*, *Fructus Citriimmaturi*, *Indigo Naturalis* and *Herba Scutellariae Barbatae*. Liver cancer featured in blood stasis and Qi stagnation was treated with No. 2 formula composed of raw *Concha Ostreae*, *Squama Manitis*, *Scolopendra*, *Eupolyphaga Sen Steleophaga*, *Rhizoma Corydalis*, *Flos Genkwa* and *Nidus Vespaee*. Liver cancer with damp heat and virulent heat was treated with No.3 formula composed of *Herba Artemisiae Scopariae*, *Radix et Rhizoma Rhei*, *Fructus Gardeniae*, *Rhizoma Curcumae Longae*, *Rhizoma Paridis*, *Fructus Forsythiae*, *Herba Lysinachiae*, *Herba Taraxaci*, *Radix Phytolaccae* and *Rhizoma Smilacis Glabrae*. Liver cancer with weakness of Liver and Spleen was treated with No.4 formula composed of *Radix Pseudostellariae*, *Radix Glehniae*, *Radix Angelicae Sineasis*, *Semen Pharbitidis*, *Herba Lobeliae Chinensis*, *Herba Artemisiae Annuae*, *Cortex Moutan* and *Cortex Magnoliae*. These types of liver cancer were treated at the same time with No.5 formula composed of *Radix Ginseng*, *Cornu Cervi Pantotrichum*, *Placenta Hominis*, *Moschus*, *Realgar*, *Flos Carthami*, *Rhinoceros Bicornis*, *Cornu Antelopis*, *Borneolum*, *Endothelium*

*Corneum Gigeriae Galli*, *Hirudo*, *Calculus*, *Semen Strychnis*, *Vennenum Bufonis*, *Sanguis Draconis*, *Radix Kansui*, *Carapace Trionvcis*, *Radix Aconiti* and *Squama Manitis*. Most of the patients showed improvement and decreased size of diseased livers. Some of the patients had decreased or even negative results of AFP. The longest survival time was 17.4 months, averaging 7.4 months. Lin Zong-Guang<sup>[11]</sup> treated 44 cases of primary liver cancer of advanced stage with the therapy of strengthening body resistance and activating blood flow and softening hard mass which greatly improved the patients symptoms and quality of life with 1-year, 3-year and 5-year survival rates of 50%, 27.2% and 4.5%. Zhang Li-Ying<sup>[12]</sup> treated 34 cases of primary liver cancer by the formula of replenishing Qi, activating blood flow, eliminating stasis, softening hard mass and removing symptoms, as a result, 10 patients survived more than one year. Liu Bian-Lin<sup>[13]</sup> treated 30 patients with traditional dialectical therapy, who were divided into four types of Qi stagnation and blood stasis, dampness and heat of Liver and Gole, weakness of Spleen and Stomach, weakness of Liver and Kidney, with an average survival time of 15 months and two cases remained alive for over two years. Xu Yi-Yu's<sup>[14]</sup> medication was composed of *Concha Arcae*, *Pingdimu*, *Shiyan* and *Radix Rhapontici seu Echinopsis*, dispersing mass in combination of chemical therapy and surgery. Of the 212 primary liver cancer patients treated, 8 lived over 10 years, 9 lived over 5 years, 39 cases over 3 years and 93 over one year. Liu Ji-Fang<sup>[15]</sup> treated 201 cases of middle or advanced primary liver cancer with tablets of resolving masses, 198 showed obvious clinical improvement and 89.12% of them prolonged their lives for 10 to 36 months, 6.5% had temporary recovery, the total effective rate being 58.8%. The medication also showed reduction of AFP. In five cases of liver cancer treated with Liu's medication, electronic microscopic observation revealed that the tissues of their tumor lumps had been destroyed and a large amount of lymphocytes and phagocytes infiltrated and the proportion of nucleic shrank. According to Cai Xin's<sup>[16]</sup> medication, 20 g beehouse boiled in the liquid was taken once daily for the treatment of two cases of primary liver carcinoma. One showed shrinkage of the tumor lump and AFP turned negative, with 6 years of prolonged life. The other case treated in combination of other medications for three months also showed shrinkage of tumor lump and AFP turned negative. Now the patient is still alive. Zhou Jia-Xi<sup>[17]</sup> treated two patients with Liu Lu-Ming's medication, which included disinfection and invigoration, by using Pianzaihuang to disperse

mass and remove stagnation, and *radix Quinguefolium* to strengthen the body resistance, as a result, one of the patients continue to live for three years and the other for five years. Li Ya-Lin<sup>[18]</sup> treated 36 patients with liver cancer who could not be operated on with the traditional medication with the effect of soothing the Liver, activating the blood, removing stagnation and strengthening the Spleen. Pharmaceutical analysis showed that the components of the medication like *Rhizoma Zedoariae* had suppressing effect on the cancer lumps and the other components had different degree of resistant effect on liver cancer. Treated with this medication, 94.4% of patients remained in stable conditions and 5.5% showed improvement. Cheng Ning's<sup>[19]</sup> analysis of the results achieved in the recent ten years in TCM treatment of primary liver cancer showed that 92.1% of the TCM medications used in treatment of liver cancer were characterized by activation of the blood and removal of stagnation, 84.3% by elimination of heat and toxin, and 79.9% by enhancement of the Spleen. All this indicates that the major pathological cause and mechanism of primary liver cancer in the middle or advanced stage were accumulation of blood stasis, heat and toxin and the weakness of the Spleen.

#### **Treatment of complications**

**Pain.** The pain caused by liver cancer is most common among the advanced cases and is most difficult to control according to TCM such pain is caused by the stagnation of the Liver and blood stasis or by the blocking of blood vessels that resulted from body's internal damp heat. In TCM therapy both oral administration and external application were adopted, which was superior to the modern medicine, being more convenient and effective but with less side effects. ① Oral administration. Liu Lu-Ming<sup>[20]</sup> applied Xiangsha Liujunzi Tang, Jishi Xiaopi Pills, Chaihu Suge San, Wendan Tang, Yiguang Jian and Simoyinzi to 169 cases of liver cancer pain, with an effective rate of 46.1%. If complemented with anti-inflammatory drugs and salicylic acids, it can relieve 93.5% of pain. Li Ya-Lin treated 36 patients with liver cancer who could not be operated on, with the traditional medication with the effect of soothing the Liver, activating the blood, removing stagnation and strengthening the Spleen. Pharmaceutical analysis showed that the components of the medication like *Rhizoma Zedoariae* had suppressing effect on the cancer lumps and the other components had different degree of resistant effect on liver cancer. Treated with this medication, 94.4% of patients

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**Ascites.** Hu An-Bang's<sup>[28]</sup> medication made from *Cornu Rhinoceri*, *Radix Rehmanniae*,

*Rhizoma Imperatae* and *Cortex Moutan*, each 30 g, and *Cortex Cinnamomi*, *Rhizoma Anemarrhenae*, *Cortex Phellodendri*, *Radix Vladimiriae* and *Pericarpium Arecae*, each 15g showed good effect in reducing ascites. Li Pei-Wen's<sup>[29]</sup> formula composed of *Radix Astragali*, *Semen Coicis*, *Semen Pharbitidis*, *Radix Scutellariae* and *Radix Aristolochiae* produced good effect when administered externally. Lin Zhong-Guang's<sup>[30]</sup> medication aimed to strengthen the resistance and to soften the cancer lump. In 22 cases of ascite patients treated with this medication, 15 were improved in their condition, with an effective rate of 72.7%.

**Cancer fever.** The mechanism of cancer fever is not yet clear and there has been no specific method for it. Patients with such symptom are usually treated with anti-inflammatory drugs in modern medicine, which has more side effects and long-term usage might do harm to patients' health. Li Zuo-Qing<sup>[31]</sup> treated 68 of such cases with traditional Chinese dialectical therapy which are classified into three kinds of blood stagnation caused by Yin deficiency, blood stagnation caused by heat toxin and simple blood stagnation, and achieved an effective rate of 86%. Xu Ji-Ping's<sup>[32]</sup> medication showed better effect than dexamethasone and aminopyrine in treatment of the fever caused by malignant carcinoma. His medication consists of 15 g *Flos Lonicera*, 40 g *Cornus Bubalis*, 30 g *Pseudobulbus Cremastreae Appendiculatae*, 30 g *Radix Tetrastigmae*, 12 g dried toad skin and so on, which was cooked and taken twice a day. According to Wang Qing-Cai's<sup>[33]</sup> dialectical analysis, such fever can be divided into four groups of Qi weakness, Yin deficiency, blood stasis and heat toxin. He treated 70 cases according to his theory with the medications of Buzhong Yinqi decoction, Zhen Yi Tang, Xuefu Zuyu decoction and Qingwen Baidu decoction for ten days with an effective rate of 78.5%.

### Comprehensive therapy

In treatment of primary carcinoma at advanced stage, the combination of traditional Chinese and modern medicine is usually advocated to obtain higher clinical effective rate and survival rate. Dong-Wei-Yan's<sup>[34]</sup> combined therapy includes a traditional medication of *Radix Codonopsis Pilosulae*, *Rhizoma Atractylodis*, *Herba Patrinia*, *Herba Agrimoniae*, *Radix Pseudostellariae*, *Herba Taraxaci* and *Herba Solani Lyrati*, etc. and chemical treatment of 5-FU hepato-artery infusion. In a year's treatment of 36 cases, their survival rates of 1, 3, and 5 years were 57.9%, 20% and 16.6% respectively. Hu Bing<sup>[35]</sup> used combined TCM of

strengthening the Spleen and chemical therapy of adriamycine in treatment of primary carcinoma of advanced stage. The TCM consists of the liquid made from *Radix Astragalis*, *Rhizoma Astrsactylodis*, *Radix Ginseng*, *Semen Dolichoris*, *Rhizoma Dryopteris Crassirhizomae*, *Fructus Citri*, *Radix Raphani*, etc. The infusion of the combined medication was administered to 16 cases once every three weeks for nine weeks. After treatment, two cases were completely relieved of symptoms, 3 partially relieved, 8 remained stable and 3 deteriorated, with a relieving rate of 31%. Their median survival period was 12.6 months. Yu Er-Xin<sup>[36]</sup> believed that the combination of chemical therapy and TCM can increase the therapeutic effect. Generally, in patients with stage II liver cancer treated by chemical therapy alone, the 1-year survival rate was about 30%, and the 5-year survival rate was only 5%, while the combined therapy can increase the one-year survival rate to 70% and five-year survival rate to 10%-20%. Ling Hong-Ying<sup>[37]</sup> used traditional herbal liquid of *Bai Nian Le* and levamisole and cimetidine to treat 30 cases of primary carcinoma. The result showed that the medication could highly increase the activity of NK cells. Wang Xiao-Xian<sup>[38]</sup> used combined oral administration and external application to 17 primary carcinoma patients. The TCM Ganai decoction consisting of *Fu* and traditional herbal liquid of *Rhizoma Smilacis Glabrae*, *Carapax Trionycis*, *Concha Ostreae*, *Radix Astragali*, *Endothelium Corneum Gigeriae Galli*, *Herba Lycopi*, etc. was taken orally together with Xiaoji pills or Xiaoji liquid. Paste made from *Venennum Bufonis*, *Caulis Fibraureas*, etc. was applied externally to the diseased spots. The result showed that, 14 of 17 cases, died within 5 months, 3 survived, tumor lump disappeared in one of them who has now survived 49 months; the other two survived 35 and 38 months with tumor still existing. Wu Jun-Yu<sup>[39]</sup> found in his 6-year clinical observation of 18 patients with primary liver cancer who were treated with TCM therapy that TCM could increase human immunity, slow down the growth of tumor lumps and even shrink them. Such an effect would make it possible to perform the second stage operation on the cancer patients and to prevent to a certain degree the transmission of the tumor. As a result, 50% of the patients survived 3 years and 33.33% survived 5 years.

### REFERENCES

- 1 Yu Ex, Lu LN. Research in treatment and mechanism of primary cancer through strengthening the Spleen and replenishing Qi. *JTCM*, 1987;28(6):28-30
- 2 Song MZ, Yu EX. Observation on cyclic nucleoside phosphate in the plasma of dialectically classified primary cancer. *Shanghai JTCM*, 1981;(4):40-42

- 3 Wang X, Zhang S, Zhong H. Clinical observation on 24 cases of primary cancer treated by way of activating the blood flow and removing the blood stasis. *J Beijing Trad Chin Coll*, 1992;15(3):31
- 4 Ying DR. Research in treatment of carcinoma through promoting the blood circulation and removing the blood stasis. *J TCM*, 1988;(8):60
- 5 Liu JF, Tuo CW, Wang SD, Wang SL, Din L, Li BJ. Experimental research in the effect of traditional dissolving lumps tablets on tumors. *J Integrated Trad Chin West Med*, 1990;10(7):429-432
- 6 Lu GZ, Huang YC, Gao Y, Peng L, Liang YY, Xu YF. The effect of compound black nightshade injection on the cells of liver cancer. *J Integrated Trad Chin West Med*, 1987;7(2):97-99
- 7 Pan MJ. Advantages of TCM in treatment and prevention of tumors. *Fujian TCM*, 1989;20(1):39-40
- 8 Qui JX, Yang JK. Clinical observation and experimental research in treatment of liver cancer at advanced stage by traditional medication of strengthening the Spleen, replenishing Qi, clearing away heat and toxic material. *J Integrated Trad Chin West Med*, 1987;7(5):275-277
- 9 Chen K, Li XM. The effect of TCM of strengthening the body's resistance against tumor on the immunity of mice with transplanted tumor. *J Integrated Trad Chin West Med*, 1993;13(3):171-172
- 10 Wang ZG, Zhang XZ, Wang HY. Observation on treatment of 110 cases of primary liver cancer by traditional Chinese therapy. *Beijing J TCM*, 1990;(5):32-34
- 11 Lin ZG. Treatment of 44 cases of primary liver cancer at advanced stage by the medication of strengthening the body's resistance and softening hard masses. *J TCM*, 1992;33(2):23-24
- 12 Zhang LY, Ding HQ, Xu ZW, Tang JF. Quantitative observation on AFP of stage II primary liver cancer treated by traditional Chinese medication. *Shanghai J TCM*, 1989;(3):16
- 13 Liu BL. Dialectical treatment of 30 cases of primary liver cancer. *Shanxi J TCM*, 1992;13(1):17-18
- 14 Xu YY, Yu EX. Clinical observation on treatment of primary liver cancer by self prescribed medication. *Shanghai J TCM*, 1993;(12):3-4
- 15 Liu JF, Zhao HM, Li BJ, Wang ZR, Wang SD. The therapeutic effect of traditional dissolving lumps tablets on liver cancer at advanced stage. *J China Med Univ*, 1993;2(1):40-41
- 16 Cai X. Clinical use of beehive in treatment of tumors. *Jiangsu J TCM*, 1993;14(16):33
- 17 Zhong JX, Shi ZY, Chen WH, Liu SC. A report on treatment of liver cancer. *J New TCM*, 1992;(12):15-16
- 18 Li YL. Principles in TCM dialectical treatment of advanced-stage liver cancers. *J Chin Clin Tumor Res*, 1994;21(6):449
- 19 Cen Y. Usage of traditional herbal medicine in treatment of primary liver cancer at middle and advanced stages. *Shanxi J TCM*, 1995;16(2):87
- 20 Liu LM, Yu EX. Dialectical analysis of the pain caused by liver cancer at advanced stage in 169 cases. *J TCM*, 1989;(2):15-17
- 21 Huang WX. Initial observation of 18 cases of liver cancer treated by injection of Venenum Bufonis. *J Carcinoma*, 1990;9(3):239-245
- 22 Zhao XM, Kan N. A report on the effect of traditional decoction for killing pain caused by cancer in 50 patients. *Shandong J TCM*, 1992;11(5):20-21
- 23 Guo RX, Zhang LY, Gong YM, Zhang BF. Clinical observation on the effect of traditional pain killing oral liquid on the pain caused by cancer. *Sichuan J TCM*, 1993;(1):27-28
- 24 Duan FW. A clinical report on several kinds of external treatment of tumors. *Heilongjiang J TCM*, 1984;(4):19-21
- 25 Wang BF. Topical application of turtle shells and amarantaceae to severe carcinomatous pain of liver cancer. *Jiangsu J TCM*, 1986;(4):4
- 26 Guo FH, Hao WG, Gao YE, Wang ZY. Therapeutic effect of Guo's paining killing paste on carcinomatous pain of 103 patients. *J Tumor Prev*, 1992;19(4):258-260
- 27 Yang GG, Liu JC, Xie J, Jiang SG, Song ML, Li HT. Clinical research in the pain killing paste on severe carcinomatous pain. *J TCM*, 1992;33(7):30-31
- 28 Hu AB. A research on treatment of 4 cases of liver cancer. *J TCM*, 1986;(12):20-21
- 29 Li PW, Zhang DZ, Wang SF, Li SL, Yan SC. Research in traditional flatulence relieving topical paste for treatment of carcinomatous ascit. *J TCM*, 1991;(7):28-30
- 30 Lin ZG. A report on treatment of 44 cases of cancer at advanced stage with therapy of strengthening the body resistance and softening hard masses. *J TCM*, 1992;33(2):23-24
- 31 Li ZQ, Zhu JX. Clinical observation on traditional treatment of 68 cases of carcinomatous fever. *Guangxi J TCM*, 1991;14(5):197-198
- 32 Xu JP, Qou WY, Guo DQ. Observation on the effect of Jin Nu Jian on fever caused by malignant cancer. *J TCM*, 1988;(7):46-47
- 33 Wang QC. Clinical analysis of dialectical treatment of 70 cases of carcinomatous fever. *Liaoning J TCM*, 1992;19(9):34-35
- 34 Dong WY, Wang XH, Chen Y, Ou SQ. Treatment of 36 cases of primary liver cancer through combined therapy of traditional Chinese medicine and western medicine. *J Integrated Trad Chin West Med*, 1990;10(9):553
- 35 Hu B, Yuan TW. A report on treatment of 36 cases of advanced liver cancer with the traditional medication of strengthening the Spleen and replenishing the Qi with Amei Su. *J Integrated Trad Chin West Med*, 1990;10(12):746-747
- 36 Yu EX. Prolonging long-term therapeutic effect on liver cancer by using the combination of radiotherapy and traditional herbal medicine. *Applied Tumor J*, 1988;3(1):5-7
- 37 Ling HY, Wang NZ, Li JC, Zhu ZH, Liang AM, Qin WX. Research in combined therapy of traditional and western medicine for treatment of hepatocellular carcinoma. *J Integrated Trad Chin West Med*, 1989;9(6):348-349
- 38 Wang XG. Clinical observation on treatment of 17 cases of primary liver cancer with comprehensive therapy. *Hubei J TCM*, 1993;15(6):22-23
- 39 Wu ZY, Wang LG. A clinical observation on treatment of primary liver cancer with combination of traditional Chinese and modern medicines. *Beijing J TCM*, 1995;(1):16

# Changes of p53 protein blood level in esophageal cancer patients and normal subjects from a high incidence area in Henan, China \*

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**Subject headings** esophageal neoplasms; p53 protein; p53 gene; enzyme-linked immunosorbent assay

Esophageal cancer remains the leading cause of cancer-related death. Previous studies by us and others indicated that esophageal carcinogenesis is a multiple stage process. Abnormal cell hyperproliferation may be an early indicator for esophageal carcinogenesis. Although the molecular basis for esophageal carcinogenesis is still poorly understood, the recent studies showed that p53 protein accumulation and p53 gene mutation occur more frequently in the esophageal precancerous and cancerous lesions from the subjects at high incidence areas for esophageal cancer in Henan, China. The frequency of p53 protein accumulation and p53 gene mutation increased as the lesions progressed to cancer<sup>[1-4]</sup>. Therefore, it is much desirable to correlate the p53 protein changes in blood from esophageal cancer patients and normal subjects in this area. The present study was undertaken to further characterize the changes of p53 protein blood level in patients with esophageal cancer and to correlate the changes with those with normal esophageal epithelium.

## MATERIALS AND METHODS

### Subjects

All the 31 subjects were from Linzhou City (originally known as Linxian), Henan Province, a high incidence area for esophageal cancer. Histopathological examination showed that 20 subjects had primary esophageal squamous cell carcinoma and 11 had normal squamous epithelium. None of the cancer patients had received any chemotherapy or radiation therapy before

operation.

### Blood collection

Ten ml blood was obtained from each subject with empty stomach. The whole blood was centrifuged, compartmentalized and preserved in liquid nitrogen for p53 protein analysis.

### p53 protein analysis with ELISA

Reagent. ELISA Kit for p53 protein of both wild and mutated type (Oncogene Science, Inc., USA); normal serum of rat; anti-rabbit IgG labeled by peroxidase; ELISA panel (96 wells) coated by monoclonal antibody of p53 protein (PAb1801); labeled p53 protein; automatic ELISA counter (Fisher Co., USA); buffer solution (pH 7.4).

Procedure. The main procedures are based on the method provided by the Oncogene Science Inc. to define the standard curve of p53; diluted p53 to six different concentrations, the serum was diluted to 1:10 with buffered solution; added 100  $\mu$ l normal mouse serum in each well on the ELISA panel to block non-specific reaction; added 100  $\mu$ l working solution of p53 and diluted serum in the well, and incubated for 2 hours under room temperature, washed with buffer solution for three times, then added sheep-anti-rabbit IgG labeled by peroxidase, incubated for one hour under room temperature, washed with buffer solution for three times, the substrate was added and incubated for 30 minutes.

## RESULTS AND DISCUSSIONS

Our study showed that the mean value of p53 protein in the serum of 11 normal cases and 20 cases of esophageal cancer were  $0.15 \mu\text{g/L} \pm 0.09 \mu\text{g/L}$  ( $\bar{x} \pm s$ ) and  $0.23 \mu\text{g/L} \pm 0.04 \mu\text{g/L}$  ( $\bar{x} \pm s$ ) respectively. The p53 protein level in the serum of esophageal cancer patients was significantly higher than that of normal group from the same area ( $P < 0.05$ ). These results were consistent with the previous observation of the high frequency of p53 protein accumulation in the early stage of esophageal carcinogenesis, suggesting that the changes of protein level in the blood may be a sensitive indicator for molecular changes in tissue

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Project supported by the National Natural Science Foundation of China, No.39670296 and No.39770296.

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Received 1998-07-06

level. Although the molecular basis for these changes was still unclear, it might be a promising circulating biomarker for esophageal carcinogenesis. Considering the fact that the esophageal and gastric cardia cancer are the most frequent tumor in Linzhou City, the changes of p53 protein blood level observed in these patients may reflect the actual response to esophageal and gastric cardia carcinogenesis. Recent studies have indicated that circulating p53 protein detected with ELISA method was mostly mutant type. Further studies should be undertaken to determine the levels of two types of p53 proteins to elucidate the biological significance of p53 gene alteration in esophageal carcinogenesis. It is also worthy to correlate the changes of p53 protein in blood and tissue during the multiple stage of esophageal carcinogenesis through long-term follow-up studies.

It is worth note that serum p53 protein was detected in the 11 cases with normal esophageal

epithelium, which is consistent with the previous results that the accumulation of p53 protein and p53 gene mutation occurred in the nearly normal esophageal epithelium. Long-term follow-up studies for these subjects may not only shed a light on the mechanism of esophageal carcinogenesis, but also be of great potential for clinical application in early diagnosis and prevention of esophageal cancer.

## REFERENCES

- 1 Wang LD, Hong JY, Qiu SL, Gao HK, Chung CS. Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Res*, 1993;53(8):1783-1781
- 2 Gao HK, Wang LK, Zhou Q, Hong JY, Huang TY, Yang CS. p53 tumor suppressor gene mutation in early esophageal precancerous lesions and carcinoma among high-risk population in Henan, China. *Cancer Res*, 1994;54(16):4342-4346
- 3 Wang LD, Zhou Q, Hong JY, Qiu SL, Yang CS. p53 protein accumulation and gene mutation in multifocal esophageal precancerous lesions from symptom free subjects in a high incidence area for esophageal carcinoma in Henan, China. *Cancer*, 1996;77(7):1244-1249
- 4 Shi ST, Bo F, Yang Y, Wang LD, Yang CS. Immunohisto-selective sequencing (IHSS) of p53 tumor suppressor gene in human esophageal precancerous lesions. *Carcinogenesis*, 1996;17(10):2131-2136

# Functional dyspepsia of ulcer-dysmotility type: clinical incidence and therapeutic strategy

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**Subject headings** dyspepsia/drug therapy; famotidine/therapeutic use; cisapride/therapeutic use; peptic ulcer; gastrointestinal motility

Functional dyspepsia is a commonly occurring chronic digestive disorder affecting 20%-40% of the general population<sup>[1]</sup>. It is a syndromic term applied to patients who complain of symptoms presumably arising from the upper abdomen, often in response to meal ingestion, but with absence of organic abnormalities demonstrable by conventional diagnostic tests. Although functional dyspepsia is very common in the community, the classification and the clinical therapy are still uncertain. Therefore this study deals with the clinical incidence and the therapeutic strategy of functional dyspepsia of the ulcer-dysmotility mixed type.

## MATERIALS AND METHODS

### *Diagnostic criteria*<sup>[2]</sup>

The patients who fulfilled the following criteria were selected: postcibal abdominal fullness or bloating with other associated symptoms, including early satiety, upper abdominal pain, nausea, and vomiting; symptoms of moderate to severe intensity, and of more than 3 months duration; absence of clinical, biochemical, and morphological evidence of gastrointestinal, biliary, and systemic diseases assessed by negative results of anamnesis, physical examination, laboratory tests, upper gut endoscopy and ultrasonography; and no previous abdominal surgery.

### *Classification*

Functional dyspepsia is now conventionally divided into ulcer (those with symptoms suggestive of peptic ulceration), dysmotility (those with gastric stasis including upper abdominal bloating, abdominal fullness, early satiety, belching, nausea, and

vomiting), reflux (those with symptoms of gastroesophageal reflux), and unspecified dyspepsia (the remainder)<sup>[1]</sup>. Because many of subjects with dyspepsia could be classified into more than one group, we classified the patients with overlapped symptoms of peptic ulceration and gastric stasis into ulcer dysmotility mixed type of functional dyspepsia.

### *Treatment*

The patients with ulcer-dysmotility mixed type of functional dyspepsia were randomly divided into three groups: group 1 received famotidine (gaster, 40mg qd) for 3 weeks; group 2 received cisapride (prepulsid, 5mg tid) for 3 weeks; and group 3 treated with both famotidine and cisapride for 3 weeks. No additional medication was given. One month after termination of the treatment, the patients were followed up for symptoms disappearance and side-effects.

### *Statistics*

Statistical analyses were made using  $\chi^2$  test.

## RESULTS

A total of 220 patients with functional dyspepsia (122 males and 98 females; aged 19-62 years, averaging 36 years) were included in this study. All patients fulfilled the diagnostic criteria mentioned above. According to the symptoms, 59 cases (26.8%) were classified as ulcer type; 57 cases (25.9%), dysmotility type; 2 cases (0.9%) of reflux type; and 102 cases (46.4%), ulcer-dysmotility mixed type.

The ulcer-dysmotility mixed type (59 males and 43 females, aged 21-61 years, mean 39 years) were randomly allocated to three groups (34 patients each group) and treated with famotidine, cisapride, and famotidine plus cisapride respectively. One month after termination of the treatment, the symptom disappearance of the patients are shown in Table 1. Three patients withdrew from the treatment because of diarrhea caused by cisapride. The results demonstrated that a single drug (famotidine or cisapride) could not efficiently eliminate the symptoms of the patients, while the combined therapy with famotidine plus cisapride seem to be an effective treatment for those patients.

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Received 1998-05-04

**Table 1 Symptom disappearance after treatment**

Groups	n	Ulcer-line	Dysmotility-like	Both
		symptoms(%)	symptoms(%)	symptoms(%)
Famotidine	34	33 (97.1)	10 (29.4) <sup>a</sup>	10 (29.4) <sup>a</sup>
Cisapride	32	9 (28.1) <sup>a</sup>	30 (93.8)	9 (28.1) <sup>a</sup>
Famotidine plus cisapride	33	33 (100)	32 (97.0)	32 (97.0)

<sup>a</sup>*P*<0.01 vs famotidine plus cisapride group.

## DISCUSSION

Like other functional disorders of the gastrointestinal system such as irritable bowel syndrome and gastro-oesophageal reflux, our understanding of the pathophysiological mechanisms underlying this condition still remains elusive. Motor, neurohumoral, and sensory abnormalities in both the stomach and small bowel have been demonstrated in some patients with functional dyspepsia<sup>[3]</sup>. It has been proposed that the previous symptom classification in patients with functional dyspepsia may reflect, to a certain extent, different pathophysiological entities, but distinct symptom classification can not be accomplished. So the investigation and treatment of functional dyspepsia can be benefited by the symptom classification<sup>[4]</sup>. Our study indicated that the ulcer and dysmotility (46.4%) type was overlapped in patients with functional dyspepsia, therefore we suggested the concept of ulcer-dysmotility mixed type which may be beneficial to the classification and treatment of

those patients.

In present study, a total of 102 patients with ulcer-dysmotility mixed type of functional dyspepsia were randomly divided into three groups and treated with famotidine, cisapride, and famotidine plus cisapride respectively. The results demonstrated that famotidine plus cisapride could more efficiently eliminate the symptoms of the patients as compared with the group using famotidine or cisapride alone. Although the previous study suggested that the peripheral kappa agonist fedotozine could modify both sensory and motor responses to stimuli and effectively relieve the key symptoms associated with functional dyspepsia including ulcer and dysmotility-like symptoms<sup>[5]</sup>, famotidine plus cisapride may be an effective and economic therapy for the ulcer-dysmotility mixed type of functional dyspepsia based on the modified symptom related classification.

## REFERENCES

- 1 Talley NJ, Zinsmeister AR, Schleck CD, Melton LJ. Dyspepsia and dyspepsia subgroups: a population-based study. *Gastroenterology*, 1992;102(4):1259-1268
- 2 Coffin B, Azpiroz F, Guarner F, Malagelada JR. Selective gastric hypersensitivity and reflex hyporeactivity in functional dyspepsia. *Gastroenterol*, 1994;107(5):1345-1351
- 3 Lemann M, Dederding JP, Flouric B, Franchisseur C, Rambaud JC, Jian R. Abnormal perception of visceral pain in response to gastric distention in chronic idiopathic dyspepsia: the irritable stomach syndrome. *Dig Dis Sci*, 1991;36(9):1249-1254
- 4 Drossman DA, Thompson WG, Talley NJ, Funch-Jensen P, Janssens J, Whitehead WE. Identification of subgroups of functional gastrointestinal disorders. *Gastroenterol Intern*, 1990;3(1):159-172
- 5 Read NW, Abitbol JL, Bardhan KD, Whorwell PJ, Fraitag B. Efficacy and safety of the peripheral kappa agonist fedotozine versus placebo in the treatment of functional dyspepsia. *Gut*, 1997;41(5):664-668

# New treatments for inflammatory bowel disease

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**Subject headings** Crohn's disease; ulcerative colitis; inflammatory bowel disease; corticosteroids; mesalazine; immunosuppressive drugs; cytokines

## INTRODUCTION

Although inflammatory bowel disease (IBD) is apparently still relatively rare in the East, evidence from Hong Kong suggests that over recent decades the incidence of both ulcerative colitis (UC) and Crohn's disease (CD) may be rising there<sup>[1-3]</sup>. Indeed, it seems likely that as the prevalence of enteric infections falls, and urbanisation and diagnostic awareness increase, IBD will become increasingly common in Asia, as it has in the last fifty years in Europe and North America. It therefore seems appropriate to review advances in the medical therapy of these often refractory diseases.

In this paper, I shall not attempt to review extensive earlier evidence about the efficacy of corticosteroids, aminosalicylates or, in patients refractory to these drugs, immunosuppressive agents; nor shall I discuss the role of surgery. Furthermore, rather than describing the management of the "whole" patient with IBD<sup>[4,5]</sup>, I shall concentrate primarily on drugs which have recently gained an established place in, or appear very promising for, the treatment of IBD. I shall also outline the use of liquid formula diets in active CD, and mention the possible but unproven place of traditional medical modalities.

At the outset, it is worth emphasizing some of the problems encountered in the evaluation of new treatments for IBD. UC and CD have fluctuating courses which depend on both site and extent of disease, as well as, in CD, previous surgery and the time since the most recent relapse. In both diseases, objective measurement of disease activity and definition of trial endpoints is difficult. In CD, for example, measures of disease activity, such as the Crohn's disease activity index (CDAI), colonoscopic appearances and laboratory variables, do not always move in parallel<sup>[6]</sup>.

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Received 1998-07-21

In addition to these difficulties, the design, conduct and interpretation of trials in IBD is complicated by the size and variability of the placebo response<sup>[7]</sup>, the too frequent use of small numbers of patients with consequent Type II statistical errors, and the confounding effects of concurrent or recently discontinued therapy.

Finally, none of the numerous experimental animal models of bowel inflammation resembles human IBD sufficiently closely to enable valid extrapolation of results in such studies to the clinical arena. A possible exception to this generalisation is the spontaneous colitis to which the cotton-top tamarin in captivity is prone<sup>[8]</sup>, but the animal's rarity and expense prevents its utilisation for large scale therapeutic trials. In this review, therefore, attention will be focussed primarily on results obtained in human studies.

## AETIOPATHOGENESIS OF IBD

Although the aetiology of IBD remains obscure, recent studies have begun to shed light on its pathogenesis. In brief, it appears that an initiating factor, for example a microbial or dietary product or antigen, triggers an inappropriately severe and prolonged intestinal mucosal inflammatory response in genetically predisposed individuals<sup>[9]</sup>. The inflammatory response is amplified and perpetuated by recruitment of leucocytes from the gut vasculature which, with upregulation of the expression of nuclear transcription factors such as NFκB<sup>[10]</sup>, leads to excessive release locally of cytokines<sup>[9]</sup>, eicosanoids<sup>[11]</sup>, reactive oxygen metabolites<sup>[12]</sup> and other mediators. In CD, in particular, a procoagulant diathesis and multifocal granulomatous intestinal microinfarction may occur early in the disease process<sup>[13]</sup>. Elucidation of the pathogenesis of IBD has improved our understanding of the possible modes of action of conventional treatment (Table 1) and has led to the development of entirely new therapeutic approaches, to be discussed in the second half of this review (Table 2).

## NEW FORMULATIONS AND APPLICATIONS OF EXISTING DRUGS

### *Corticosteroids (Table 1)*

Corticosteroids are the most useful conventional agents in the treatment of active IBD, whether given orally, intravenously or topically<sup>[4,5]</sup>. They have multiple potentially beneficial actions on the

**Table 1 New formulations and applications of existing therapies for IBD**

Treatment	References
Corticosteroids	
Budesonide	14-18
Aminosalicylates	
Mesalazine (enteric-coated pH-released & slow-release), olsalazine, balsalazide	4,5,19-22
Immunomodulatory agents	
Azathioprine & 6-mercaptopurine	23-29
Cyclosporine	30,31
Methotrexate	32,33
Mycophenolate mofetil	34
Antibiotics	
Metronidazole	35-37
Other	38
Liquid formula diet	39,40

**Table 2 Potential new treatments for IBD aimed at specific pathophysiological targets**

Target	Agent	References
Colonic bacterial flora	Non-pathogenic <i>E coli</i>	41,42
Epithelium	Short chain fatty acids*	43-46
Leucocytes		
Reduce numbers	Apheresis, anti-CD4 antibodies, bone marrow transplant	47-50
Reduce migration	Adhesion molecule antibodies or antisense oligonucleotide	51
Cytokines		
Reduce pro-inflammatory cytokines	NFkB antisense oligonucleotide	10
Antagonise inflammatory cytokines	Anti-TNF antibodies*, IL-1 receptor antagonist	52-55
Increase cytokines	anti-inflammatory IL-10* , interferon alpha or beta, IL-11, TGF beta	56
Mediators	Cytoprotective prostaglandins COX2 inhibition	11
	Synthesis inhibitors and receptor antagonists of leucotrienes, thromboxanes, PAF	11,57-60,67
	Antioxidants	12,61
	Inducible NOS inhibition	62
	Fish oil (EPA)	63-66
Vasculature	Heparin*	67,68
Enteric nerves	Local anaesthetics*	69-71
Unknown targets	Nicotine (UC)*, stopping smoking (CD)*	72-75

\*Denotes current or imminent option

inflammatory process, but which of these is, or are, of predominant importance in IBD is unclear. The anti-inflammatory actions of steroids include inhibition of leucocyte migration and activation; inhibition of the expression of cellular adhesion molecules; suppression of synthesis of cytokines (interleukins-1, -2, -6, -8 and tumour necrosis factor(TNF)), at least in part by inhibition of the activation of NFkB; reduction of release of lipid mediators ( leucotrienes, thromboxanes, prostaglandins and platelet activating factor (PAF)); and inhibition of phospholipase A2, cyclo-oxygenase 2 and inducible nitric oxide synthase.

The numerous side-effects of systemic corticosteroids, particularly when given longterm (e. g. moon face, acne, purpura, dysphoria, opportunistic infection, hypertension, diabetes

mellitus, weight gain, osteopaenia and growth retardation in children), have prompted a search for safer formulations. To this end, a number of corticosteroid preparations have recently been assessed which when given orally are either poorly absorbed from the gut or very rapidly metabolised in the intestinal wall or liver: one of these, budesonide, has so far achieved clinical application.

When given in an oral controlled ileal release (CR) formulation to patients with active ileocaecal CD, budesonide was not significantly less effective than prednisolone in inducing remission in one study<sup>[14]</sup> and substantially better than placebo in another<sup>[15]</sup>. Side-effects were much fewer in patients given budesonide CR than prednisolone, as was adrenal suppression assessed by measurement of plasma cortisol levels<sup>[14]</sup>. When evaluated for maintenance of remission over 12 months in CD, budesonide CR delayed the onset of relapse when compared to placebo, but overall relapse rates at 1 year (60-70%) were, disappointingly, not improved<sup>[16,17]</sup>. In a trial in patients with UC, an oral colonic release preparation of budesonide showed benefit similar to that obtained with prednisolone in patients with active disease; particularly if extensive, disease; improved efficacy for patients with left-sided disease may depend upon the development of formulations providing even more delayed release of the active drug, or the use of higher doses<sup>[18]</sup>. Further studies are needed to clarify the role of oral budesonide in IBD. In particular, we require reassurance that to achieve a clinical result equivalent to that of prednisolone, it will not be necessary to use so great a dose of budesonide as to produce an incidence of systemic side-effects and adrenal suppression similar to that caused by conventional steroids.

### Aminosalicylates (Table 1)

Suphasalazine is firmly established in the management of active colitis (both UC<sup>[4]</sup> and CD<sup>[5]</sup>), and in the maintenance of remission in UC<sup>[4]</sup>; the same applies to its sulphapyridine (and therefore relatively side-effect) free derivatives, whether in bacterially-liberated (olsalazine, balsalazide), enteric-coated pH-released [Asacol, Claversal, Salofalk (mesalazine e. c. )] or slow-release [Pentasa (mesalazine s. r.)] formulations.

Like corticosteroids, aminosalicylates have a wide variety of anti-inflammatory effects, although which of these explain their efficacy in IBD is not known. These actions include inhibition of leucocyte migration and cytotoxicity; reduced activation of NFkB; inhibition of the synthesis of lipid mediators ( leucotrienes, thromboxanes, prostaglandins, PAF ) and of interleukin-1; reduction of prostaglandin degradation; antioxidant

effects; TNF antagonism; and in epithelial cells, induction of heat shock proteins and inhibition of apoptosis and MHC Class II expression.

In active ileocaecal CD, recent interest has focussed on the use of mesalazine slow-release (Pentasa), a preparation which delivers high concentrations of 5-aminosalicylic acid to the small bowel as well as colon. In one study, this preparation, given in high dose (4 g/day) for 16 weeks, resulted in a remission rate of 43% in patients with active ileocaecal CD, while lower doses (1 g and 2 g) were no more effective than placebo (remission rates 18% - 24%)<sup>[19]</sup>.

While several individual studies in the last 10 years have suggested that aminosalicylates may also have a role in maintaining remission in inactive CD, a recent meta-analysis has shown this effect to be minimal, with a reduction in relative risk of symptomatic relapse at 1 year in patients on mesalazine as opposed to placebo of only 6%, and in post-operative patients of 13%<sup>[20]</sup>. Cost-benefit issues in relation to longterm use of these relatively expensive drugs in inactive CD clearly need resolution.

Aminosalicylates have a dose-related therapeutic role in moderately active (although not acute severe) UC and, particularly, in patients with inactive disease, in whom they reduce annual relapse rates to about 25% compared with 75% in placebo-treated patients. In patients with left-sided active UC, clinical trials support the theoretical proposal that drugs delivering 5-aminosalicylate (5-ASA) primarily to the colon (olsalazine, balsalazide) may be more effective than those from which 5-ASA is released more proximally, e. g. Asacol<sup>[21,22]</sup>.

### **Immunomodulatory drugs (Table 1)**

**Azathioprine and 6-mercaptopurine** The benefits of azathioprine and its metabolite, 6-mercaptopurine, as second-line agents in the management of chronic IBD are now widely accepted<sup>[23]</sup>. These agents inhibit purine nucleotide biosynthesis and appear thereby to modify Tlymphocyte function.

Azathioprine is useful in maintaining remission of both UC<sup>[24]</sup> (in patients failing to respond adequately to aminosalicylates) and CD<sup>[25]</sup>, and as a steroid-sparing drug in the minority of patients with either disease who relapse repeatedly on steroid withdrawal<sup>[23]</sup>; it may also play special roles in accelerating remission and healing ileal lesions when given in combination with prednisone in active CD<sup>[26,27]</sup>, and in Crohn's patients with perianal disease<sup>[23]</sup>.

Apart from their side-effects (nausea, vomiting, headache, joint pains, rash, fever, and, more seriously, bone marrow depression, acute

pancreatitis, chronic hepatitis and possible malignancy in longterm users), the main disadvantages of a zathioprine and 6-mercaptopurine are that they take up to 4 months to act when given orally. Trials to assess the possibility of accelerating the clinical response in active CD using intravenous azathioprine are in progress<sup>[28]</sup>. If results prove successful, use of this route of administration will demand prior assay of red blood cell concentration of 6-thiopurine methyltransferase (6-TPMT), homozygous deficiency of which can be fatal as a result of failure of inactivation of 6-mercaptopurine.

A French report showed that, in patients with inactive CD, the risk of relapse after 4 years of successful treatment with azathioprine (or 6-mercaptopurine) was similar whether the immunosuppressive agent was continued or stopped; The authors suggested that, in view of the potential toxicity of long-term use of azathioprine, its withdrawal should be considered in patients who had remained in remission after 4 years' treatment<sup>[29]</sup>.

**Cyclosporine** Cyclosporine inhibits helper-and cytotoxic T-lymphocyte function and proliferation, mainly through inhibition of interleukin-2 gene transcription. It also reduces interferon-gamma, interleukin-3 and interleukin-4 production.

Interest in the use of intravenous cyclosporine in active CD was stimulated by Brynskov's provocative report in 1989<sup>[30]</sup>, but subsequent trials have cast a dampener on the use of this agent, at least in low dose, in Crohn's.

In contrast, in a single small controlled study<sup>[31]</sup>, the results of which have been largely confirmed by subsequent experience elsewhere, intravenous (4 mg/kg-day) followed by oral (5 - 8 mg·kg/·day) cyclosporin, given in addition to continuing corticosteroids, averted colectomy in the acute phase in about 80% of patients with acute severe UC who had failed to respond to 5-7 days of intravenous steroids.

Enthusiasm for this approach, however, needs to be tempered both by the frequency of relapse necessitating colectomy (up to 50%) that follows withdrawal of cyclosporine, and by its serious side-effects. Acutely, these include opportunistic infections, particularly pneumocystis carinii pneumonia; renal impairment including a 20% reduction in glomerular filtration rate in most patients, and an often irreversible interstitial nephritis in up to 25% patients; hypertension, hepatotoxicity, epileptic fits, hyperkalaemia, hyperuricaemia, hypertrichosis and paraesthesiae. Longterm oral use may predispose to lymphoma. The side-effects of cyclosporine necessitate frequent

monitoring of cyclosporine blood levels and serum biochemistry in treated patients. At present, use of cyclosporine in acute severe UC should probably be restricted to clinical trials and specialist centres familiar with its use. Further studies are needed to define which patients should be given the drug intravenously, and what continuing oral therapy, for example cyclosporin, azathioprine or 6-mercaptopurine, they should be prescribed thereafter. It is possible, however, that intravenous cyclosporine, perhaps given with prophylactic trimethoprim/sulphamethoxazole, may turn out to be very useful, in the minority of patients with steroid-refractory acute severe UC, for buying time for improving their nutrition prior to, and/or preparing them psychologically for surgery.

**Methotrexate** Methotrexate is an immunosuppressive agent widely used in difficult rheumatoid arthritis and psoriasis. A North American group reported that this drug, given once a week as a 25 mg intramuscular injection, was superior to placebo in improving symptoms (remission rates at 16 weeks 40% and 20%, respectively) and reducing requirements for prednisone in steroid-dependent CD<sup>[32]</sup>. In chronic steroid-dependent UC, in contrast, a lower dose of methotrexate (12.5 mg), given orally once weekly, was no more effective than placebo in the induction of remission or its maintenance over a 9 month period<sup>[33]</sup>. Whether use of methotrexate in IBD becomes widespread will depend on the success of rival agents with fewer side-effects.

**Mycophenolate mofetil** Very recently, a preliminary study suggested that mycophenolate mofetil, a T-cell inhibitor used increasingly in the prevention of transplant rejection, may be of value in refractory Crohn's disease<sup>[34]</sup>. This drug appears to be relatively non-toxic, although expensive, and is currently undergoing controlled trial in CD.

#### **Antibiotics (Table 1)**

**Metronidazole** Metronidazole acts not only against a range of anaerobes and protozoa, but also has immunomodulatory effects. Controlled trials have shown that oral metronidazole (800 mg/day) has moderate benefit in ileocolonic CD<sup>[35]</sup>, and in preventing recurrence after ileal resection<sup>[36]</sup>, while open studies indicate that it may also be effective in perianal CD<sup>[37]</sup>. Treatment needs to be given for up to 3 months, and may be complicated by nausea, vomiting and unpleasant reactions when combined with alcohol; more importantly, it may induce a peripheral neuropathy not always reversible on its discontinuation.

**Other antibiotics** Anecdotal reports and uncontrolled trials have suggested possible roles for clarithromycin and ciprofloxacin in CD, the latter particularly for perianal disease, and for trimethoprim/sulphamethoxazole in acute severe UC. Tobramycin given orally in addition to steroids and sulphasalazine improved remission rate in active UC<sup>[38]</sup>, and some gastroenterologists use this or another broad-spectrum antibiotic as prophylaxis against bacteraemia and endotoxic shock in severely ill patients with acute severe UC.

#### **DIETARY THERAPY (Table 1)**

There is no specific dietary therapy for patients with UC, although a few (<5%) may improve with avoidance of cow's milk, and some with proctitis and proximal constipation may benefit from fibre supplementation. Patients with stricturing small bowel CD should avoid high residue foods (e.g. citrus fruit segments, nuts, uncooked vegetables) which might cause bolus obstruction. All patients with IBD, particularly if it is active or extensive, are at risk of nutritional deficiencies which need replacement as necessary.

Over the last 20 years, it has become clear that in children with CD, as well as in adults with extensive small bowel disease and in those who respond poorly to, or prefer to avoid corticosteroids, an alternative therapy is a liquid formula diet. This can either be elemental (aminoacid-based), protein hydrolysate (peptide-containing) or polymeric (containing whole protein and not therefore hypoallergenic), and is given for 4-6 weeks as the sole nutritional source<sup>[39,40]</sup>. This approach is probably as effective as corticosteroid therapy in the short-term, about 60% patients achieving remission. Unfortunately, after the resumption of a normal diet, many patients relapse (50% at 6 months): whether this can be prevented by selective and gradual reintroduction of particular foods to which individual patients are not intolerant, or by the intermittent use of further enteral feeding for short periods, remains to be proven.

The success of enteral nutrition as a primary treatment for CD is also limited by its cost, the unpleasant taste of some of the available preparations, the need often to give the feed by nasogastric tube, and the poor compliance of many patients in adhering to it. Such therapy does, nevertheless, offer a valuable alternative in the well-motivated minority of patients for whom it is appropriate.

#### **NEW THERAPIES AIMED AT SPECIFIC PATHOPHYSIOLOGICAL TARGETS (Table 2)**

Elucidation of the pathogenesis of IBD has led to

the evaluation in experimental animal models, and to a lesser extent in the human disease, of several different therapeutic approaches aimed at specific pathophysiological targets (Table 2). Where substantial data in humans, will now be briefly discussed.

#### ***Non-pathogenic escherichia coli***

There is some evidence that patients with UC have increased proportions of adhesive and enterohaemorrhagic *E coli* in their large bowel. Two preliminary reports suggest that oral administration of capsules containing non-pathogenic *E coli* may have a role in maintaining remission in patients with inactive UC<sup>[41,42]</sup>, but further work is required to confirm the efficacy of this or other (e.g., lactobacillus) probiotic approaches.

#### ***Short chain fatty acids (SCFA)***

Normal colonic epithelial cells depend for their energy metabolism on a luminal supply of SCFA, derived from bacterial flora. In UC, colonocytes inadequately utilise SCFA; low luminal SCFA levels in UC exacerbate this metabolic defect<sup>[43]</sup>. Efforts to remedy the defect by treatment of patients with distal UC with enemas containing SCFA, principally butyrate, have unfortunately not proved uniformly successful<sup>[44-46]</sup>; furthermore, the appeal of this very safe therapy is restricted by the unpleasant smell of the enemas.

#### ***Modifying leucocyte numbers and function***

Depleting leucocyte numbers, by use of leucocyte apheresis, antiCD4 antibodies or bone marrow transplantation, has been shown in uncontrolled reports to suppress activity of CD<sup>[47-49]</sup>; a similar effect is seen in AIDS when the CD4 count falls<sup>[50]</sup>. Furthermore, trials are in progress to assess the clinical efficacy in IBD of inhibiting leucocyte migration into the gut mucosa using antibodies or antisense oligonucleotides to adhesion molecules such as ICAM-1<sup>[51]</sup>. As with other major immunomodulatory therapies, it is not yet clear whether the benefits of such approaches will outweigh their cost, complexity and, particularly, toxicity in relation to the risks of infection and malignancy.

#### ***Modulation of cytokine activity***

Recognition of altered cytokine expression in IBD has prompted therapeutic trials using interleukin-1 receptor antagonist, interferon-alpha and gamma, anti-TNF-alpha antibody and interleukin-10 (IL-10): of these, the last two are the most promising.

**Anti-TNF-alpha antibody** Controlled trials have

shown that intravenous infusions of either mouse/human chimeric (cA2) or 95% humanised (CDP571) anti-TNF-alpha antibody induced remission in active refractory CD<sup>[52,53]</sup> and healed Crohn's fistulae<sup>[54]</sup>; uncontrolled studies suggest efficacy in UC too<sup>[55]</sup>. The published results are impressive, mucosal lesions healing completely in many instances. However, the relative merits of cA2 and CDP571 require clarification in relation to their efficacy, safety and cost. Reassurance is needed that repeated usage will not lead to adverse effects as a result of host antibody induction, or of immunosuppression with consequent opportunistic infection or malignancy. Definition of which patients are most likely to benefit from this very specialist treatment is also needed: this may relate not only to their disease phenotype (e.g., fistulating disease), but also their genotype (e.g., TNF microsatellite subtype).

**Interleukin-10** IL-10 is an anti-inflammatory and immunosuppressive cytokine. A recent placebo-controlled trial of recombinant human IL-10 gave promising results in steroid-refractory CD<sup>[56]</sup>, and further reports are imminent.

**Antisense oligonucleotide to NFkB.** The upregulation of NFkB in IBD tissue may play a central role in its pathogenesis as a result of stimulation of the synthesis of proinflammatory cytokines such as TNF, IL-1 and IL-6<sup>[10]</sup>. It remains to be seen whether trials of antisense oligonucleotides to NFkB will prove as effective and safe in human IBD as they appear to be in experimental colitis in mice<sup>[10]</sup>.

#### ***Modifying the effects of lipid mediators***

Reducing synthesis of proinflammatory prostaglandins with non-selective non-steroidal anti-inflammatory drugs (NSAIDs) has an adverse rather than beneficial effect in IBD, perhaps because of the concomitant suppression of cytoprotective prostaglandins<sup>[11]</sup>. The efficacy and safety of selective cyclooxygenase-2 (COX2) inhibitors have not yet been formally assessed in IBD. Trials with inhibitors of the synthesis of the extremely potent inflammatory mediator, leucotriene B4, in UC have shown at best a very modest benefit<sup>[57,58]</sup>. Ridogrel, a dual thromboxane synthesis inhibitor and receptor antagonist<sup>[59]</sup>, has been shown to induce remission in over 40% patients with moderately active UC<sup>[60]</sup>, and is under trial in active Crohn's. Antagonists to platelet activating factor (PAF) have been ineffective in active UC.

#### ***Antioxidants***

While enhanced mucosal production of reactive

oxygen metabolites is well established<sup>[12]</sup>, published trials of antioxidant therapy in human IBD are limited to one open study of patients with steroid-resistant CD who appeared to benefit from intramuscular injections of superoxide dismutase<sup>[61]</sup>. Despite the lack of controlled data available, many patients with IBD in the West use over the counter antioxidant drugs in an effort to ameliorate their disease.

Although increased mucosal generation of nitric oxide may contribute to the pathogenesis of IBD<sup>[62]</sup>, there is no data yet to support the hypothesis that selective inhibition of inducible nitric oxide synthase may be beneficial.

#### **Fish oil (eicosapentaenoic acid, EPA)**

EPA, the active ingredient of fish oil capsules, decreases synthesis of leucotriene B<sub>4</sub>, thromboxane A<sub>2</sub>, prostaglandin E<sub>2</sub>, platelet activating factor and interleukin-1. Although these actions should make it a useful anti-inflammatory agent, trials in UC have shown that high doses produce only modest clinical improvement<sup>[63-65]</sup>; in addition, the strong fishy odour on the breath associated with consumption of EPA preparations is likely to inhibit their widespread use.

More recently, an enteric-coated fish oil preparation, which is better tolerated than standard formulations, although is not yet commercially available, has been reported to reduce substantially the relapse rate in patients with inactive CD (relapse rate at 1 year 28% on fish oil, 69% on placebo)<sup>[66]</sup>. This exciting result, if confirmed by other groups, could become a very useful and, probably, safe form of maintenance therapy in CD: the apparently beneficial cardiovascular effects of EPA would contribute to its popularity with patients.

#### **Modulation of procoagulant state**

Active IBD is characterised by a procoagulant diathesis which may contribute not only to the increased risk of systemic thromboembolism characteristic of the disease<sup>[59,67]</sup>, but also to the intramucosal inflammatory process<sup>[13]</sup>. Several recent pilot studies suggest that intravenous heparin may have a beneficial effect on disease activity in both UC and CD<sup>[68]</sup>, and controlled studies are in progress. Mechanisms of action of heparin in IBD are likely to include interference with leucocyte-endothelial cell adhesion and of platelet activation as well as its anticoagulant effects<sup>[68]</sup>.

#### **Modulation of enteric nerve function**

Neuronal hyperplasia, hypertrophy and degeneration, together with abnormalities of neurotransmitter content, have been described in

the gut mucosa of patients with IBD. In open studies, Bjorck *et al* have reported clinical and sigmoidoscopic improvement in 90% of UC patients treated with lidocaine enemas for up to 12 weeks<sup>[69]</sup>; similar uncontrolled results using ropivacaine gel rectally have been published more recently<sup>[70]</sup>. This approach needs to be validated by controlled trials; whether any beneficial effect of lidocaine or other local anaesthetics is due to modulation of enteric nerve function or to inhibition of production by mucosal leucocytes of inflammatory mediators<sup>[71]</sup> is unclear.

#### **Smoking: Nicotine**

Smoking is rare in patients with UC and anecdotal reports have suggested that some individuals can control their disease by judicious indulgence in this otherwise undesirable habit. Two controlled studies have confirmed that nicotine patches can induce remission in active UC<sup>[72,73]</sup>, although, surprisingly, cannot maintain it<sup>[74]</sup>. Studies are in progress to assess the efficacy of alternative formulations of nicotine (for example in oral pH-release capsules or enemas) which, by allowing first-pass hepatic metabolism of nicotine will avert the systemic side effects produced by skin patches, and allow the use of higher doses. The mechanism of the therapeutic effect of nicotine in UC, some of the pharmacological effects of which appear to be pro-inflammatory, is unknown: possibilities include increased colonic mucus secretion, alterations of cell-mediated immunity, and reductions in gut permeability, prostaglandin E<sub>2</sub> production and rectal mucosal blood flow.

Smoking has an adverse effect on the natural history of CD<sup>[75]</sup>, including the reoperation rate: patients with CD who smoke should be advised to stop.

#### **TRADITIONAL MEDICINE**

In the West, a substantial minority of patients with IBD, dissatisfied with conventional pharmacological treatment, resort to alternative therapies including herbal medications such as aloe vera, relaxation, aromatherapy, acupuncture and homeopathy (Lakeman M & Rampton DS, unpublished)<sup>[76,77]</sup>. Unfortunately, however, there appear to be very few reports of the efficacy of such therapy, at least in the English language literature<sup>[78]</sup>: controlled studies of traditional medical treatment of IBD are urgently needed.

#### **CONCLUSIONS**

There is still no entirely effective, safe, cheap treatment for the suppression of IBD, let alone its cure.

Amongst the conventional alternatives, the

major recent advances are the development of oral corticosteroids, such as budesonide, with few systemic side-effects, and of new aminosalicylates, such as mesalazine slow-release for CD and balsalazide for UC. The side-effects of existing immunomodulatory agents, with the possible exception of azathioprine, make them unlikely to achieve a major role in patients with uncomplicated disease. Liquid formula diets are effective and safe in patients with active small bowel CD, but the value of traditional medical treatment in IBD is as yet unproven.

Of the drugs designed to rectify specific pathophysiological abnormalities in IBD, short chain fatty acids and lidocaine enemas are occasionally useful in patients with refractory distal UC. Injections of anti-TNF antibody and interleukin-10 may prove to be a major step forward in patients with difficult IBD, but their safety in the long- as well as short-term requires confirmation. Other “designer-drugs” have proved disappointing to date, perhaps because their effects on the complex inflammatory response are, unlike those of corticosteroids and aminosalicylates, too accurately focussed on specific, but redundant mediator pathways. We still need to learn what makes the inflammatory response in the mucosa of patients with UC and Crohn’s persist chronically: it is possible that identification of this key abnormality will, in the absence of the discovery of a reversible primary cause, offer the best hope of developing an effective new therapy for patients with IBD.

## REFERENCES

- Sung JY, Chan KL, Hsu R, Liew CT, Lawton JWM. Ulcerative colitis and antineutrophil cytoplasmic antibodies in Hong Kong Chinese. *Am J Gastroenterol*, 1993;88:864-869
- Lai CL, Wu PC, Wong KL *et al.* Clinical features of ulcerative proctocolitis in Hong Kong Chinese: a review of three decades. *Am J Proctol Gastroenterol Colon Rectal Surg*, 1985;1:14-19
- Sung JY, Hsu RKK, Chan FKL, Liew CT, Lau JWY, Li AKC. Crohn’s disease in the Chinese population. An experience from Hong Kong. *Dis Colon Rectum*, 1994;37:1307-1309
- Kornbluth A, Sachar DB. Ulcerative colitis practice guidelines in adults. *Am Gastroenterol*, 1997;92:204-211
- Hanauer SB, Meyers S. Management of Crohn’s disease in adults. *Am J Gastroenterol*, 1997;92:559-566
- Modigliani R, Mary JY, Simon JF *et al.* Clinical, biological and endoscopic picture of attacks of Crohn’s disease: evaluation on prednisone. *Gastroenterology*, 1990;98:811-818
- Meyers S, Janowitz HD. “Natural history” of Crohn’s disease. An analytic review of the placebo lesson. *Gastroenterology*, 1984;87:1189-1192
- Chalifoux LV, Bronson RT. Colonic adenocarcinoma associated with chronic ulcerative colitis in cotton top marmosets. *Gastroenterology*, 1981;80:942-947
- Podolsky DK. Inflammatory bowel disease. *New Eng J Med*, 1991;325:928-937 & 1008-1016
- Neurath MF, Pettersson S, Meyer Zum Buschenfelde KH, Strober W. Local administration of antisense phosphothiolate oligonucleotides to the p65 subunit of NF- $\kappa$ B abrogates established experimental colitis in mice. *Nature Medicine*, 1996;2:998-1004
- Rampton DS, Hawkey CJ. Prostaglandins and ulcerative colitis. *Gut*, 1984; 25: 1399-1413
- Simmonds NJ, Rampton DS. Inflammatory bowel disease—a radical view. *Gut*, 1993;34:865-868
- Wakefield AJ, Sankey EA, Dhillon AP. Granulomatous vasculitis in Crohn’s disease. *Gastroenterology*, 1991;100:1279-1287
- Rutgeerts P, Lofberg R, Malchow H. A comparison of budesonide with prednisolone for active Crohn’s disease. *New Eng J Med*, 1994;331:842-845
- Greenberg GR, Feagan BG, Martin F. Oral budesonide for active Crohn’s disease. *New Eng J Med*, 1994;331:836-841
- Greenberg GR, Feagan BG, Martin F. Oral budesonide as maintenance treatment for Crohn’s disease: a placebo controlled, dose ranging study. *Gastroenterology*, 1996;110:45-51
- Lofberg R, Rutgeerts P, Malchow H. Budesonide prolongs time to relapse in ileal and ileocaecal Crohn’s disease. A placebo-controlled one year study. *Gut*, 1996;39:82-86
- Lofberg R, Danielsson A, Suhr O. Oral budesonide versus prednisolone in patients with active extensive and left-sided ulcerative colitis. *Gastroenterology*, 1996;110:1713-1718
- Singleton JW, Hanauer SB, Gitnick GL. Mesalamine capsules for the treatment of active Crohn’s disease: result of 16 week trial. *Gastroenterology*, 1993;104:1292-1301
- Comma C, Giunta M, Rosselli M, Cottone M. Mesalazine in the maintenance treatment of Crohn’s disease: a meta-analysis adjusted for confounding variables. *Gastroenterology*, 1997;113:1465-1473
- Courtney MG, Nunes DP, Bergin CF. Randomised comparison of olsalazine and mesalazine in prevention of relapses in ulcerative colitis. *Lancet*, 1992; 339:1279-1281
- Green JRB, Lobo AJ, Holdsworth CD. Balsalazide is more effective and better tolerated than mesalamine in the treatment of acute ulcerative colitis. *Gastroenterology*, 1998;114:23-28
- Pearson DC, May GR, Fick GH, Sutherland LR. Azathioprine and 6-mercaptopurine in Crohn’s disease: a meta-analysis. *Ann Intern Med*, 1995;122: 132-142
- Hawthorne AB, Logan RFA, Hawkey CJ. Randomised controlled trial of aza thioprine withdrawal in ulcerative colitis. *BMS*, 1992;305:20-22
- O’Donoghue DP, Dawson AM, Powell-Tuck J, Bown RL, Lennard-Jones JE. Double blind withdrawal trial of azathioprine as maintenance treatment for Crohn’s disease. *Lancet*, 1978;2:955-957
- Ewe K, Press AG, Sing CC. Azathioprine combined with prednisone or monotherapy with prednisone in active Crohn’s disease. *Gastroenterology*, 1993; 105:367-372
- D’Haens G, Geboes K, Ponette E, Penninckx F, Rutgeerts P. Healing of severe recurrent ileitis with azathioprine therapy in patients with Crohn’s disease. *Gastroenterology*, 1997;112:1475-1481
- Sandborn WJ, van Os EC, Zins BJ. An intravenous loading dose of azathioprine decreases the time to response in patients with Crohn’s disease. *Gastroenterology*, 1995;109:1808-1817
- Bouhnik Y, Lemann M, Mary JY. Long-term follow-up of patients with Crohn’s disease treated with azathioprine or 6-mercaptopurine. *Lancet*, 1996; 347:215-219
- Brynskov J, Freund L, Rasmussen SN. A placebo-controlled, double-blind randomised trial of cyclosporine therapy in active chronic Crohn’s disease. *New Eng J Med*, 1989;321:845-850
- Lichtiger S, Present DH, Kornbluth A. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *New Eng J Med*, 1994;330:1841-1845
- Feagan BG, Rochon J, Fedorak RN. Methotrexate for the treatment of Crohn’s disease. *New Eng J Med*, 1995;332:292-297
- Oren R, Arber N, Odes S. Methotrexate in chronic active ulcerative colitis: a double blind, randomised, Israeli multi-centre trial. *Gastroenterology*, 1996;110:1416-1421
- Neurath MF, Duchmann R, Peters M. Mycophenolate mofetil/cortisone versus azathioprine/cortisone for treatment of active inflammatory bowel disease: preliminary results of a prospective randomised study. *Gut*, 1997;41(suppl3): A224
- Rosen A, Ursing B, Alm T. A comparative study of metronidazole and sulphasalazine for active Crohn’s disease: the Cooperative Crohn’s Disease Study in Sweden. II Result. *Gastroenterology*, 1982;83:550-562
- Rutgeerts P, Hiele M, Geboes K. Controlled trial of metronidazole for prevention of Crohn’s recurrence after ileal resection. *Gastroenterology*, 1995; 108:1617-1621
- Bernstein LH, Frank MS, Brandt LJ, Boley SL. Healing of perineal Crohn’s disease with metronidazole. *Gastroenterology*, 1980;79:357-365
- Burke DA, Axon ATR, Clayden SA, Dixon MF, Johnston D, Lacey RW. The efficacy of tobramycin in the treatment of ulcerative colitis. *Aliment Pharmacol Therap*, 1990;4:123-129
- Fernandes Benares F, Cabre F, Esteve-Comas M, Gassull MA. How effective is enteral nutrition in inducing clinical remission in active Crohn’s disease: a meta-analysis of the randomised clinical trials. *J Parent Enteral Nutr*, 1995; 19:356-364
- Griffiths AM, Ohlsson A, Sherman PM, Sutherland LR. Meta-analysis of enteral nutrition as a primary treatment of active Crohn’s disease. *Gastroenterology*, 1995;108:1056-1067
- Rembacken BJ, Snelling AM, Hawkey PM, Axon ATR. A double-blind trial of non pathogenic *E coli* vs mesalazine for the treatment of ulcerative colitis. *Gut*, 1997;41(suppl 3):A70
- Kruis W, Schutz E, Fric P, Fixa B, Jud, aier G, Stolte M. Double-blind comparison of an oral *Escherichia coli* preparation and mesalazine in maintaining remission of ulcerative colitis. *Aliment Pharmacol Therap*, 1997;11:853-858
- Roediger WE. The colonic epithelium in ulcerative colitis: an energy deficiency disease. *Lancet*, 1980;2:712-715
- Breuer RI, Soergel KH, Lashner BA. Short chain fatty acid rectal irrigation for left sided ulcerative colitis: a randomised, placebo-controlled trial. *Gut*, 1997;40:485-491
- Scheppach W, Sommer H, Kirchner T. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology*, 1992;103:51-56
- Vernia P, Marcheggiano A, Caprilli R. Short-chain fatty acid topical treatment in distal ulcerative colitis. *Aliment Pharmacol Therapy*, 1995;9:309-313

- 47 Stronkhorst A, Radema S, Yong S-L. CD4 antibody treatment in patients with active Crohn's disease: a phase I dose finding study. *Gut*, 1997;40:320-327
- 48 Lopez-Cubero SO, Sullivan KM, McDonald GB. Course of Crohn's disease after allogeneic marrow transplantation. *Gastroenterology*, 1998;114:433-440
- 49 Bicks RO, Groshart KD. Editorial: the current status of T-cell lymphocyte apheresis (TLA) treatment of Crohn's disease. *J Clin Gastroenterol*, 1989;11:136-138
- 50 James SP. Remission of Crohn's disease after human immunodeficiency virus infection. *Gastroenterology*, 1988;95:1667-1669
- 51 Koizumi M, King N, Lobb R, Benjamin C, Podolsky DF. Expression of vascular adhesion molecules in inflammatory bowel disease. *Gastroenterology*, 1992;103:840-847
- 52 Targan SR, Hanauer SB, van Deventer SJH. A short-term study of chimeric monoclonal antibody cA2 to tumour necrosis factor alpha for Crohn's disease. *New Eng J Med*, 1997;337:1029-1035
- 53 Stack WA, Mann SD, Roy AJ. Randomised controlled trial of CDP571 antibody to tumour necrosis factor alpha in Crohn's disease. *Lancet*, 1997;349:521-524
- 54 Van Deventer SJH, van Hogezaand R, Present DH. Controlled study of anti-TNF-alpha for enterocutaneous fistulae complicating Crohn's disease. *Gut*, 1997;41(suppl 3):A2
- 55 Evans RC, Clarke L, Heath P, Stephens S, Morris AI, Rhodes JM. Treatment of ulcerative colitis with an engineered human anti-TNF-alpha antibody CDP571. *Aliment Pharmacol Therapy*, 1997;11:1031-1035
- 56 Van Deventer SJH, Elson CO, Fedorak RN. Multiple doses of intravenous interleukin 10 in steroid refractory Crohn's disease. *Gastroenterology*, 1997;113:383-389
- 57 Laursen LS, Lauritsen K, Bukhave K. Selective 5 lipoxygenase inhibition by Zileuton in the treatment of relapsing ulcerative colitis: a randomised double-blind, placebo-controlled multicentre trial. *Eu J Gastroenterol Hepatol*, 1994; 6:209-215
- 58 Hawkey CJ, Dube LM, Rountree LV. A trial of Zileuton versus mesalazine or placebo in the maintenance of remission of ulcerative colitis. *Gastroenterology*, 1997;112:718-724
- 59 Rampton DS, Collins CE. Thromboxanes in inflammatory bowel disease—pathogenic and therapeutic implications. *Aliment Pharmac Therapy*, 1993; 7:357-367
- 60 Wright J, Schenowitz G, Adler G, Schneider H. Ridogrel for the treatment of mild to moderate ulcerative colitis: a placebo-controlled trial. *Gut*, 1996;39(suppl 3):A188
- 61 Emerit J, Pelletier S, Tosoni-Verilgnue D, Mollet R. Phase II trial of copper-zinc superoxide dismutase (Cu Zn SOD) in the treatment of Crohn's disease. *Free Rad Biol Med*, 1989;7:145-149
- 62 Boughton-Smith NK, Evans SM, Hawket CJ. Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet*, 1993;342:338-340
- 63 Stenson WF, Cort D, Rodgers J. Dietary supplementation with fish oil in ulcerative colitis. *Ann Intern Med*, 1992;116:609-614
- 64 Aslan A, Triadafilopoulos G. Fish oil supplementation in active ulcerative colitis: a double blind, placebo controlled crossover study. *Am J Gastroenterol*, 1992;87:432-437
- 65 Hawthorne AB, Daneshmend TK, Hawkey CJ. Treatment of ulcerative colitis with fish oil supplementation: a prospective 12 month randomised controlled trial. *Gut*, 1992;33:922-928
- 66 Belluzi A, Brignola C, Campieri M, Pera A, Boschi S, Miglioli M. Effect of an enteric coated fish-oil preparation on relapses in Crohn's disease. *New Eng J Med*, 1996;334:1557-1560
- 67 Collins CE, Rampton DS. Platelet dysfunction: a new dimension in inflammatory bowel disease. *Gut*, 1995;36:5-8
- 68 Evans RC, Shim Wong V, Morris AI, Rhodes JM. Treatment of corticosteroid-resistant ulcerative colitis with heparin—a report of 16 cases. *Aliment Pharmacol Therapy*, 1997;11:1037-1040
- 69 Bjorck S, Dahlstrom A, Ahlman H. Treatment of distal ulcerative colitis with lidocaine. *Can J Gastroenterol*, 1993;7:179-181
- 70 Arlander E, Ost A, Stahlberg D, Lofberg R. Ropivacaine gel in active distal ulcerative colitis and proctitis—a pharmacokinetic and exploratory clinical study. *Aliment Pharmac Therapy*, 1996;10:73-81
- 71 Sinclair R, Eriksson AS, Gretzer C, Cassuto J, Thomsen P. Inhibitory effects of amide local anaesthetics on stimulus-induced human leucocyte metabolic activation, leucotriene B4 release and interleukin-1 secretion in vitro. *Acta Anaesthesiol Scand*, 1993;37:159-165
- 72 Pullan RD, Rhodes J, Ganesh S. Transdermal nicotine for ulcerative colitis. *New Eng J Med*, 1994;330:811-815
- 73 Sandborn WJ, Tremaine W, Offord KP. Transdermal nicotine for mildly to moderately active ulcerative colitis: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med*, 1997;126:364-371
- 74 Thomas GAO, Rhodes J, Mani V. Transdermal nicotine as maintenance therapy for ulcerative colitis. *New Eng J Med*, 1995;332:988-992
- 75 Sutherland LR, Ramcharan S, Bryant, Fick G. Effect of cigarette smoking on recurrence of Crohn's disease. *Gastroenterology*, 1990;98:1123-1128
- 76 Rawsthorne P, Cronin CC, Shanahan F, Anton PA, Lofberg R, Bernstein CN. An international survey of the use and attitudes regarding alternative medicine by patients with inflammatory bowel disease. *Gastroenterology*, 1998; 114:A1067
- 77 Moody GA, Eaden JA, Phakta P, Sher K, Mayberry JF. The role of complementary medicine in European and Asian patients with inflammatory bowel disease. *Public Health*, 1998; in press
- 78 Chen Z. Treatment of ulcerative colitis with acupuncture. *J Tradit Chin Med*, 1995;15:231-233

# Progress in studies of tetrandrine against hepatofibrosis

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**Subject headings** liver cirrhosis/therapy; liver cirrhosis/pathology; liver cirrhosis, experimental/therapy; tetrandrine/therapeutic use

Tetrandrine (Tet) is the main alkaloid isolated from the lumpy root of *Stephania tetrandra* s. *Moore*. Its molecular formula is  $C_{33}H_{42}N_2O_6$  and its chemical structure belongs to a dibenzy-isoquinidine. Modern pharmacological studies have proved that Tet is a  $Ca^{2+}$  antagonist, which acts mainly on the calcium channel of cell to block the cross-membrane transportation of calcium ions as well as their intracellular distribution and utilization. In recent years, the actions of Tet in preventing and treating hepatofibrosis have gradually attracted attention of more investigators. With the establishment of the technique of liver cell isolation and culture and advance in technology of molecular biology, studies of the antihepafibrotic effects of Tet have probed into the cellular, subcellular and molecular levels. This article is to give a brief review of such researches over the past few years.

## EFFECT OF TETRANDRINE ON EXPERIMENTAL HEPATOFIBROSIS IN RATS

The effects of Tet on  $CC_{14}$  induced rat hepatofibrosis model showed that the serum contents of hyaluronic acid (HA) and serum ALT activities among the Tet treated groups in different stages were all lower than those in the untreated control model ( $P < 0.01$ ). At the end of the 3rd week, the degrees of liver cell degeneration and necroses and inflammatory cell unvasion in the treated group were all lower than those of the control model. At the end of the 12th week, the control model rats showed an increase in fibroblast proliferation to grade 2.8 with pseudolobule formation on HE and VG staining; however in the corresponding treatment group, the lobular structure was still fairly well preserved, although there were some proliferation of fibroblasts and increase in collagen. These indicate that Tet may improve liver function, reduce liver damage and inhibit the extracellular matrix (ECM) formation<sup>[1]</sup>. Tet could suppress the proliferation

and transformation of fat storing cells (FSC or Ito cells), reduce the deposition of type IV collagen and remarkably decrease the number of FSCs in rat with hepatofibrosis, as compared with the saline treated controls ( $P < 0.01$ ). Moreover, the area of rough endoplasmic neticulum of FSCs in the treated group was less than that of the control group ( $P < 0.05$ )<sup>[2,3]</sup>. In rats with liver cirrhosis and portal hypertension, Tet could reduce liver cell damage and fibroses, and lower the serum ALT, alkaline phosphatase (ALP) and total bilirubin (STB) to normal<sup>[4]</sup>. Tet could also lower the portal pressure and systemic arterial pressure in portal hypertensive rats<sup>[5]</sup>. In comparison with the traditional antifibrotic drug colohicine, Tet had more or less similar effects in reducing serum procollagen III peptide (P III P), HA and intrahepatic inflammation and had better effect than colohicine and less toxic side-effects<sup>[6]</sup> in suppression of proliferation and transformation of FSCs as well as the degree of collagen deposition.

## CYTOLOGICAL STUDY OF THE ANTIFIBROTIC EFFECT OF TET

Liver fibrosis is a progressive proliferation of connective tissue in the liver parenchyma caused by various chronic liver diseases, and is an intermediate in the course of ultimate development to cirrhosis. Cells participating in liver fibrosis mainly include fibroblasts, Ito cells, hepatocytes, endothelial cells, Kupffer cells, etc. These cells, stimulated by cytokines of some immune active cells, may synthesize and secrete excessive amount of collagen and other extracellular matrix (ECM), which are deposited in the liver and thus lead to liver fibrosis.

### *Effects of Tet on the growth and proliferation of fibroblasts*

Fibroblasts can synthesize and secrete large amounts of collagen, fibronectin, HA, laminin (LN) and other components of ECM. Consequently, their growth and proliferation have innegligible effects on liver fibrosis. It was observed by means of flowcytometry that Tet in various concentrations (10, 20, 30, 40, 50 and 60 mg/L) had the following actions on the growth of 3T6 fibroblasts: remarkably delaying the progress of G1 to S stage, suppressing the increase of DNA contents in S stage while increasing the protein contents in G1 and G2, leading to an unbalance in cell growth till their

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Received 1998-06-25

death. Moreover, Tet showed a dose-effect relationship. The addition of  $\text{Ca}^{2+}$  and ATP proved that the effect of Tet on 3T6 growth was not related to a block of  $\text{Ca}^{2+}$  inflow<sup>[7,8]</sup>. Other studies reported that Tet in 1.0 mg/L could deter the cells from passage from G1 to S, Tet in 1.5 and 2.0 mg/L could remarkably increase G2+M cells, lower their RNA content, increase protein content, but cause no change in DNA content. These indicate that Tet may cause an unbalance of 6T3 growth and have a dose-dependent inhibition effect on their proliferation. The  $^3\text{H}$  proline incorporation test indicated that Tet at 10 mg/L - 50 mg/L could remarkably inhibit collagen synthesis by 6T3 cells and the effect had a positive correlation with Tet concentration. Under reverse microscopy, the cells were found growing without well obvious morphological changes, indicating that the effects of Tet was not a direct cell-killing, but exerting an action at a molecular level. Tet may also suppress the proliferation of human embryonic lung fibroblasts in a concentration-dependent state, and the degree of suppression increases with a prolonged duration<sup>[9]</sup>.

#### ***Influence of Tet on hepatocytic proliferation***

Hepatocytes (or hepatic parenchymal cells) constitute over 90% of the liver. They can synthesize types I and II collagen, chondroitin sulfate, HA and some other ECMs. In the physiological state, its collagen synthesis is low, but under certain pathological conditions collagen synthesis is markedly promoted. The addition of  $\text{CCl}_4$  to the *in vitro* hepatocyte culture caused cell damage and an elevation of intracellular  $\text{Ca}^{2+}$  concentration. As a result, Tet could increase the viability of the cell, stabilize intracellular  $\text{Ca}^{2+}$  concentration, reduce the release of lactic dehydrogenase and preserve the state of flow in cell membrane, thus protecting the cell against noxious agents<sup>[11]</sup>. Flow cytometric study of the action of Tet on the growth and proliferation of rat RBL liver cell demonstrated that with increasing concentrations of Tet from 10 mg/L - 60 mg/L, the progress from G1 to S was hastened, and the DNA contents in cells in S stage and protein contents in G1 and G2 stages were all increased with a positive concentration-effect relationship. The addition of  $\text{Ca}^{2+}$  and ATP into the above preparations indicated the promoting effect of Tet on hepatocyte growth and proliferation was not related to an influx of  $\text{Ca}^{2+}$  into the cells<sup>[12]</sup>. Nevertheless, there were also contradictory conclusions<sup>[10,13]</sup>. In addition, with 3H-TdR and 3H-proline incorporation tests, Tet in concentrations of 10 mg/L - 15 mg/L could markedly inhibit the synthesis of DNA and collagen of both human embryonic hepatocytes and rat liver

cells, and the degree of inhibition was positively correlated with the concentration of Tet.

#### ***Effect of Tet on Ito cells***

Ito cells are located in the perisinusoidal space of the liver and have the function of storing fat and vitamin A, and generating collagen and some other components of ECM. It is considered that Ito cells belong to a group of inactive fibroblasts which under certain circumstances may transform into myofibroblasts and fibroblasts, and thus play an important role in the development of liver cirrhosis. In the  $\text{CCl}_4$  induced rat hepatic fibrosis model, Tet could remarkably inhibit the proliferation and transformation of Ito cell<sup>[2,6]</sup>. The addition of Tet to Ito cell culture markedly suppress the synthesis of DNA and collagen by the cell, and the degree of suppression is positively correlated with the concentration of Tet. Furthermore, Tet could block the action of platelet-derived growth factor (PDGF) in promoting cell proliferation and collagen synthesis of Ito cell. This suggests that in addition to a direct action of Tet on Ito cells, it may also exert an indirect anti-hepatofibrotic action through inhibiting the effect of PDGF<sup>[14]</sup>.

#### **STUDY OF ANTIHEPATOFIBROTIC EFFECTS OF TET AT SUBCELLULAR LEVEL**

Liver (parenchymal) cells contain mitochondria (Mc), microsomes (Ms), lysosomes (Ls) and other organelle in abundance. The functions of Mc and Ms are not limited to energy metabolism and protein synthesis, but also participate in modulating the intracellular free  $\text{Ca}^{2+}$  ion concentration. Impairment of the latter function may be one of the important factors leading to liver cell damage, eventually causes liver fibrosis. The monoamine oxidase (MAO) in the mitochondrion is an enzyme participating in collagen synthesis; and an elevation of serum MAO activity may be a sensitive parameter of liver fibrosis. N-acetyl- $\beta$ -D-glucosaminidase (NAG) is another Mc enzyme involved in decomposing matrix proteoglycans and this is also related to liver fibrosis. Elevated serum NAG indicates an activity of both synthesis and decomposition of matrix. Our experiment in  $\text{CCl}_4$  treated rats showed that small dose of Tet could lower the activities of mitochondria MAO and NAG, and increase the active uptake but reduce the passive release of  $\text{Ca}^{2+}$  by Mc. Moreover, Tet enhanced the flow over the inner membrane as well as the flow in the middle of lipid-bilayer of Mc, and there was an increase in sulfhydryl group content<sup>[10,11]</sup>. These suggest that Tet may protect the Mc against injury and also may have an antihepatofibrotic effect.

## MOLECULAR BIOLOGICAL STUDY OF ANTIHEPATOFIBROTIC ACTION OF TETRANDRINE

In the 3T6 fibroblast culture, molecular hybridization of nucleic acid was done with the cDNA probe. The results demonstrated that Tet could remarkably suppress the expression levels of types I, III and IV procollagen mRNAs of 3T6 cells ( $P < 0.05 - 0.01$ ), suggesting the inhibiting effects of Tet on the expressions of types I, III and IV collagen genes being at the level of transcription. In liver fibrosis, Tet could also lower the expression levels of mRNAs of PDGF, PDGF receptor  $\beta$  (PDGFR  $\beta$ ) and transforming growth factor  $\beta$ 1 (TGF  $\beta$ 1), suggesting that Tet indirectly reduces collagen synthesis through suppression of gene expressions of TGF  $\beta$ 1, PDGF and other hepatofibrosis-related growth factors.

## CLINICAL OBSERVATIONS OF THE EFFECTS OF TET ON LIVER CIRRHOSIS AND CHRONIC LIVER DISEASE

In 33 patients with liver cirrhosis, who received oral Tet for 3 months, there was obvious improvement in liver function, including the IV tryptophan load test; after 18 months continuous Tet therapy, serum P III P level fell remarkably ( $P < 0.01$ )<sup>[19]</sup>. In another series of 54 cirrhosis cases, oral Tet therapy for 18 months resulted in marked decrease of serum P III P, HA and types I and III collagen in the liver tissue ( $P < 0.05 - 0.01$ ). There was statistically significant difference in comparison with the glucurone treatment group as a control ( $P < 0.01$ ). Patients in the Tet treatment group showed different degrees of improvement in liver and kidney function<sup>[20]</sup>. In 115 cases of chronic liver disease treated with oral Tet for 6 months, their serum HA and P III P levels were remarkably lower than those before treatment ( $P < 0.01$ ). Among them, liver fibrosis disappeared in 14 (15.4%) cases, much reduced in 54 (58.1%) cases and slightly reduced in 19 (20.4%) cases. In addition, there were reductions in inflammatory cell infiltration and numbers of Ito cells in the Tet treated patients, as compared with the glucurone

controls ( $P < 0.001$ ).

In conclusion, the antihepatofibrotic effect of tetrandrine has been well proven by the results of experimental and clinical studies. With the ever-increasing in-depth studies about the pharmacodynamics, pharmacokinetics, reasonable combinations of drugs, the best preparation form and dosage of the drug and courses, tetrandrine is expected to be an effective antihepatofibrotic agent.

## REFERENCES

- 1 Fan LY, Kong XT, Gao Feng. Studies on tetrandrine against experimental liver fibrosis. *Med J Chin PLA*, 1994; 19(6): 444-446
- 2 Wang YJ, Sun ZQ, Li DG, Wang YJ, Che JT, Qi F, et al. Effects of tetrandrine on collagen IV and fat-storing cells in experimental hepatic fibrosis. *J New Dig*, 1994; 2(2): 78-79
- 3 Quan QZ, Sun ZQ, Li DG, Wang YJ, Che JT, Qi F, et al. Experimental and clinical studies of  $Ca^{2+}$  antagonists in antihepatic fibrosis. *J New Dig*, 1994; 2(4): 214-217
- 4 Mu Yi, Shen YZ, Chu YF. Effects of tetrandrine on gastric mucosal and hepatic lesions in cirrhotic portal hypertensive rats. *China J Dig*, 1997; 16(4): 213-216
- 5 Liu TB, Lin HC, Huang YT. Portal hypotensive effects of tetrandrine and verapamil in portal hypertensive rats. *J Pharm Pharmacol*, 1997; 49(1): 345-347
- 6 Sun ZQ, Wang YJ, Quan QZ, Zhang ZI. Comparative study of antifibrotic effects of tetrandrine and colchicine. *Chin Pharmacol Bull*, 1996; 12(4): 345-347
- 7 Liu YL, Li DG, Lu HM, Xu QF. The effects of calcium antagonists on 3T6 fibroblasts. *Beijing Med J*, 1996; 18(1): 26-29
- 8 Liu YL, Li DG, Lu HM. Effects of tetrandrine on RBL cells and 3T6 fibroblasts. *Intergr Trad West Med Liver Dis*, 1994; 4(4): 21-23
- 9 Liu XS, Li DG, Lu HM, Yu QF. Effects of tetrandrine and verapamil on the proliferation of fibroblasts. *J New Dig*, 1997; 5(2): 82-83
- 10 Fan LY, Kong XT, Gao Feng, Hou Jian. Effects of tetrandrine on the DNA and collagen synthesis of the fibroblasts and human calf hepilocytes. *Chin J Chin Hepatol*, 1995; 11(1): 25-26
- 11 Chen XH, Hu YM, Liao YQ. Protective Effect of tetrandrine on CCL<sub>4</sub>-injured Hepatocyte. *Acta Pharmacol Sinica*, 1996; 17(14): 348-350
- 12 Liu YL, Li DG, Lu HM, Xu QF. Effects of tetrandrine on hepatocytes proliferation. *Shanghai Second Med Uni Acta*, 1995; 15(3): 212-215
- 13 Fan LY, Kong XT, Gao CF. Effects of tetrandrine on the DNA and collagen of rat hepatocytes and fat-storing cells. *Chin J Dig*, 1994; 14(5): 281-283
- 14 Tian ZB, Liu SL, Li DG, Lu HM. Blocking action of tetrandrine on the cell proliferation induced by PDGF in human lung fibroblasts and liver Ito cell of rats. *Natl Med J China*, 1997; 77(1): 50-53
- 15 Li DG, Liu YL, Lu HM, Jiang ZM, Xu QF. Effects of tetrandrine on mitochondria of hepatofibrosis rats. *Chin J Dig*, 1994; 14(6): 339-341
- 16 Liu YL, Li DG, Lu HM, Jiang ZM, Xu QF. Subcellular study of calcium antagonists in treating hepatic fibrosis. *J New Dig*, 1996; 4(1): 3-5
- 17 Liu XS. Effects of  $Ca^{2+}$  antagonists on the collagen gene expression of rats in experimental hepatofibrosis. *Doctor's Degree Thesis* (to be published)
- 18 Tian ZB. Effects of growth factor on the pathogenesis of hepatic fibrosis. *Doctor's Degree Thesis* (to be published)
- 19 Li DG, Xia WX, Lu HM, Li XH, Jiang ZM, Quan QZ, et al. Significance of serum procollagen III peptide in reflecting the therapeutic effects of calcium-channel blockers on hepatic fibrosis. *Chin J Int Med*, 1990; 29: 453-456
- 20 Quan QZ, Xu JUN, Li DG, Xu Jun, Qi Feng et al. Effects of tetrandrine on serum hyaluronate and type III procollagen peptide in patients with cirrhosis. *Shanghai Med J*, 1993; 16(10): 562-564

# Nutritional status in non-alcoholic sub-clinical porto-systemic encephalopathy

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**Subject headings** liver cirrhosis; portal-systemic encephalopathy; nutritional status; albumin; somatosensory evoked potentials

## Abstract

**AIM:** To understand the role of nutritional status in cirrhotic patients without clinical porto-systemic encephalopathy (PSE).

**METHODS:** Fifty-one non-alcoholic patients with cirrhosis without PSE were studied prospectively and compared with 20 healthy volunteers. The nutritional evaluation included serum prealbumin, albumin, transferrin, body mass index (BMI), mid-arm muscle circumference (MAMC), and grip power. The occurrence of subclinical PSE (SPSE) was defined when N20 - N65 inter-peak latencies of median nerve-stimulated somatosensory evoked potentials were > 2.5 standard deviations of control means. Blood chemistries were tested within 12h of somatosensory evoked potentials test and nutritional evaluation.

**RESULTS:** Twenty-five, 17 and 9 cirrhotic patients were graded as Child-Pugh class A, B, and C, respectively. Twenty-four (47.1%) patients developed SPSE. Cirrhotic patients with SPSE had lower serum albumin (2.8 mg/L  $\pm$  5 mg/L vs 31 mg/L  $\pm$  7 mg/L,  $P < 0.001$ ) levels than those without SPSE. Prealbumin (106 mg/L  $\pm$  57 mg/L vs 125 mg/L  $\pm$  58 mg/L), transferrin (1.64 g/L  $\pm$  0.46 g/L vs 1.78 g/L  $\pm$  0.58 g/L), BMI (23.7 kg/m<sup>2</sup>  $\pm$  2.7 kg/m<sup>2</sup> vs 25.3 kg/m<sup>2</sup>  $\pm$  3.6 kg/m<sup>2</sup>), MAMC (22.2 cm  $\pm$  2.6 cm vs 22.7 cm  $\pm$  3.5 cm), and grip power (26.3 kg  $\pm$  6.4 kg vs 26.9 kg  $\pm$  6.8 kg) were not different between cirrhotic patients with and without SPSE. N20-N65 inter-peak latencies were correlated with serum albumin levels ( $P = 0.01$ ) but not with prealbumin, transferrin, BMI, MAMC, or grip power. Serum albumin, prealbumin and transferrin levels were different among cirrhotic

patients with Child-Pugh classes A, B, and C ( $P < 0.05$ ). BMI, MAMC, and grip power were not different among Child-Pugh classes A, B and C.

**CONCLUSION:** Our data suggest that serum albumin level is a simple test in the evaluation of nutritional status in patients with cirrhosis.

## INTRODUCTION

Protein-calorie malnutrition is common in patients with cirrhosis<sup>[1-4]</sup>. Many factors, including reduced nutrient ingestion, malabsorption, increased demand of nutrients, and alteration of energy metabolism have been suggested as a cause of malnutrition in cirrhotic patients<sup>[2]</sup>. In patients with cirrhosis, malnutrition may increase the number of complications and deteriorate liver functions<sup>[1]</sup>. Malabsorption can result in muscle wasting, amino acid imbalance, hypoalbuminemia, and zinc deficiency, which are thought to be involved in the pathogenesis of portosystemic encephalopathy (PSE)<sup>[5]</sup>.

In our previous studies, N20-N65 inter-peak latencies (IPLs) of somatosensory evoked potentials (SEPs) were useful in the detection and monitoring of PSE<sup>[6-8]</sup>. The SEPs in patients with chronic liver disease without clinical PSE showed minimal dysfunction in central and peripheral conduction, with delayed peak and inter-peak latencies (IPLs)<sup>[7-10]</sup>. In patients with cirrhosis, malnutrition and increased circulating toxic substances may result in diffuse neuropathy<sup>[11,12]</sup>. Our subsequent study showed that N20-N65 IPLs of SEPs were helpful in the assessment of subclinical PSE (SPSE), and about a half of cirrhotic patients had SPSE<sup>[13]</sup>. The role of nutritional status in cirrhotic patients with SPSE remains uncertain. Thus, we conducted this study.

## PATIENTS AND METHODS

We prospectively studied 51 documented patients (mean age: 58  $\pm$  11 years; 29 men, 22 women) with non-alcoholic cirrhosis (hepatitis B virus, 24; hepatitis C virus, 23; hepatitis B virus and hepatitis

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Received 1998-08-08

C virus, 2; hepatitis B virus and hepatitis D virus, 1; cryptogenic, 1) without clinical PSE at the Cathay General Hospital from 1995 through 1997. Twenty healthy volunteers served as control subjects. Cirrhosis was diagnosed when a patient developed chronic hepatitis, sonographic findings of small liver as well as splenomegaly, and the presentation of moderate or severe degrees of esophageal varices. Chronic hepatitis was defined when serum alanine aminotransferase (ALT) levels were 1.5 times higher than the upper normal limit (normal ALT activity <35 IU/L) over a period of more than 6 months.

Patients with neurological diseases and metabolic disorders such as alcoholism, diabetes mellitus and end-stage renal disease were excluded to avoid coexistent neuropathy or other brain dysfunction. Patients with fever, sepsis or shock were also excluded to avoid variations caused by body temperature. All cirrhotic patients had blood tests and were tested for grading of PSE and SEPs.

The degree of PSE of each patient was semiquantified into grades from 1 to 4, based on the mental changes proposed by Conn<sup>[14]</sup> immediately prior to the SEP test. The SEP recordings were carried out in a quiet and dimly lit room with the subjects in a supine position using a Dantec Counterpoint (Dantec Elektronik Medicinsk, Skovlunde, Denmark) by a method as described in previous studies<sup>[6,13]</sup>. Median nerve evoked cortical responses were recorded for N20-N65 inter-peak latencies (IPLs). The occurrence of SPSE in cirrhotic patients was defined when N20-N65 IPLs of median nerve stimulated SEPs were greater than 2.5 of standard deviations of control means.

For the assessment of nutritional status, biochemical analyses and anthropometric measurements were made within 4h of SEP test. Biochemical analyses included serum prealbumin, albumin and transferrin levels. Anthropometric measurements included weight, height, mid arm circumference (MAC), triceps skin fold (TSF) and mean grip power taken by the same dietitian<sup>[15]</sup>. The body mass index (BMI, kg/m<sup>2</sup>) was calculated using the equation:  $BMI = \text{weight}/\text{height}^2$ <sup>[15]</sup>. The mid-arm muscle circumference (MAMC, cm), an index of the body's total skeletal muscle mass, was calculated using the equation:  $MAMC = MAC - (0.314 \pm TSF)$ <sup>[15]</sup>.

Peripheral blood was collected after overnight fasting and within 4 h of SEP test at our central laboratory using conventional methods. Serum was also measured for prealbumin and transferrin levels using a Beckman Array<sup>TM</sup> Protein System (Vigil PR<sub>x</sub><sup>TM</sup>, Beckman Instruments Inc., Fullerton, California). Venous ammonia levels were tested shortly after the SEP test. Thirty-five patients had

15 min indocyanine green clearance tests within one week of SEP testing. Clinical grading of the severity of liver disease was based on Child Pugh score<sup>[16]</sup>.

Peripheral blood was assayed for leukocyte subsets. One hundred  $\mu\text{L}$  of blood was mixed and incubated with two or three of the 20  $\mu\text{L}$  fluorochrome labeled (FITC, PE or PerCP) monoclonal antibodies against T cells (CD3+, CD4+, CD8+, IL-2R+, HLA-DR+), B cells (CD19+), or monocytic phagocytes (CD14+, Becton Dickinson, Immuno-cytometry Systems, San Jose, California) at room temperature for 15 min. The red blood cells were lysed with 2 mL 1X FACS lysing solution for 5 min. Centrifugation, washing and dilution of the pellet were performed with PBS. Ten thousand leukocytes were measured using a FACSCAN flow cytometer for leukocytes containing fluorochrome labeled (FITC, PE, PerCP and 2,7 dichlorofluorescein) monoclonal antibodies within 6h of collection.

The study protocol was reviewed and approved under the guidelines of the 1975 Declaration of Helsinki by the Institutional Review Board of our hospital. Statistical analyses were made using the appropriate commercially available software (Microsoft Excel 97SR-1 and SPSS for Windows Release 7.0).

## RESULTS

Clinical and biochemical data are shown in Table 1. All the following data are shown as mean  $\pm$  standard deviation. The patients with cirrhotics with and without SPSE and the controls were sex and age matched. Cirrhotic patients had increased serum ALT levels (non-SPSE: 60 IU/L  $\pm$  76 IU/L,  $P = 0.004$ ; SPSE: 89 IU/L  $\pm$  100 IU/L,  $P = 0.001$ ) and total bilirubin levels (non-SPSE: 44.6  $\mu\text{mol/L}$ ,  $\pm$  18.81  $\mu\text{mol/L}$ ,  $P = 0.007$ ; SPSE: 64.98  $\mu\text{mol/L}$   $\pm$  106.02  $\mu\text{mol/L}$ ,  $P < 0.001$ ). Cirrhotic patients also had prolonged prothrombin time (non-SPSE: 1.9s  $\pm$  1.5s,  $P = 0.001$ ; SPSE: 2.8s  $\pm$  1.9s,  $P < 0.001$ ) and decreased serum albumin levels (non-SPSE: 31 g/L  $\pm$  7 mg/L,  $P = 0.02$ ; SPSE: 28 g/L  $\pm$  5g/L,  $P < 0.001$ ). The mean venous ammonia level (non-SPSE: 29.27  $\mu\text{mol/L}$   $\pm$  15.71  $\mu\text{mol/L}$ ; SPSE: 32.84  $\mu\text{mol/L}$   $\pm$  14.28  $\mu\text{mol/L}$  was within normal limits. None of the cirrhotic patients or control subjects had clinical PSE or abnormal venous ammonia levels. The mean percentage of the indocyanine green clearance test results was increased (non-SPSE: 27%  $\pm$  19%,  $P < 0.001$ ; SPSE: 35%  $\pm$  17%,  $P < 0.001$ ). Sixteen patients had indocyanine green clearance test by  $> 30\%$ . Cirrhotic patients with SPSE had higher total serum bilirubin ( $P = 0.03$ ), higher serum albumin ( $P < 0.001$ ), and more prolonged prothrombin time ( $P = 0.03$ ) than cirrhotic patients without SPSE.

**Table 1 Vital and laboratory data of cirrhotic patients with and without SPSE**

	Controls ( ) (n = 20)	SPSE	
		(-) (n = 27)	(+) (n = 24)
Age (yrs)	53±13	57±9	59±12
Sex (M/F)	14/ 6	16/11	18/ 6
Total bilirubin (mg/ L)	8.5±5.1	22.2±18.8 <sup>a</sup>	64.9±106.0 <sup>bc</sup>
Albumin (g/ L)	39±7	31±7 <sup>a</sup>	28±5 <sup>bc</sup>
ALT (IU/L)	19±9	60±76 <sup>a</sup>	89±100 <sup>a</sup>
Prolonged prothrombin time (s)	0.9±0.5	1.9±1.5 <sup>a</sup>	2.8±1.9 <sup>bc</sup>
Indocyanine green clearance (%)	4±3	27±19 <sup>b</sup>	35±17 <sup>bc</sup>
Ammonia (μmol/ L)	15.7±7.14	29.3±15.7	32.8±14.3
Child Pugh (A/B/C)		17/6/4	8/11/5 <sup>b</sup>
N20-N65 inter peak latencies (ms)	40.2±3.0	45.9±8.5	50.2±9.6 <sup>bd</sup>

<sup>a</sup>*P* < 0.01, compared with control subjects; <sup>b</sup>*P* < 0.001, compared with control subjects; <sup>c</sup>*P* < 0.05, compared with cirrhotic patients without SPSE, and <sup>d</sup>*P* < 0.001, compared with cirrhotic patients without SPSE.

Twenty-five, 17 and 9 cirrhotic patients were graded as Child-Pugh class A, B, and C, respectively (Table 2). Age, sex, serum ALT levels, venous blood ammonia levels, and N20-N65 IPLs were not different among patients in Child-Pugh classes A, B and C. Patients in Child Pugh classes B and C had lower serum albumin levels (*P* < 0.001), higher total serum bilirubin levels (*P* < 0.001), more prolonged prothrombin time (*P* < 0.001), and less clearance of indocyanine green (B vs A: *P* < 0.05, C vs A: *P* < 0.001) than patients in Child Pugh class A.

**Table 2 Vital and laboratory data of cirrhotic patients in Child-Pugh classes A, B and C**

	Child-Pugh		
	A (n=25)	B (n=17)	C (n=9)
Age (yrs)	59±9	61±6	50±11
Sex (M/F)	16/ 9	11/6	7/2
Total bilirubin (μmol/ L)	15.4±6.8	27.4±12.9 <sup>a</sup>	148.8±109.4 <sup>ab</sup>
Albumin (g/dL)	34±5	26±3 <sup>a</sup>	24±2 <sup>a</sup>
ALT (IU/L)	58±56	103±59	102±83
Prolonged prothrombin time (s)	1.5±0.9	2.3±1.1 <sup>a</sup>	4.6±1.5 <sup>ab</sup>
Ammonia (μmol/ L)	27.8±10.0	29.3±14.3	34.4±7.9
Indocyanine green clearance (%)	20±17	35±12 <sup>c</sup>	45±9 <sup>a</sup>
N20-N65 inter peak latencies (ms)	45.9±8.5	50.2±7.4	48.0±3.7

<sup>a</sup>*P* < 0.001, compared with Child-Pugh class A; <sup>b</sup>*P* < 0.001, compared with Child-Pugh class B; <sup>c</sup>*P* < 0.05, compared with Child-Pugh class A.

The mean N20 - N65 IPLs of the control subjects was 40.2±3.0 millisecond (ms); which was compatible with that of general Taiwanese population<sup>[17]</sup>. All control subjects had normal N20 - N65 IPLs. Twenty four (47.1%) cirrhotic patients had abnormal N20 - N65 IPLs. Cirrhotic patients with SPSE (50.2 ms ± 9.6 ms, *P* < 0.001) had prolonged N20 - N65 IPLs as compared with

cirrhotic patients without SPSE (45.9ms ± 8.5ms) and controls. The N20 - N65 IPLs of cirrhotic patients without SPSE were not prolonged (*P* < 0.001), which were not different among patients in Child-Pugh classes A, B and C (*P* = NS).

Cirrhotic patients ( non-SPSE: 4507/ μL ± 1546/ μL, *P* < 0.001; SPSE: 4686/ μL ± 1380/ μL, *P* < 0.001) had a lower number of leukocytes than control subjects (7088/ μL ± 1557/ μL) (Table 3). The number of leukocytes were not different between the patients with and without SPSE (*P* = 0.06). Cirrhotic patients had lower numbers of polymorphonuclear neutrophils (non-SPSE: 2398/ μL ± 1089/ μL, *P* < 0.001; SPSE: 2714/ μL ± 1904/ μL, *P* < 0.001), and CD3+ leukocytes (non-SPSE: 586/ μL ± 325/ μL, *P* < 0.001; SPSE: 618/ μL ± 420/ μL, *P* < 0.001) than control subjects. The number of monocytes, eosinophils, basophils, IL-2R+ leukocytes, HLA-DR+ leukocytes, CD14+ leukocytes, and CD19+ leukocytes were not different between control subjects and cirrhotic patients. All the leukocyte subsets were not different between cirrhotic patients with and without SPSE and among the patients in Child-Pugh classes A, B and C (*P* = NS) (Table 4).

**Table 3 Leukocyte subsets (μL) of cirrhotic patients with and without SPSE**

	Controls	SPSE	
		(-)	(+)
Leukocytes	7088±1557	4507±1546 <sup>a</sup>	4686±1380 <sup>a</sup>
Polymorphonuclear neutrophils	4658±1668	2398±1089 <sup>a</sup>	2714±1904 <sup>a</sup>
Lymphocytes	1842±747	1250±644 <sup>a</sup>	1485±603 <sup>a</sup>
Monocytes	376±120	305±137	307±167
Eosinophils	204±231	99±70 <sup>a</sup>	116±88 <sup>a</sup>
Basophils	41±28	30±15	24±24
CD3+ leukocytes	1231±557	586±325 <sup>a</sup>	618±420 <sup>a</sup>
CD4+/CD8+	1.5±0.8	1.9±0.9 <sup>a</sup>	1.9±0.9 <sup>a</sup>
IL-2R+ leukocytes	94±61	63±58	104±106
HLA-DR+ leukocytes	30±20	76±129 <sup>a</sup>	66±59 <sup>a</sup>
CD14+ leukocytes	368±149	366±469	350±310
CD19+ leukocytes	218±134	199±114	169±113

<sup>a</sup>*P* < 0.05, compared with control subjects.

**Table 4 Leukocyte subsets (μL) of cirrhotic patients with Child-Pugh calss A, B and C**

	Child-Pugh		
	A	B	C
Leukocytes	4152±1237	3951±1097	5669±1299
Polymorphonuclear neutrophils	2306±758	2231±674	3824±1037
Lymphocytes	1418±595	1371±567	1180±422
Monocytes	280±130	245±92	442±182
Eosinophils	113±84	95±57	114±59
Basophils	31±22	29±12	38±14
CD3+ leukocytes	569±300	619±316	655±316
CD4+/CD8+	2.0±1.0	2.0±0.7	1.7±0.6
IL-2R+ leukocytes	77±83	45±29	112±68
HLA-DR+ leukocytes	59±68	124±115	48±27
CD14+ leukocytes	326±379	327±185	510±310
CD19+ leukocytes	178±107	187±91	200±107

The data of BMI, MAMC and grip power were not different between control subjects and cirrhotic patients without and with SPSE ( $P = \text{NS}$ ) (Table 5), and among patients in Child-Pugh classes A, B and C (Table 6).

**Table 5** Nutritional profiles of cirrhotic patients with and without SPSE

	Controls	SPSE	
		(-)	(+)
Albumin (m/L)	39±4	31±7 <sup>a</sup>	28±5 <sup>bc</sup>
Prealbumin (mg/L)	313±57	125±58 <sup>d</sup>	106±57 <sup>d</sup>
Transferrin (mg/dL)	215±50	178±58 <sup>a</sup>	164±46 <sup>d</sup>
BMI (kg/m <sup>2</sup> )	24.2±3.5	25.3±3.6	23.7±2.7
MAMC (cm)	22.1±3.6	22.7±3.5	22.2±2.6
Grip power (kg)	28.8±10.6	26.9±6.8	26.3±6.4

BMI: body mass index, MAMC: mid arm muscle circumference. <sup>a</sup> $P < 0.05$ , compared with control subjects; <sup>b</sup> $P < 0.005$ , compared with control subjects; <sup>c</sup> $P < 0.001$ , compared with cirrhotic patients without SPSE; <sup>d</sup> $P < 0.001$ , compared with control subjects.

**Table 6** Nutritional profiles of cirrhotic patients in Child-Pugh classes A, B and C

	Child-Pugh		
	A	B	C
Albumin (m/L)	34±5	26±3 <sup>a</sup>	24±2 <sup>a</sup>
Prealbumin (mg/L)	152±52	93±31 <sup>a</sup>	60±19 <sup>ab</sup>
Transferrin (mg/dL)	1.96±35	1.54±39 <sup>c</sup>	1.35±38 <sup>c</sup>
BMI (kg/m <sup>2</sup> )	24.3±2.6	24.9±2.5	22.9±2.1
MAMC (cm)	22.3±2.7	22.2±2.4	22.9±2.1
Grip power (kg)	30.0±8.5	27.9±6.8	22.5±6.4

BMI: body mass index, MAMC: mid arm muscle circumference. <sup>a</sup> $P < 0.001$ , compared with Child-Pugh class A; <sup>b</sup> $P < 0.001$ , compared with Child Pugh class B; <sup>c</sup> $P < 0.005$ , compared with Child-Pugh class A.

The serum prealbumin and transferrin levels were lower in cirrhotic patients without (12.5 mg/L ± 58 mg/L,  $P < 0.001$ ; 1.78 g/L ± 58 g/L,  $P < 0.001$ ) and with (106 mg/L ± 57 mg/L,  $P < 0.001$ ; 1.64 g/L ± 0.46 g/L,  $P = 0.01$ ) SPSE than control subjects (313 mg/L ± 57 mg/L and 2.15 g/L ± 0.50 g/L). Neither serum prealbumin ( $P = 0.12$ ) nor transferrin ( $P = 0.17$ ) levels were different between patients with SPSE and without SPSE. Patients in Child-Pugh class B had lower serum prealbumin and transferrin levels than patients in Child-Pugh class B ( $P < 0.001$ ).

Based on simple linear regression, N20 - N65 IPLs of the cirrhotic patients were correlated with serum albumin levels ( $r = 0.34$ ,  $n = 51$ ,  $P = 0.01$ ), but not with ammonia ( $P = 0.35$ ), total bilirubin ( $P = 0.50$ ), ALT ( $P = 0.70$ ), prealbumin ( $P = 0.07$ ), transferrin ( $P = 0.88$ ), prothrombin time ( $P = 0.26$ ), Child-Pugh score ( $P = 0.21$ ), or indocyanine green clearance ( $P = 0.80$ ) N20-N65 IPLs did not

correlated with white cell count ( $P = 0.58$ ), leukocyte subsets ( $P = 0.56$ ), BMI ( $P = 0.20$ ), MCAC ( $P = 0.07$ ), or grip power ( $P = 0.79$ ).

**DISCUSSION**

PSE is one of the major complications in patients with cirrhosis. The clinical course of PSE is often unstable, and close observation is usually required to assess it. Some cirrhotic patients may have SPSE capable of disturbing daily life but without obvious impairment of mental status<sup>[14]</sup>. Psychometric tests have been widely used for the clinical assessment of SPSE. However, poorly educated and aged subjects tend to be abnormal in psychometric tests<sup>[13,18]</sup>. Our earlier study showed that SEPs were not affected by education and age and were more sensitive than psychometric tests in the assessment of SPSE in better-educated cirrhotic patients<sup>[13]</sup>. In the present study, 24 (47.1%) of 51 cirrhotic patients developed SPSE, the data confirmed the results of our previous studies that the occurrence of SPSE was common in cirrhotic patients without clinical PSE<sup>[7,8,13]</sup>.

Our data showed that cirrhotic patients in Child-Pugh classes B and C had more jaundice, lower serum albumin levels, higher serum ALT activities, more prolonged prothrombin time, and less clearance of indocyanine green than those in class A. Cirrhotic patients with poor liver function tended to develop PSE. This may explain why the occurrence of SPSE was mainly in patients in Child-Pugh class B and non-SPSE mainly in patients in class A.

Several parameters including body weight, anthropometry, and biochemical measurement are often used to assess the degree of malabsorption in cirrhotic patients<sup>[1,2,15]</sup>. In the present study, cirrhotic patients with SPSE had higher total serum bilirubin, higher serum albumin, more prolonged prothrombin time, and less clearance of indocyanine green than those without SPSE. Serum prealbumin and transferrin levels were different among cirrhotic patients in Child-Pugh classes A, B and C. Cirrhotic patients with SPSE had lower serum prealbumin and transferrin levels than patients without SPSE. Our data was compatible with the study in alcoholic cirrhosis that serum albumin, prealbumin, and transferrin levels correlated with the degree of liver damage<sup>[19]</sup>. However, our data showed that only serum albumin levels were correlated with N20 - N65 IPLs. The low albumin levels reflect the impaired hepatic synthetic functions<sup>[1,4]</sup>. The good correlation between N20 - N65 IPLs and serum albumin labels suggested that serum albumin levels are helpful in the evaluation of nutritional status in cirrhotic patients with SPSE.

It is well known that malnutrition may impair immunocompetence and increase susceptibility to infection, and leukocyte count is one of the indicators of immune status<sup>[20,21]</sup>. Peripheral blood

of cirrhotic patients had lower leukocyte count. Our data confirmed the lower leukocyte count in cirrhotic patients, and the decrease was mainly polymorphonuclear neutrophils and leukocytes. The study of leukocyte subsets further showed that the decrease of leukocytes were mainly CD3+, CD4+, and CD8+ leukocytes. The ratio of CD4+ and CD8+ was higher in cirrhotic patients compared with those of control subjects. The change of the number of leukocyte subsets was not significant among patients in Child-Pugh classes A, B and C. The number of leukocyte subsets were not different between cirrhotic patients with and without SPSE.

In this study, BMI, MAMC and grip power were not different among patients in Child-Pugh classes A, B and C; N20-N65 IPLs did not correlate with BMI, MAMC, and grip power. Our findings were compatible with recent studies<sup>[22,23]</sup> that midarm muscle area and mid-arm fat area did not relate to mortality rate of cirrhotic patients. The role of anthropometry is limited in the prediction of SPSE in cirrhotic patients.

Recent studies suggested that nutritional therapy might be helpful in the reversal of malnutrition, decreasing morbidity and nutrition, and improvement of PSE<sup>[24,25]</sup>. Thus, the early assessment of malnutrition is important. The measurement of albumin level is simple and inexpensive, and it is available to most of the clinical laboratories. It is likely that lower serum albumin levels in cirrhotic patients may suggest the possible occurrence of SPSE, and early nutritional therapy may be important to correct the malnutrition.

**ACKNOWLEDGMENTS** The authors wish to thank Ms. Chi-Hsao Chen, Ms. Shiao-Fun Wei, Ms. Shiao-Ping Dai, and Ms. Pi-Chen Huang for their excellent technical assistance.

## REFERENCES

- Muller MJ. Malnutrition in cirrhotics. *J Hepatol*, 1995;23(suppl 1):31-35
- Crawford DHG, Cuneo RC, Shepherd RW. Pathogenesis and assessment of malnutrition in liver disease. *J Gastroenterol Hepatol*, 1993;8:89-94
- Italian Multicentre Cooperative Project on Nutrition in Liver Cirrhosis. Nutritional status in cirrhosis. *J Hepatol*, 1994;21:317-325
- McCullough AJ, Tavill AS. Disordered energy and protein metabolism in liver disease. *Semin Liver Dis*, 1991;11:265-277
- Mullen KD, Weber FL. Role of nutrition in hepatic encephalopathy. *Semin Liver Dis*, 1991;11:292-304
- Yang SS, Chu NS, Liaw YF. Somatosensory evoked potentials in hepatic encephalopathy. *Gastroenterology*, 1985;89:625-630
- Chu NS, Yang SS. Somatosensory and brainstem evoked potentials in alcoholic liver disease with and without encephalopathy. *Alcohol*, 1987;4:225-230
- Chu NS, Yang SS. Portal systemic encephalopathy: alterations in somatosensory and brainstem auditory evoked potentials. *J Neuro Sci*, 1988;84:41-50
- Yang SS, Chu NS, Wu CH. The role of somatosensory evoked potentials on hepatic encephalopathy. *Biomed Eng Appl Basis Comm*, 1997;9:154-157
- Chu NS, Yang SS, Liaw YF. Evoked potentials in liver disease. *J Gastroenterol Hepatol*, 1997;12:s288-293
- Yang SS, Hughes RD, Williams R. Digoxin like immunoreactive substances in severe acute liver disease due to viral hepatitis and paracetamol overdose. *Hepatology*, 1988;8:93-97
- Yang SS, Korula J, Sunderheimer JE, Keyser AJ. Digoxin-like immuno-reactive substances in chronic liver disease. *Hepatology*, 1989;9:363-366
- Yang SS, Wu CH, Chiang TR, Chen DS. Somatosensory evoked potentials in sub-clinical porto-systemic encephalopathy: a comparison with psychometric tests. *Hepatology*, 1998;27:357-361
- Conn HO. Trailmaking and number-connection tests in the assessment of mental state in portal systemic encephalopathy. *Am J Digest Dis*, 1977;22:541-550
- Anonymous. Nutrition assessment. In: Whitney EN, Cataldo CB, Rolfe SR, eds. Understanding normal and clinical nutrition. 3rd ed. St. Paul: West Publishing Co. 1991:602-642
- Pugh RNH, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg*, 1973;60:646-649
- Chu NS. Somatosensory evoked potentials: correlation with height. *Electroencephal Clin Neurophysiol*, 1986;65:169-176
- Zeneroli ML, Cioni G, Ventura P, Russo AM, Venturini I, Casalgrandi G et al. Interindividual variability of the number connection test. *J Hepatol*, 1992;15:263-264
- Merli M, Romiti A, Riggio O, Capocaccia L. Optimal nutritional indexes in chronic liver disease. *J Parenteral Enteral Nutr*, 1987;11:130s-134s
- Cunningham-Rundles S. Effect of nutritional status on immunological function. *Am J Clin Nutr*, 1982;35:1202-1210
- O'Keefe SJ, El-Zayadi AR, Carraher TE, Davis M, Williams R. Malnutrition and immune competence in patients with liver disease. *Lancet*, 1980;ii:615-617
- Merli M, Riggio O, Dally L, PINC. Does malnutrition affect survival in cirrhosis. *Hepatology*, 1996;23:1041-1046
- Prijatmoko DWI, Strauss BJG, Lambert JR, Sievert W, Stroud DB, Wahlqvist ML et al. Early detection of protein depletion in alcoholic cirrhosis: role of body composition analysis. *Gastroenterology*, 1993;105:1839-1845
- Munoz SJ. Nutritional therapies in liver disease. *Semin Liver Dis*, 1991;11:278-291
- Morgan TR, Moritz TE, Mendenhall CL, Haas R, VA Cooperative Study Group #275. Protein consumption and hepatic encephalopathy in alcoholic hepatitis. *J Am Coll Nutr*, 1995;14:152-158

# *Helicobacter pylori* acquisition of metronidazole resistance by natural transformation *in vitro*

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**Subject headings** *Helicobacter pylori*; metronidazole; drug resistance, microbial; transformation, bacterial

## Abstract

**AIM** To study whether *Helicobacter pylori* is naturally transformable.

**METHODS** Transformation was performed in BHI broth supplemented with horse serum and yeast extract. Genomic DNA extracted from a metronidazole resistant *H. pylori* strain was added to *H. pylori* broth culture. The mixture was incubated at microaerophilic atmosphere. The DNA-treated cells were plated on blood agar containing 8mg/L metronidazole to select for transformants. Sterile distilled water was used as a negative DNA control. The DNA profiles of transformants were compared with that of their parent strains by randomly amplified polymorphic DNA (RAPD) fingerprinting.

**RESULTS** Transformation of *H. pylori* with DNA from a metronidazole resistant strain as a marker was demonstrated. Out of the 12 strains of *H. pylori* tested, 9 (75%) strains were found to be transformable. The transformation frequencies ranged from  $3.4 \times 10^{-6}$  to  $2.4 \times 10^{-4}$ . By RAPD, DNA fingerprints of the transformants and their parent strains showed no change in DNA profiles though transformants were all resistant to metronidazole as compared with their metronidazole-sensitive parent strains.

**CONCLUSION** *Helicobacter pylori* is naturally transformable which might be one of the ways that *H. pylori* develops resistance to metronidazole.

## INTRODUCTION

Genetic transformation is a process by which a cell takes up naked DNA from the surrounding medium and incorporates it into its own genomic DNA to acquire an altered genotype. Natural transformation is widely distributed among bacteria. This process may enable bacteria to get advantageous mutations to escape and survive under unfavourable conditions. *Helicobacter pylori* is recognized as a major factor in the development of gastritis and peptic ulcer<sup>[1]</sup>. This bacterium can be eradicated from stomach by antibiotics. However, effective treatment of *H. pylori* has proved difficult with the development of resistance to some antimicrobials. An increase in prevalence of metronidazole resistant *H. pylori* has been reported<sup>[2]</sup>. In this study we test *in vitro* whether natural transformation could be one way for *H. pylori* to acquire metronidazole resistance.

## MATERIALS AND METHODS

### Strains

*H. pylori* isolates obtained from patients with gastroduodenal diseases were used in this study. The strain was isolated on chocolate blood agar No. 2 medium supplemented with 5% horse blood and incubated at 37°C under microaerophilic environment. The strain was identified by standard procedures as stated by Goodwin *et al*<sup>[3]</sup>.

### DNA extraction

Plate culture of *H. pylori* was transferred into an Eppendorf tube and 1.5 mL volume of TE buffer (100 mM Tris-HCL and 1mM EDTA) was added. The suspension was centrifuged at  $8000 \times g$  and washed once with TE buffer. The pellet was suspended in 800 µl TE buffer. The bacterial suspension was incubated in 100 µl of 10 g/L lysozyme (Sigma) at 37°C for 30 minutes, and then lysed with 100 µl of 10% sodium deodecyl sulfate for another 30 minutes at 37°C. Following the addition of 5 µl of 10 g/L proteinase K (Boehringer), the mixture was incubated for 1 hour at 56°C. DNA was purified by extracting twice with equal volume of phenol and once with equal volume of chloroform, DNA was then precipitated overnight with two volumes of absolute ethanol and 20 µl 3M sodium acetate at -20°C. The DNA precipitate was washed once with 70% ethanol. The pellet was vacuum-dried using speed-vac (Savant) and resuspended in

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Received 1998-08-08

200 $\mu$ l sterile distilled water. This served as target DNA for PCR-based RAPD. DNA concentration was measured at  $\lambda$  260nm.

### PCR-based RAPD fingerprinting

Universal primer for PCR-based RAPD was randomly chosen according to Akopyanz *et al* (1992)<sup>[4]</sup> to allow for the fingerprinting of the whole DNA content of cells. The primer used in this study was 5-AAGAGCCCGT-3. PCR reaction was carried out in 25 $\mu$ l volume. Fifty ng of *H. pylori* genomic DNA, 2 mM MgCl<sub>2</sub>, 20 pmol primer, 1 unit of Taq DNA polymerase and 250 mM each of dGTP, dCTP, dATP and dTTP were placed in standard PCR incubation buffer containing 10 mM Tris-HCl, 50 mM KCl, 2 mM MgCl<sub>2</sub> and 0.01% gelatin (Promega, USA). The reaction mixture was overlaid with a drop of mineral oil to prevent evaporation. PCR was performed with a thermal cycler (Amplifon, USA) consisting of an initial step of denaturation of target DNA at 94°C for 5 minutes. This was followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 minutes. The microliters of the PCR products were electrophoresed in 1% horizontal agarose gels for 2 hours at 80 V in TBE buffer. The gels were stained with ethidium bromide (1 mg/L) and photographed with filtered UV illumination on Polaroid type 667 film.

### Transformation experiment

*H. pylori* transformation was performed in BHI broth supplemented with horse serum and yeast extract. Briefly, DNA was extracted from a metronidazole-resistant *H. pylori* strain, H38. Twelve metronidazole-sensitive strains of *H. pylori* grown respectively in Brain-heart infusion broth supplemented with 10% horse serum and 0.4% yeast extract were incubated under microaerobic conditions at 37°C for 24 hours. Aliquots of 50  $\mu$ g DNA of H 38 were added into 1mL test *H. pylori* broth culture. The mixture was incubated at 37°C for 6 hours. The DNA-treated cells were plated on blood agar containing 8 mg/L metronidazole to select transformants. The transformation frequencies were calculated by dividing the number of transformants from the total number of viable cells on chocolate blood agar. Sterile distilled water was used as a negative DNA control. The DNA profiles of transformants were compared with that of their parent strains by RAPD fingerprinting.

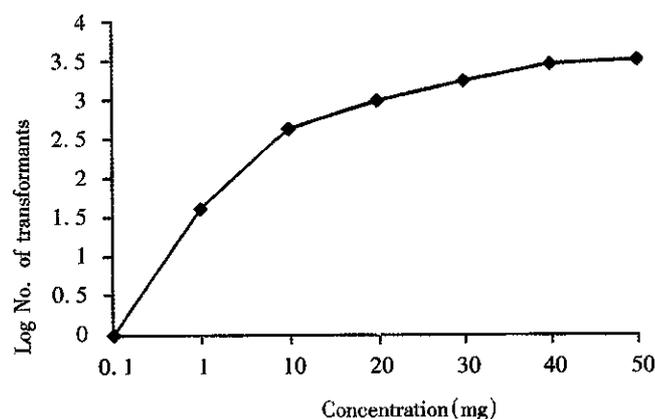
## RESULTS

Transformation of *H. pylori* using metronidazole resistance as a marker was demonstrated. *H. pylori* H38 DNA (metronidazole resistance) was used as a

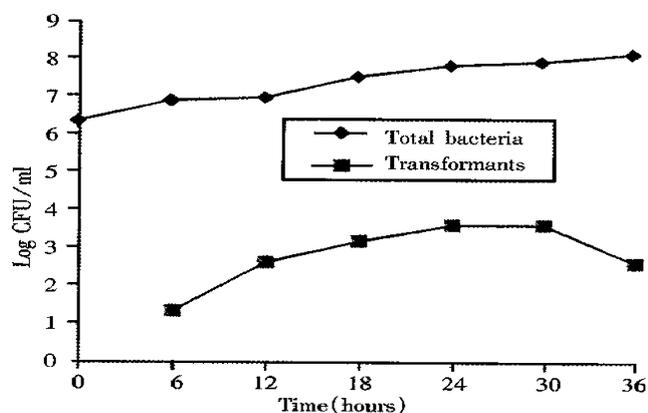
donor to test for natural transformation competence in broth cultures. To optimize conditions for transformation of *H. pylori*, DNA of H38 was added to NCTC 11637 broth at 6-36 hours after initial inoculation. Transformants were obtained at frequencies ranging from  $2.8 \times 10^{-6}$  to  $5.9 \times 10^{-5}$  (Figure 1 and Table 1). The highest number of transformants and frequency of transformation were found when DNA was added at 24 hours. It was interesting to note that the transformation frequency of NCTC 11637 increased with increasing donor DNA concentration (Figure 2).

**Table 1** Effect of time on transformants of *H. pylori* NCTC 11637

	6 hours	12 hours	18 hours	24 hours	30 hours	36 hours
Total bacteria	$7.1 \times 10^6$	$8.1 \times 10^6$	$2.8 \times 10^7$	$5.4 \times 10^7$	$6.3 \times 10^7$	$9.8 \times 10^7$
Transformants	20	370	1300	3200	3100	290
Frequencies	$2.8 \times 10^6$	$4.6 \times 10^5$	$4.6 \times 10^5$	$5.9 \times 10^5$	$4.9 \times 10^5$	$3.0 \times 10^6$



**Figure 1** Effect of growth phase on the competence of natural transformation in *H. pylori*. H38 DNA (50mg/L) was added to NCTC 11637 at the indicated time intervals. Total cell number and transformants were enumerated.



**Figure 2** Dependence of transformation frequency on the concentration of donor H38 DNA. H38 DNA with different concentration was added to 108 NCTC 11637. Number of transformants was enumerated.

Using DNA of metronidazole resistant strain, H38, as a marker for transformation, 9 (75%) of 12 *H. pylori* strains tested were found to be transformable. The transformants were all resistant to metronidazole as compared with their metronidazole-sensitive parent cells. The transformation frequencies ranged from  $3.4 \times 10^{-6}$  to  $2.4 \times 10^{-4}$  (Table 2). By RAPD, the DNA fingerprints of the transformants and their parent strains showed no change in DNA profiles (Figure 3).

**Table 2** Transformation of different strains of *H. pylori*

Recipient	Donor	Total bacteria	Transformants	Frequencies
H 1	H 38	$1.8 \times 10^8$	43000	$2.4 \times 10^4$
H 9		$5.2 \times 10^7$	8900	$1.7 \times 10^4$
H 11		$9.3 \times 10^8$	No	
H 13		$8.4 \times 10^7$	4100	$4.9 \times 10^5$
H 29		$5.6 \times 10^6$	No	
H 41		$8.3 \times 10^5$	40	$4.8 \times 10^5$
H 43		$5.1 \times 10^6$	30	$5.9 \times 10^6$
H 46		$5.8 \times 10^6$	20	$3.4 \times 10^6$
H 50		$3.2 \times 10^8$	4500	$1.4 \times 10^5$
H 53		$3.5 \times 10^7$	320	$9.1 \times 10^6$
H 62		$3.9 \times 10^5$	No	
H 68		$8.8 \times 10^7$	740	$8.4 \times 10^6$



**Figure 3** Comparison of DNA profiles of transformants and parent strains by RAPD. PCR products were run on 1% agarose gel. Strains H13, H41 and H43 were metronidazole sensitive. Lanes 1 & 8.  $\lambda$  DNA digested with Hind III. Lanes 2 & 3. H13 and its transformant. Lanes 4 & 5. H41 & its transformant. Lanes 6 & 7. H43 and its transformant.

## DISCUSSION

Natural transformation competence was found among prokaryotes, such as *Streptococcus pneumoniae*<sup>[5]</sup>, *Haemophilus influenzae*<sup>[6]</sup> and *Bacillus subtilis*<sup>[7]</sup>. Stewart<sup>[8]</sup> reported that competence was internally regulated and was in a

stable state once developed. Natural transformation in *H. pylori* has been demonstrated by Nedenskow-S-rensén *et al*<sup>[9]</sup> and Wang *et al*<sup>[10]</sup>. This study has confirmed that *H. pylori* can acquire resistance to metronidazole by natural transformation. Nine (75%) of 12 strains were found to be transformable in this study. The transformation frequencies ranged from  $3.4 \times 10^{-6}$  to  $2.4 \times 10^{-4}$ . Furthermore, we examined the DNA fingerprints of recipient cell and its progeny by RAPD. DNA fingerprinting showed that no significant DNA profile change occurred. This is not unexpected. The size of metronidazole resistance gene may be insignificant compared with the entire chromosomal length. RAPD only can detect a small fraction of target DNA. It is possible that the universal primer used for RAPD in study could not recognise this slight difference especially if a point mutation is involved.

Ling *et al*<sup>[11]</sup> found that *H. pylori* strains resistance to metronidazole increased from 29% in 1991 to 73% in 1995 in Hong Kong. We believe that the densely populated environment in Hong Kong and the increased use of metronidazole and other imidazoles in the population had contributed to this phenomenon. This study shows a 75% transformable frequency *in vitro*. The results indicate that natural transformation of metronidazole resistance may play an important role in the development of antibiotic resistance. Natural transformation might promote *H. pylori* in acquiring advantageous genes from other strains in order to adapt and survive in some particular environments.

In this study natural transformation of *H. pylori* was demonstrated *in vitro*. It might be one of the means by which *H. pylori* develops resistance to metronidazole.

## REFERENCES

- Dick JD. *Helicobacter (Campylobacter) pylori*: a new twist to an old disease. *Ann Rev Microbiol*, 1990;44:249-269
- Glupczynski Y, Burette A, Dekoster E, Nyst JE, Deltenre M, Cadranet S *et al*. Metronidazole resistance in *Helicobacter pylori*. *Lancet*, 1990;1(8695): 976-977
- Goodwin CS, Armstrong JA. Microbiological aspects of *Helicobacter pylori (Campylobacter pylori)*. *Eur J Clin Microbiol Infect Dis*, 1990;9(1):1-13
- Akopyanz N, Bukanov No, Westblom TU, Kresovich S, Berg DE. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res*, 1992;20(19):5137-5142
- Morrison DA, Mannarelli B, Vijayakumar MN. Competence for transformation in *Streptococcus pneumoniae*: an inducible high-capacity system for genetic exchange. In: Schlessinger D, ed. *Microbiology*. Am Soc Microbiol: Washington DC, 1982:136-138
- Albritton WL, Setlow JK, Thomas M, Sottnek F, Steigerwalt G. Heterospecific transformation in the genus *Haemophilus*. *Mol Gen Genet*, 1984;193(2): 141-146
- Harford N, Mergeay M. Interspecific transformation of rifampicin resistance in the genus *Bacillus*. *Mol Gen Genet*, 1973;120(2):151-155
- Stewart GJ. The biology of natural transformation. *Annual Reviews Microbiol*, 1986;40:211-235
- Nedenskow-S-rensén P, Bukholm G, B-vre K. Natural competence for genetic transformation in *Campylobacter pylori*. *J Infect Dis*, 1990;161(2):365-366
- Wang Y, Roos KP, Taylor DE. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. *J Gen Microbiol*, 1993; 139 (10): 2485-2493
- Ling KW, Cheng AFB, Sung JY, Yiu PYL, Chung SSC. An increase in *Helicobacter pylori* strains resistant to metronidazole: a five-year study. *Helicobacter*, 1996;1(1):57-61

# Gene therapy for human colorectal carcinoma using human CEA promoter controlled bacterial ADP-ribosylating toxin genes: PEA and DTA gene transfer \*

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**Subject headings** colorectal neoplasms; gene therapy; gene transfer; carcinoembryonic antigen; pseudomonas exotoxin A; diphtheria toxin A

**therapeutic genes, are suitable for the tissue-specific gene therapy for colorectal carcinoma.**

## Abstract

**AIM** To establish a tissue-specific gene therapy for colorectal carcinoma using bacterial ADP-ribosylating toxin genes.

**METHODS** Pseudomonas exotoxin A domain II + III (PEA) was cloned from genomic DNA of *Pseudomonas aeruginosa*. PEA and diphtheria toxin A chain gene (DTA) were modified to express eukaryotically. After sequencing, the toxin genes under the control of human carcinoembryonic antigen (CEA) promoter were cloned into retroviral vectors to construct CEAPEA and CEADTA respectively. *In vitro* cotransfection of the constructs with luciferase vectors and *in vivo* gene transfer in nude mice were subsequently carried out.

**RESULTS** Both CEAPEA and CEADTA specifically inhibited the reporter gene expression in the CEA positive human colorectal carcinoma (CRC) cells *in vitro*. Direct injection of CEAPEA and CEADTA constructs into the established human tumors in BALB/c nude mice led to significant and selective reductions in CRC tumor size as compared with that in control groups.

**CONCLUSION** The toxin genes, working as

## INTRODUCTION

A major requirement for *in vivo* gene therapy for cancer is to target the therapeutic gene expression to malignant tissues by selecting potent therapeutic genes. *Pseudomonas* exotoxin A and diphtheria toxin A chain, which kill tumor cells by irreversible ADP-ribosylation of elongation-2, may become potent therapeutic genes. PE is made of three structural domains: the N-terminal domain (I) is responsible for the binding of toxin to its receptor on the cells; the middle domain (II) plays a role in the translocation of toxin across the membrane, and the C-terminal domain (III) has the ADP-ribosylation activity<sup>[1]</sup>. DT is secreted from *Corynebacterium diphtheriae* as a single polypeptide chain containing two major domains: A (amino-terminal, 193 residues), which carries the active site for ADP-ribosylation of elongation factor-2, and B (carboxyl-terminal, 342 residues), which promotes binding of toxin to cells and the entry of A into the cytosolic compartment. Immunotoxins have been engineered by fusing domains II and III of PE and DTA chain to specific antibodies. Immunotoxin therapy is challenged by limited accessibility of antibodies or antibody conjugates to solid tumors<sup>[2]</sup>. Expression of the toxin genes in mammalian cells may result in the inhibition of further protein biosynthesis, and consequent cell death.

*In vivo* gene therapy relies on unique tissue-specific or tumor-specific transcriptional elements to drive the expression of the toxic protein only in those cells containing transcription factors capable of activating the promoter elements. CEA is a tumor-associated cell surface antigen overexpressed in tumor cells such as colorectal carcinoma cells. CEA promoters were cloned both from normal and CEA-positive human colonic carcinoma genomic DNA<sup>[3]</sup>. Analysis of the CEA promoters showed more than 8-fold increase in expression in CEA-positive cells, as compared with CEA-negative cells<sup>[4]</sup>. We are working on an alternative means of exploiting a natural toxin which does not depend on cell surface molecules. Instead, DNA coding for the

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Presented at the Annual Meeting of American Association for Cancer Research, Philadelphia, 24-28 March, 1998.

\*Supported by the National Natural Science Foundation of China, No.39770834.

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Received 1998-07-06

toxin genes driven by CEA promoter is introduced into tumor cells. The study may provide some opportunities for tissue-specific gene therapy for CEA-positive tumors.

## MATERIALS AND METHODS

### Cells and animals

CEA-positive LoVo and SW1463 human colorectal carcinoma cell line, HeLa cell line and murine embryo fibroblast NIH3T3 were originally obtained from ATCC. CEA-negative human renal cell carcinoma cell line RC9406 was established by our laboratory. BALB/c nude mice were from the Shanghai Tumor Research Institute (Shanghai, China). All cells line and animals were maintained under standard conditions.

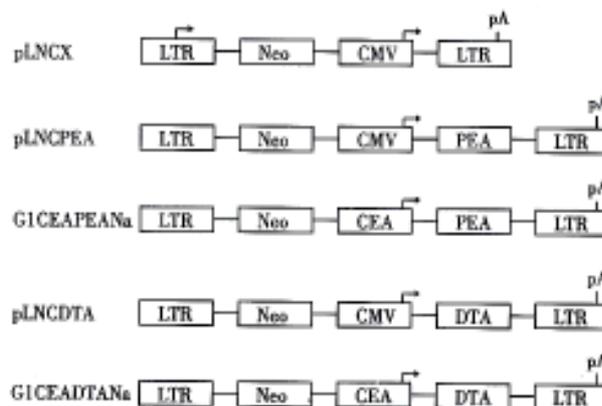
### Cloning of PEA and DTA genes

*Pseudomonas aeruginosa* strains were kept in our department. The sequence of oligonucleotide primers for cloning PEA domain II + III gene and DTA gene were obtained from published data<sup>[1,5]</sup>, introducing Hind III site and kozak sequence at the 5' terminus and strong termination signal and Cla I site at the 3' terminus, and commercially synthesized (Promega). Inner primers for cloning PEA gene had the sequence of GG AAG CTT GCC GCC ACC ATG GAG ACT TTC ACC CGT CAT CGC CA and GG ATC GAT TTA CTT CAG GTC CTC GCG CGG C. Outer primers for cloning PEA gene had the 5' sequence of ACG GTC ATC AGT CAT CGC CTG CA and shared the same 3' sequence with the inner primer. Oligonucleotide primers for cloning DTA had the sequences of GG AAG CTT GCC GCC ACC ATG GGC GCT GAT GAT GTT GTT GA and GG ATC GAT TTA TCC GAC ACG ATT TCC TGC ACA G. Preparation of the bacterial genomic DNA and Nest-PCR employed to clone the PEA gene and plasmid pET15b-DT (kindly provided by Dr. RJ Collier<sup>[5]</sup>), were performed as previously described<sup>[3]</sup>, with some modification. Genomic DNA (0.1 µg) and Elongase (0.5U) (GIBCO) were used. The PCR products were digested with Cla I and Hind III, purified by the Wizard PCR product purification system (Promega), and then subcloned into pSP72 vectors to create pSP72-PEA and pSP72-DTA. Sequences of the cloned PEA and DTA fragments were analyzed using the double-stranded DNA sequencing system (Promega). The pSP72-PEA, pSP72-DTA, Sp6 and T7 primers (Promega) served as templates and sequencing primers respectively. Automatic DNA sequencing was commercially performed.

### Construction of recombinant retroviral vectors

Sph I site of plasmid pGEM-CEA<sup>[3]</sup>, which contained 400bp human CEA promoter cloned from genomic DNA of the CEA positive colorectal carcinoma tissues, was changed to Hind III. The Sal I/Hind III digested CEA promoter was released

from pGEM-CEA, and subcloned into polylinker site of G1Na retroviral vector to create G1CEANa. The Hind III/Cla I digested PEA and DTA fragments were subcloned to G1CEANa and pLNCX<sup>[6]</sup> to create resulting constructs G1CEAPEANa, G1CEADTANa, pLNCDTA and pLNCPEA respectively. The structure of the constructs is shown in Figure 1.



**Figure 1** Structure of the retroviral constructs

LTR: long terminal repeat region; Neo: neomycin resistance gene; CMV: human cytomegalovirus immediate early promoter; CEA: human carcinoembryonic antigen promoter; PEA: *Pseudomonas* exotoxin domain II + III fragment; DTA: diphtheria toxin A.

### Cotransfection and luciferase assays

Each transfection contained 1pmol pGL2 control (Promega) and 1pmol retroviral constructs. For transient luciferase assays, cells were transfected with DOTAP (Boehringer Mannheim). Transfection and luciferase assay were made as previously described<sup>[7]</sup>. All values were normalized to the expression of luciferase transfected with pGL2-control alone. All plasmids (pLNCX, G1CEAPEANa, G1CEADTANa, pLNCPEA and pLNCDTA) were tested in SW1463, LoVo, NIH3T3, RC9406 and HeLa cell lines.

### Tumor xenograft in BALB/c nude mice and in vivo gene transfer

Two million LoVo and RC9406 cells were s. c. injected into the back of the BALB/c nude mice. When established, the tumors were resected and sheared to 0 mm - 5 mm<sup>3</sup>, and s. c. reimplanted into the BALB/c nude mice of next generation under sterile conditions. After 30 generations of reimplantation, the tumors grew steadily in the nude mice.

After purification with column chromatography, the constructs (20 mg/L) were mixed with the same volume of lactated Ringer's solution. An aliquot of the mixture was added to the same volume of a cationic liposome (composed of TMAG, DLPC and DOPE, with a molar ratio of 1: 2: 3) in lactated Ringer's solution in a separate

sterile vial at room temperature. After incubation for 15min, 0.1 mL DNA liposome solution was injected into the tumors, or in the presence of 107 units per ml adenovirus (Ad2), under sterile conditions. The injection was administered 6 times at an interval of 2 days. The tumor weight was estimated as previously described<sup>[8]</sup>. The mice were killed at 42 h following the last injection of the constructs, and then the treated tumors were resected. Total RNA was extracted with TriZol (GIBCO), and subsequently digested with RNase-free DNase I (GIBCO). RT-PCR for detection of the transcription of PEA and DTA gene were performed with the inner pairs of the related primers to confirm the toxin gene expression.

### Statistics

The Student's *t* test and  $\chi^2$  test were used. A *P* value of <0.05 was considered significant.

## RESULTS

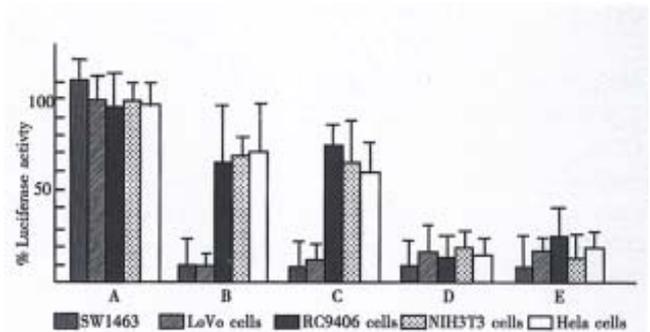
### PEA and DTA gene cloning

The modified PEA and DTA gene were obtained. DNA sequencing demonstrated that the introduced Kozak sequence, termination code and restriction sites did not destroy the open reading frame of PEA and DTA coding regions. The PEA gene had no mutation in the aminoacid sites 485-492, 553 and 612, which were considered to be key positions for expressing ADP-ribosylation activity. Base-replacement mutation was found in the aminoacid positions 412 and 420. Roles of these mutations remained to be investigated. No mutation was found in the DTA gene.

### In vitro specificity of the PEA and DTA gene expression

*In vitro* experiments showed that the CEA promoter conferred a selective expression of PEA and DTA gene in CEA positive human colorectal carcinoma cell lines LoVo and SW1463. The levels of luciferase expression in LoVo and SW1463 cells were inhibited by 90% - 97% and 80% - 85% at 36 h after transfection with an equal mass of G1CEAPEANa and G1CEADTANa respectively. Concentration dependent inhibition was also observed, the inhibition being substantial with as little as 0.1  $\mu$ g. In contrast, cotransfection with a plasmid lacking the PEA and DTA genes did not inhibit luciferase expression (Figure 2). The basal transcription level of the PEA and DTA from the CEA promoter was inferred from a partial translation inhibition effect of the cotransfected luciferase gene in the CEA negative RC9406 and HeLa cells. The results indicate that the translation inhibition of luciferase was due to active toxic protein production and level of the inhibition was closely associated with PEA and DTA gene

expression.



**Figure 2** Inhibition of luciferase gene expression by the toxin gene cotransfection

A: Transfection with pGL2-Control only; B: Cotransfection with pGL2-Control and G1CEAPEANa; C: Cotransfection with pGL2-control and G1CEADTANa; D: Cotransfection with pGL2-control and pLNCPEA; E: Cotransfection with pGL2-control and pLNCDTA.

### Selective arrest of tumor growth by the *in vivo* toxin gene transfer

The growth rate of LoVo tumor in the groups treatment with G1CEAPEANa and G1CEADTANa appeared to be reduced as compared with the liposome controls at day 7 of the treatment. Growth of RC9406 tumors did not significantly respond to G1CEAPEANa or G1CEADTANa administration ( $P > 0.05$ , compared to the liposome control). The selective anti-colorectal carcinoma effect of G1CEAPEANa was stronger than G1CEADTANa ( $P < 0.05$ ,  $n = 7$ ). pLNCPEA and pLNCDTA *i. t.* injections with the cationic liposome were relatively capable of reducing the growth rate of both LoVo tumors and RC9406 tumors ( $P < 0.05$ , as against liposome control). Intratumoral administration of G1CEAPEANa in the presence of the adenovirus (Ad2 type) and cationic liposome resulted in marked regression of the established colorectal carcinoma, as compared with G1CEAPEANa and the liposome groups. The ADP-ribosylating bacterial toxin genes, under the control of the CEA promoter, showed strong and selective antitumor activities for CEA positive CRC tumor (Table 1). RT-PCR employed for the detection of the toxin gene expression in the treated tumor bodies showed transcription of the toxin genes in G1CEAPEANa treated or G1CEADTANa treated LoVo tumors and pLNCPEA treated or pLNCDTA treated LoVo and RC9406 tumor, but not in G1CEAPEANa or G1CEADTANa treated RC9406 tumor bodies. The results demonstrated that expression of the toxin genes was responsible for the growth inhibition of the tumor bodies.

**Table 1** *In vivo* growth inhibition of human carcinoma by the toxin genes transfer

Days following gene therapy	Estimated tumor weight (mg) of tumor xenograft following toxin genes transfer ( $\bar{x}\pm s$ , $n = 7$ )										
	LoVo (CEA/PEA)	RC9406 (CEA/PEA)	LoVo (CEA/DTA)	RC9406 (CEA/DTA)	LoVo (CMV/PEA)	RC9406 (CMV/DTA)	LoVo	RC9406	LoVo	RC9406	LoVo (CEA/PEA)+Ad
0	486±160	472±201	504±232	516±164	480±200	496±320	480±190	490±230	490±190	490±230	490±130
7	404±176	632±246	480±320	706±240	365±240	380±224	680±185	680±210	680±185	680±210	414±98
21	454±201	1246±212	645±340	1321±324	584±402	638±320	1386±440	1206±340	1386±440	1206±340	226±63
35	758±424	1840±764 ( $n = 6$ )	1084±286	1986±884 ( $n = 6$ )	906±327 ( $n = 6$ )	986±380 ( $n = 6$ )	2810±528 ( $n = 4$ )	2762±620 ( $n = 5$ )	2810±528 ( $n = 4$ )	2762±620 ( $n = 5$ )	306±45

## DISCUSSION

Both DTA and PEA coding genes can be modified to express prokaryotically and eukaryotically, and post-translational process in eukaryotic cells did not eliminate its ADP-ribosylation activity. So the toxin genes are suitable for cancer gene therapy if expression of the genes can be restricted into tumor cells or tumor bodies. In the study, cell type-specific inhibition and concentration-dependent inhibition of luciferase gene expression were demonstrated in the cotransfection assay, implying that expression level of the toxin genes played a crucial role in the growth inhibition of the established human tumor. Our previous studies suggested that tissue and tumor-specific transcriptional regulatory sequences played a central role in conferring a selective expression of prodrug activation gene<sup>[8]</sup> and cytokine genes<sup>[9-11]</sup>. We cloned CEA promoters and found that some trans-acting elements residing in the nuclei of the colorectal carcinoma cells LoVo, not in RC9406 renal carcinoma cells, proved to be responsible for the CEA overexpression in the cancer cells, and the flanking region of CEA gene lacked the conventional TATA and CAAT boxes. The results of these studies indicated that the CEA promoter might be strict enough to confer the toxin genes to selectively express in CEA positive colorectal carcinoma. From *in vitro* studies, the specific expression of the toxin genes driven by the CEA promoter was demonstrated following cotransfection with the luciferase vectors, but the basal transcription of PEA and DTA gene in CEA negative cells may exist. Tissue or cell specificity of transcriptional regulatory elements for controlling expression of these genes remains to be improved. The prokaryotic transcriptional control systems, the tetracycline system and the lactose system, have been employed to control expression of DTA gene in glioma gene therapy<sup>[12]</sup>. A more practicable control system needs to be developed.

In the present study, the retroviral constructs were respectively introduced into ecotropic and amphotropic retrovirus packaging cells in order to generate recombinant retroviruses. But we failed to obtain a stable retrovirus producing cell line. Concentration of the retrovirus was too low to be measurable, although HIV-regulated DTA transcription unit was reported to have been

packaged into recombinant retroviruses. Intratumoral injection of the unique retroviral constructs with a cationic liposome was the only alternative way for the *in vivo* gene therapy. The liposomal delivery system for the toxic gene is suitable for the *in vivo* gene therapy for preparation of liposome using cationic lipids has also become extremely popular for the delivery of pDNA, largely due to its simplicity and relative effectiveness. Cationic liposome-mediated expression of viral promoter regulated DTA gene has been used for the treatment of HIV infection<sup>[13]</sup>. In the study, the liposome delivered PEA or DTA genes under the control of the CEA promoter resulted in selective inhibition on the growth of CEA positive colorectal carcinoma in the animal models. The inhibition would be stronger if the Ad2 adenoviruses were added into the gene transfer system. The addition of adenoviruses in *trans* together with the conjugates has resulted in a significant increase in the transfection activity, probably because of its ability to aid the escape of the conjugate from the endosomal-lysosomal pathway<sup>[14]</sup>. The strategy may be useful in clinical investigation.

## REFERENCES

- Pastan I, Chaudhary V, Fitzgerald DJ. Recombinant toxins as novel therapeutic agents. *Ann Rev Biochem*, 1992;61:331-354
- Chen SY, Yang AG, Chen JD, Kute T, King CR, Collier JR *et al.* Potent antitumor activity of a new class of tumor-specific killer cells. *Nature*, 1997;385(6611):78-80
- Cao GW, Kuriyama S, Gao J, Mitoro T, Cui L, Nakatani T *et al.* Comparison of carcinoembryonic antigen promoter region from human colorectal carcinoma and normal adjacent mucosa to induce strong tumor selective gene expression. *Int J Cancer*, 1998; 76(6):in press
- Cao GW, Du P, Pan X, Zhang XQ, Cui L, Qi ZT. Identifying the role of CEA core promoter region from human colorectal carcinoma tissues in the cancer-specific cytosine deaminase gene therapy. *WJG*, 1998;in press
- Silverman JA, Mindell JA, Finkelstein A, Shen WH, Collier RJ. Mutational analysis of the helical hairpin region of diphtheria toxin transmembrane domain. *J Biol Chem*, 1994;269 (36):22524-22532
- Miller AD, Miller DG, Garcia JV, Lynch CM. Use of retroviral vectors for gene transfer and expression. *Method Enzymol*, 1993;217(4):581-599
- Richards CA, Austin EA, Huber BE. Transcriptional regulatory sequences of carcinoembryonic antigen: Identification and use with cytosine deaminase for tumor-specific gene therapy. *Hum Gene Ther*, 1995;6(7):881-893
- Cao GW, Qi ZT, Pan X, Zhang XQ, Gao J, Cui L *et al.* Gene therapy for malignant melanoma using bacterial cytosine deaminase gene under the control of tyrosinase promoter. *J Med Cell PLA*, 1998;13(3):in press
- Cao GW, Kuriyama S, Du P, Sakamoto T, Yang W, Masui K, Qi ZT. Construction of retroviral vectors to induce strong hepatoma cell-specific expression of cytokine genes. *J Gastroenterol Hepatol*, 1996;11(12):1053-1061
- Cao GW, Kuriyama S, Du P, Sakamoto T, Kong XT, Masui K *et al.* Complete regression of established murine hepatocellular carcinoma by *in vivo* tumor necrosis factor  $\alpha$  gene transfer. *Gastroenterology*, 1997;112(2):501-510
- Cao GW, Gao J, Du P, Qi ZT, Kong XT. Construction of retroviral vectors to induce a strong expression of human class I interferon genes in human hepatocellular carcinoma cells *in vitro*. *Chin Natl J New Gastroenterol*, 1997;3(3):139-142
- Paulus W, Baur I, Oberer DM, Breakefield XO, Reeves SA. Regulated expression of the diphtheria toxin A gene in human glioma cells using prokaryotic transcriptional control elements. *J Neurosurg*, 1997;87(1):89-95
- Konopka K, Harrison GS, Felger PL, Duzgunes N. Cationic liposome-mediated expression of HIV-regulated luciferase and diphtheria toxin A genes in HeLa cells infected with or expressing HIV. *Biochim Biophys Acta*, 1997;1356(2):185-197
- Seth P, Brinkmann U, Schwartz GN, Katayose D, Gress R, Pasten I *et al.* Adenovirus mediated gene transfer to human breast tumor cells: an approach for cancer gene therapy and bone marrow purging. *Cancer Res*, 1996;56(6):1346-1351

# Expression and significance of HBV genes and their antigens in human primary intrahepatic cholangiocarcinoma \*

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**Subject headings** hepatitis B virus; gene, viral; antigens, viral; *in situ* hybridization; immunohistochemistry; cholangiocarcinoma

## Abstract

**AIM** To explore the etiology and pathogenesis of human primary intrahepatic cholangiocarcinoma, the expression of HBV genes and HBV-antigens was detected in the cancerous tissue and its surrounding hepatic tissues.

**METHODS** HBV-antigens were detected by immunohistochemical technique and HBV genes were examined with *in situ* hybridization.

**RESULTS** In 20 cases of cholangiocarcinoma, the positive detection rate of HBxAg, pre-S1, pre-S2, HBsAg and HBcAg was 75%, 40%, 40%, 10% and 0%, respectively, and in the surrounding hepatic tissues of 19 cases the positive rates were 84.2%, 47.9%, 47.9%, 31.6% and 31.6%. Among 40 cases of cholangiocarcinoma, the positive rate of HBV-DNA, x-gene, pre-s gene, s gene and c gene fell on 77.5%, 70.0%, 47.5%, 40% and 42.5%, respectively, and of the surrounding hepatic tissues in 33 cases, 87.9%, 84.8%, 63.6%, 69.7% and 66.7%.

**CONCLUSION** The development of human primary intrahepatic cholangiocarcinoma bears a close relationship with chronic persistent HBV infection. Particularly, the x gene of HBV and its protein (HBxAg) might play an important role in pathogenesis of hepatic carcinoma.

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\*Project supported by the National Natural Science Foundation of China, No.39270744

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Received 1998-07-05

## INTRODUCTION

A large number of studies indicate a close relationship between human primary hepatocellular carcinoma and hepatitis B virus (HBV) infection, which is considered generally as an important factor in the development of hepatic carcinoma<sup>[1,2]</sup>. In human primary hepatic carcinoma, hepatocellular carcinoma is more frequently encountered, while intrahepatic cholangiocarcinoma (ChC), including hepatocholangiocarcinoma (HChC), is relatively less, being 8% - 10%<sup>[3]</sup>. For a long time, the etiology and pathogenesis of intrahepatic cholangiocarcinoma have been unclear. A few reports considered it to be related to infestation with clonorchiasis sinensis<sup>[4,5]</sup>, but never involved with HBV infection. We used immunohistochemical technique and *in situ* hybridization methods to detect HBV genes and their-related antigens in the tissues of intrahepatic cholangiocarcinoma and its surrounding hepatic tissues for the purpose of exploring the etiology and pathogenesis of intrahepatic cholangiocarcinoma.

## MATERIAL AND METHODS

All the 40 cases of surgically resected specimens of intrahepatic cholangiocarcinoma and hepatocholangiocarcinoma were from the Pathologic Laboratory of the Affiliated Hospital of Fourth Military Medical University. The specimens were fixed in 10% formalin, paraffin embedded and serially sectioned 5  $\mu$ m in thickness. The HBV antigens (HBxAg, HBsAg, HBcAg, pre-S1 and pre S2) were detected by immunohistochemical techniques (ABC and PAP). *In situ* hybridization method was adopted for detection of HBV genes (HBV-DNA, x gene, s gene, pre s gene and c gene). Of the main reagents of immunohistochemistry, the antibodies of anti-HBx, S1 and S2 were gained from Fox Chase Cancer Center, Philadelphia, USA, and ABC kit and PAP kit (including anti-HBs and anti-HBc) were purchased from BioGenes Lab, USA. The ABC and PAP methods were in accordance with the instructions of the kits. For *in situ* hybridization, Bio-11-dUTP was purchased from the Institute of Pharmacology of Beijing Medical University. The gene primers of S, pre S and C of HBV were

acquired from Fox Chase Cancer Center, Philadelphia, USA. The colony of *E. Coli*- C600-HBV (containing PCP 10) was supplied by the Research Institute of Liver Diseases of Beijing Medical University. The colony of PMM15-HBV was furnished by the Basic Medical Institute of Military Medical Academy, Beijing. *E. Coli*, DNA polymerase I, restriction endonuclease EcoRI, Bam HI and Bgl II were purchased from Hua Mei Biotechnology, Co. As for the preparation of probes, the plasmids of PMM15-HBV and PCP-HBV were extracted through lysis by alkali. PCP 10-HBV was cleaved by EcoRI and various sized gene fragments of 3.2 kb were harvested by freeze-squeezing method, and then, cleaved with Bam HI and Bgl II. 584 X gene fragments of nucleotide were obtained by PMM15-HBV. The probes of HBV-DNA and X gene were labeled through nicking translation, and the fragments of C gene, S gene and pre S gene of HBV were labeled with PCR. Control groups were set up including positivity, negativity, blank and replacement.

## RESULTS

### *Pathological characteristics*

In the specimens of 40 cases, 30 cases were confirmed by pathological examination as intrahepatic cholangiocarcinoma and 10 as hepato-cholangiocarcinoma. The patterns of intrahepatic cholangiocarcinoma manifested as tubulo-glandular structures, mostly the cuboidal cells arranging in small lumens and partly the high columnar cells taking the form of variously sized lumens with few mucus secretion. In some cases, the cells were in mass arrangement. The carcinoma cells were small in size with scanty cytoplasm. The fibrous interstitial tissues were more abundant. The above-mentioned tubulo-glandular structures and cellular masses showed irregular distribution within the fibrous interstitial tissue. Hepato-cholangiocarcinoma appeared as the tubulo-glandular structures mingling with hepatocellular carcinoma-cell cords or masses and the transition of each other was visible. In individual case, secretion of the bile was occasionally encountered. The carcinoma cells possessed a certain atypicality with relatively less mitotic figures. Of the 40 specimens of intrahepatic cholangiocarcinoma, the surrounding hepatic tissues were present in 33 cases, 22 of them were chronic active hepatitis (CAH), 7 chronic persistent hepatitis (CPH) and 4 liver cirrhosis (CIR).

### *Detection of HBV antigens*

HBV antigens were detected in 20 cases of intrahepatic cholangiocarcinoma and 19 cases of the

surrounding hepatic tissues. The HBV antigens included HBsAg, HBcAg, HBxAg, pre-S1 and pre-S2. Their positive results are shown in Tables 1, 2. In addition, the positive coexistence of HBxAg, S1 and S2 antigens were present in 6 cases in the cancerous tissues, and positive HBxAg and HBsAg in 1 case. In the surrounding hepatic tissues, 7 cases showed the positive coexistence of HBxAg, S1 and S2 antigens, 4 cases with positive HBxAg and HBsAg, and 3 cases with positive HBxAg and HBcAg.

**Table 1 Positive results of 5 HBV antigens in cancerous tissues**

Pathological diagnosis	Cases	HBxAg	pre-S1	pre-S2	HBsAg	HBcAg
Chc	12	8	5	5	0	0
HChC	8	7	3	3	2	0
Total(%)	20	15(75)	8(40)	8(40)	2(10)	0

Chc: intrahepatic cholangiocarcinoma

HChC: hepato-cholangiocarcinoma

**Table 2 Positive results of 5 HBV antigens in surrounding hepatic tissues**

Pathological diagnosis	Cases	HBxAg	pre-S1	pre-S2	HBsAg	HBcAg
CAH	8	8	5	5	2	5
CPH	6	5	1	1	1	0
CIR	5	3	3	3	3	1
Total(%)	19	16(84.2)	9(47.4)	9(47.4)	6(31.6)	6(31.6)

CAH: Chronic active hepatitis

CPH: Chronic persistent hepatitis

CIR: Liver cirrhosis

In the cancerous and surrounding hepatic tissues, positive HBxAg presented brown-yellow coloured evenly fine granules, mainly as in the cytoplasm (Figure 1). They gathered around the nuclei or on the cellular membranes, or in combined presence. The distribution and intensity of positive HBxAg varied in different cases, even in different places of the same case. In general, detection rate and intensity of the positive cells in the surrounding hepatic tissues were both higher than that in the cancerous tissues (Figure 2). In the above-mentioned cancerous and surrounding hepatic tissues, the positive HBxAg cells were scattered, local or diffuse in distribution. In this group, local distribution was most common and diffuse distribution was the next.

In the cancerous and surrounding hepatic tissues, HBsAg and the antigens of pre-S1 and pre-S2 appeared as brown-yellow coloured evenly fine granules as well, mainly intracytoplasmic, a few intranuclear or on the cellular membranes. The intracytoplasmic positive materials appeared

frequently in shape of an inclusion body, located at one side of the nucleus (Figure 3). In some cells nearly the whole cytoplasm was full of these materials with a few on the cellular membranes (Figure 4). These positive cells were mainly in local distribution.

HBcAg was chiefly present in the surrounding hepatic tissues, mostly seen in the nuclei, some in the cytoplasm or on the cellular membranes, even present in combination (Figure 5). The intranuclear HBcAg manifested as brown yellow coloured coarse granules, while fine granules were intracytoplasmic or on the cellular membranes. Positive HBcAg was frequently seen in chronic active hepatitis.

The detection of above-mentioned antigens was compared with several control experiments. The positive sections remained positive by repeated stainings, and the negative sections, the replacement and blank experiments were all negative.

#### Detection of HBV genes

The 5 kinds probes of were applied to detect HBV-DNA, X gene, pre S gene, S gene and C gene in the cancerous and surrounding hepatic tissues. The results are shown in Tables 3, 4.

**Table 3 Positive results of hybridization by 5 kinds of probe for HBV genes in cancerous tissues**

Pathological diagnosis	Cases	HBV-DNA	X	pre-S	S	C
Chc	30	23	20	13	12	9
HChC	10	8	8	6	4	8
Total(%)	40	31(77.5)	28(70.0)	19(47.5)	16(40.0)	17(42.5)

**Table 4 Positive results of hybridization by 5 kinds of probe for HBV genes in surrounding hepatic tissues**

Pathological diagnosis	Cases	HBV-DNA	X	pre-S	S	C
CAH	22	18	18	12	14	13
CPH	7	7	6	5	5	5
CIR	4	4	4	4	4	4
Total(%)	33	29(87.9)	28(84.8)	21(63.6)	23(69.7)	22(66.7)

On the basis of statistics of positive cases, among 40 cases of intrahepatic cholangiocarcinoma, HBV-DNA was positive in 33 cases (82.5%), X gene, 31 (77.5%), pre S gene 26 (65.0%), S gene 24 (60.0%) and C gene 27 (67.5%). In cancerous tissues, 9 cases had coexistent positive expression of X gene, pre S gene, S gene and C gene, 6 had positive X and S genes, and 4 had only positive X gene. In the surrounding hepatic tissues, 16 cases showed the coexistent expression of X gene, S gene, pre-S gene and C gene, 5 cases positive X and S

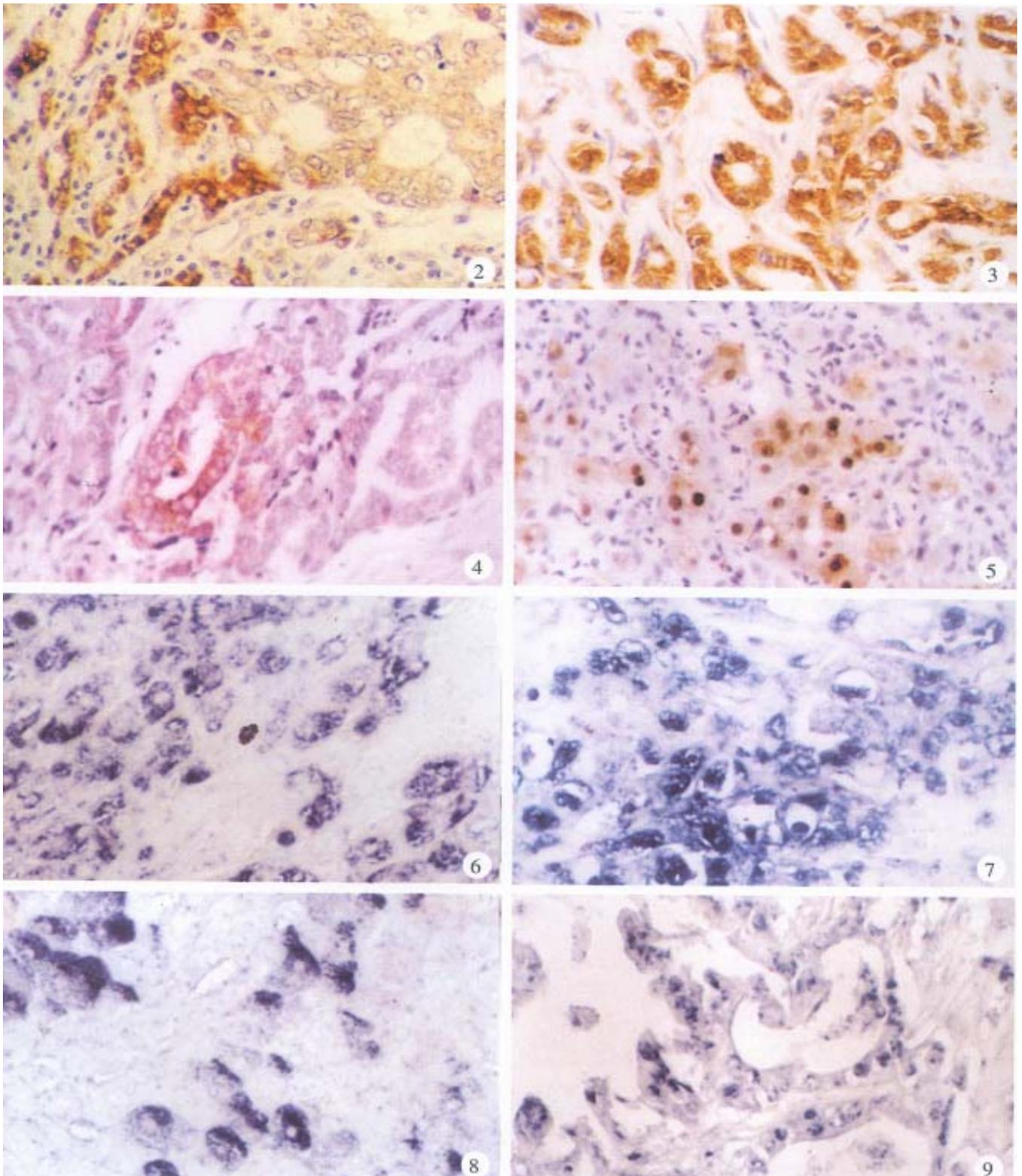
genes, and 2 cases only positive X gene.

In the cancerous and surrounding hepatic tissues, the positive hybridized signal of HBV-DNA and X gene appeared as blue-purple coloured fine granules in the cytoplasm (Figure 6) and the nuclei, or on the cellular membranes, or present in combination (Figure 7). The main manifestation was the nuclear type or nucleocytoplasmic type, while the purely cytoplasmic type was less encountered. The distribution and reaction intensity of positive hybridized signal varied in different cases, results were even different in different places of the same case. In general, the detection rate of the positive cells and intensity of positive reaction in the surrounding hepatic tissues were higher than that in cancerous tissues. The distribution of the positive cells could generally be classified into 3 types: scattered type, only individual positive cells were visible in section; local type, a small number of positive cells gathered in clusters located at certain regions in section; and diffuse type, numerous positive cells distributed diffusely in section. In this study, the local type was chiefly observed, the diffuse type was the next. In the cancerous and surrounding hepatic tissues, the positive signal of pre s gene, s gene and c gene also appeared as blue-purple coloured fine granules, mainly located in the cytoplasm or nucleocytoplasm (Figures 8 and 9), a few on the cellular membranes. Mostly, nearly the whole cytoplasm was permeated with the positive materials. Most positive cells were local in distribution, and the remainings in diffuse distribution, with scattered distribution least encountered.

The abovementioned detection of genes was compared with several control experiments. The positive sections still presented positive by repeated hybridizations. The negative sections and the blank and replacement controls were all negative in results.



**Figure 1** Cancerous surrounding hepatic tissue, HBxAg-positive substance located in the cytoplasm. ABC×400



- Figure 2** Cancerous and surrounding hepatic tissues, detection rate and intensity of the HBxAg-positive cells in the surrounding hepatic tissues were both higher than that in the cancerous tissues. ABC×200
- Figure 3** Cancerous tissue, HBsAg-positive substance located mainly in the cytoplasm. ABC×200
- Figure 4** Cancerous tissue, pre-S1 antigens located mainly in the cytoplasm, some on cellular membranes. ABC×400
- Figure 5** Surrounding hepatic tissue, HBc-Ag positive located mainly in the nuclei, some in the cytoplasm and on cellular membranes. ABC×400
- Figure 6** Surrounding hepatic tissue, HBV-DNA gene located in the cytoplasm, some in the nuclei. ISH×400
- Figure 7** Surrounding hepatic tissue, pre-s gene located in the cytoplasm, some in the nuclei. ISH×400
- Figure 8** Surrounding hepatic tissue, s gene located mainly in the cytoplasm. ISH×400
- Figure 9** Cancerous tissue, c gene located mainly in the nuclei, some in the cytoplasm. ISH×400

## DISCUSSION

In human primary hepatic carcinoma, intrahepatic cholangiocarcinoma is relatively less encountered, and few reports were involved in the study of etiology and pathogenesis. This disease was considered to be related to colloidal thorium dioxide (ChO<sub>2</sub>)<sup>[6]</sup>. Hou reported that the development of intrahepatic cholangiocarcinoma bore relationship with *Clonorchis sinensis* infection by mechanic stimulation of worm-moving and chemical irritation of the bile and tyrosinase from the worm body<sup>[6]</sup>. In our study, these patients had<sup>[5]</sup> neither taken colloidal thorium dioxide, nor infected with *Clonorchis sinensis*. Moreover, the cancerous and surrounding hepatic tissues showed no pathological pattern of parasitic infection. In the cancerous and surrounding hepatic tissues in this series, the detection rate of HBV antigens was obviously high, being 85%, especially the HBxAg, being above 80%. The detection rate of HBV-DNA was 82.5%. Among the genes of X, pre S, C and S, X gene had the highest detection rate of 77.5%. By pathological examination of the surrounding hepatic tissues, CAH was 66.7%, CPH 21.2% and CIR 12.1%. 75.0% of the patients with hepatic carcinoma had a history of chronic hepatitis. This result indicates that the development of intrahepatic cholangiocarcinoma is closely related to HBV infection. The persistent HBV infection plays an important role not only in hepatocellular carcinoma, but also in intrahepatic cholangiocarcinoma.

Up to now, the mechanism of development of intrahepatic cholangiocarcinoma by HBV is still unclear. The HBV and reverse transvirus are homologous in the origin of evolution<sup>[7]</sup>, and it is generally considered that the X gene of HBV and its protein product may play an important role in malignant transformation. It has been discovered that the sera of patients with hepatic carcinoma contained higher anti-HBx with a detection rate of 85.7%<sup>[8]</sup>. The X gene region might be the important region or HBV integrated into the chromosomes of primary hepatic carcinoma, and the integration of DNA of the host cells with HBV-

DNA was completed through the peculiar recombination in the X gene region<sup>[9]</sup>. The translation product of X gene has a transactive expression of genes<sup>[10]</sup>. The experiment of transgenic mice manifested that the HBx might directly induce primary hepatic carcinoma<sup>[11]</sup>. These facts state clearly that HBV may possess probably the direct carcinogenic effect, and HBx gene is the important factor. Thus, intrahepatic cholangiocarcinoma may be closely related to HBV infection.

About 80% hepatocellular carcinoma cases are accompanied by liver cirrhosis. It can be assumed that HBV infection may induce chronic active hepatitis, further progress to liver cirrhosis and finally result in malignant transformation. Fewer intrahepatic cholangio-carcinoma cases are complicated with liver cirrhosis<sup>[12]</sup>. In our study, only 12% of intrahepatic cholangiocarcinoma were associated with liver cirrhosis, while 87.8% with chronic active hepatitis and chronic persistent hepatitis. This indicates that intrahepatic cholangiocarcinoma is not chiefly developed through liver cirrhosis but mainly through persistent hepatitis. The precise relationship of HBV and development of intrahepatic cholangiocarcinoma remains to be further studied.

## REFERENCES

- 1 Beasley RP. Hepatitis B virus: the major etiology of hepatocellular carcinoma. *Cancer*, 1988;61:1942
- 2 London WT, Buetow K. Hepatitis B virus and primary hepatocellular carcinoma. *Cancer Invest*, 1988;6:317
- 3 Nakaajima T, Kondo Y, Miyazaki M et al. A histopathologic study of 102 cases of intrahepatic cholangiocarcinoma. *Hum Pathol*, 1988;19:1228
- 4 Hou PC. Hepatic clonorchiasis and carcinoma of the bile duct in dog. *J Path Bact*, 1965;89:365
- 5 Hou PC. The relationship between primary carcinoma of the liver and infestation with clonorchiasis sinensis. *J Path Bact*, 1956;72:239
- 6 Kew MC. Tumors of the liver. In: Zakim D and Boyer TD. *Hepatology: a textbook of liver disease*. Philadelphia WB Saunders Company, 1982:1065
- 7 Miller RH, Robinson WS. Common evolutionary origin of hepatitis B virus and retroviruses. *Proc Natl Acad Sci USA*, 1986;83:2531
- 8 Suqihara S, Kojiro M. Pathology of Cholangiocarcinoma. In: Okudak, Ishak KG, eds. *Neoplasma of liver*. Japan: Springer Verlag, 1987:143
- 9 Klatskin G. Adenocarcinoma of the hepatic duct at its bifurcation with in the porta hepatis: an unusual tumor with distinctive clinical and pathological features. *Am J Med*, 1965;38:241
- 10 Zahm P, Hofschneider PH, Koshy P. The HBV X-ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. *Oncogene*, 1988;3:1169
- 11 Kin CM, Koike K, Saito I. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature*, 1991;351:317
- 12 Ellis EF, Gordor PR, Gotelieb LS. Oral contraceptives and cholangiocarcinoma. *Lancet*, 1978;1:207

# Basaloid squamous carcinoma of esophagus: a clinicopathological, immunohistochemical and electron microscopic study of sixteen cases

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**Subject headings** esophageal neoplasms/pathology; esophageal neoplasms/ultrastructure; carcinoma, squamous cell/pathology; carcinoma, squamous cell/ultrastructure

## Abstract

**AIM** To further clarify the clinicopathological, immunohistochemical and electron microscopic features, and prognostic aspect of basaloid squamous carcinoma (BSC), a rare esophageal carcinoma.

**METHODS** We reviewed 763 documented cases of esophageal malignancies (1977-1996) from our hospital, and discovered 16 (2.1%) cases of BSC. The clinicopathological features of these cases were evaluated. Immunohistochemistry (S-P method), histochemical stains, and electron microscopy were used to further characterize the neoplasm.

**RESULTS** The tumors were classified into stages I ( $n = 1$ ), IIA ( $n = 6$ ), IIB ( $n = 2$ ), III ( $n = 5$ ), and IV ( $n = 2$ ) according to the criteria of the UICC TNM classification system of malignant tumors (1987). Most neoplasms were located in the mid third of the esophagus. Grossly, they had a similar appearance of conventional esophageal carcinoma, but showed a typical cytoarchitectural pattern of BSC histologically. The most important histologic feature of this tumor is carcinoma with a basaloid pattern, intimately associated with squamous cell carcinoma, dysplasia, or focal squamous differentiation. The basaloid cells were round to oval in shape with scant cytoplasm, arranged mainly in the form of solid, smooth-contoured lobules with peripheral palisading. A panel of immunostains were used for the basaloid component of the tumor with the

following results: CK (Pan) 14/16 (+); EMA 16/16 (+); Vimentin 4/16 (+); S-100 protein 7/16 (+). CEA and smooth muscle actin were negative. Electron microscopy (EM) revealed that the basaloid cells were poorly differentiated, with a few desmosomes and fibrils, and numerous free and polyribosome. Of the 11 patients with adequate follow-up 8 died within 2 years, with an average survival time of 16.2 months. No stage II, III or IV cases survived beyond 5 years. The one-year survival rate was 60% and two-year 20%.

**CONCLUSION** The BSC of esophagus is a distinct clinicopathological entity with poor prognosis. The cellular differentiation and biologic behavior of esophageal BSC were assumed to occupy a station intermediate between that of conventional squamous cell carcinoma and small undifferentiated cell carcinoma.

## INTRODUCTION

The term basaloid squamous carcinoma (BSC) was first proposed by Wain *et al* in 1986 to describe a rare, aggressive neoplasm with a predilection occurring in the hypopharynx, base of tongue, larynx<sup>[1]</sup>, and late in the esophagus<sup>[2]</sup>, nasal and oral cavity<sup>[3,4]</sup>, tonsil<sup>[5]</sup>, nasopharynx<sup>[6]</sup>, trachea, bronchus and lung<sup>[7-9]</sup>, and other sites including external ear<sup>[10]</sup>, anal canal, vulva and penile<sup>[11-13]</sup>. It is characterized by basaloid carcinoma intimately associated with squamous cell carcinoma, dysplasia, carcinoma *in situ*, or focal squamous differentiation. Approximately 400 cases of BSC have been reported in the world literature by the end of 1996, including 69 cases of BSC of esophagus. In a recent review of esophageal neoplasms at the Department of Pathology of our hospital, sixteen such tumors were identified. In this paper, we report about the clinicopathological and immunohistochemical features of these 16 cases of BSC of esophagus to further categorize this lesion. Electron microscopic features of seven cases, and their prognosis were also described.

## MATERIAL AND METHODS

All resected esophageal tumor slices examined over the last 20 years (1977-1996) at the Department of

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Received 1998-04-20

Pathology of our hospital were reviewed. Of 763 cases of esophageal malignancies, 16 showed the histopathologic pattern of basaloid squamous carcinoma<sup>[1]</sup>. Clinical data, including age and sex, location and size, and gross appearance were obtained from the medical records. Follow-up information was available in 11 cases. All surgical specimens were fixed in 10% formalin and processed in the usual way for paraffin embedding. In addition to routine hematoxylin and eosin (H&E) staining, periodic acid-Schiff (PAS) with or without diastase, and Alcian blue (pH 2.5) staining was performed.

Seven cases was also reviewed electron microscopically. Immunohistochemistry was performed on paraffin sections of representative portions of the tumors using the streptavidin peroxidase method (S-P method)<sup>[14]</sup>. The S-P Kit was obtained from Zymed Laboratories Inc, USA. The prediluted antibodies against cytokeratin (Pan) (CK), carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), vimentin, smooth muscle specific actin (SMA), and S-100 protein were products of Maxim Biotech, Inc, USA. The results of immunohistochemical stains were recorded as negative (-), weakly positive (less than 10% positive cells), positive (10% - 15% positive cells)

and strongly positive (more than 50% positive cells).

## RESULTS

### Demographic and clinical data

The clinicopathologic, TNM staging, therapeutic, and follow-up data are summarized in Table 1. The patients ranged in age from 42 to 72, with a mean age of 58 years (median 57 years). There were 9 males and 7 females, all were Chinese. The patients presented with progressive dysphagia. The duration of symptoms ranged from 1 to 8 months. All cases were treated with either curative or palliative esophagectomy. Eleven patients were further treated by chemotherapy. Eleven of the 16 esophageal tumors were located at the middle third of the esophagus, 4 at the lower third, and 1 at the upper third. Their size ranged from 1.3 cm to 7.0 cm in greatest diameter. The gross appearance of the tumors in this series were infiltrative lesions in 4 cases, protuberant lesion in 6, ulcerative lesion in 5, and a polypoid mass in 1. The staging of the lesions followed the criteria of the UICC TNM classification system of malignant tumors, 1987<sup>[15]</sup>. The tumors were then classified as stage I ( $n = 1$ ), stage II A ( $n = 6$ ), stage II B ( $n = 2$ ), stage III ( $n = 5$ ), and stage IV ( $n = 2$ ).

**Table 1 Clinicopathological, Staging, Therapeutic and Follow-up Data in 16 Patients with Esophageal BSC**

Case No.	Age/sex	Tumor location	Tumor size (cm)	Gross appearance	Stage	Therapy		Mitoses (per 10HPF)	Follow-up
						Surgery	Chemo-therapy		
1	42/F	Lower 1/3	2.5×1.5×0.6	Polypoid	IIA(T2N0M0)	Curative esophagectomy		198	Not available
2	56/M	Middle 1/3	2.5×2.0×0.8	Protuberant	IIA(T3N0M0)	Curative esophagectomy		51	Not available
3	50/F	Lower 1/3	4.0×2.0×1.0	Ulcerative	III(T3N1M0)	Curative esophagectomy		74	Not available
4	62/M	Middle 1/3	3.5×1.5	Protuberant	IIA(T2N0M0)	Curative esophagectomy	+	86	Died of tumor recurrence at 18 mos
5	56/M	Lower 1/3	4.3×2.8×1.8	Protuberant	I (T1N0M0)	Curative esophagectomy	+	60	Alive & well at 10
6	62/M	Upper 1/3	2.8×1.0×0.7	Ulcerative	III(T3N1M0)	Curative esophagectomy	+	45	Died of tumor, recurrence 14mo after operation
7	54/M	Middle 1/3	4.0×2.8	Infiltrative	IV(T2N0M1)	Palliative esophagectomy	+	47	Metastases to lung and pleura, died 12mos after operation
8	60/F	Middle 1/3	2.5 (in diameter)	Infiltrative	III(T3N1M0)	Curative esophagectomy	+	98	Died of metastases and recurrence 8mo later
9	72/M	Middle 1/3	5.5×2.0	Ulcerative	IIA(T3N0M0)	Curative esophagectomy		136	Not available
10	62/F	Middle 1/3	3.5×2.0×3.0	Protuberant	IIA(T2N0M0)	Curative esophagectomy		49	Not available
11	49/M	Middle 1/3	3.5×3.0	Ulcerative	IV(T4N1M1)	Palliative esophagectomy	+	54	Metastases to lung and brain, Died 9mo after operation
12	70/M	Lower 1/3	5.0×2.0×1.5	Infiltrative	III(T3N1M0)	Curative esophagectomy	+	82	Died of tumor, recurrence 4 years after operation
13	67/M	Middle 1/3	5.0×4.8×2.0	Infiltrative	IIA(T3N0M0)	Curative esophagectomy	+	104	Died 16mo after operation
14	57/F	Middle 1/3 (with two lesions)	2.0×1.5 (in diameter)	Ulcerative	IIB(T1N1M0)	Curative esophagectomy	+	60	Died 3mo after diagnosis
15	57/F	Middle 1/3	7.0×5.0×3.0	Protuberant	III(T4N1M0)	Palliative esophagectomy	+	85	Died 8mo after operation
16	52/F	Middle 1/3	4.0×2.5	Protuberant	IIB(T2N1M0)	Curative esophagectomy	+	105	Alive 4mo after operation

The criteria of the UICC TNM classification system (1987) was used.

Follow-up information was obtained from 11 cases. Nine patients died 3 to 48 months after operation. The average survival of these nine patients was 16.2 months. One patient is alive disease free at 10 years, and another patient is still on chemotherapy 4 months after operation. The survival rate was 60% at 12 months, and 20% at 24 months.

**Histopathologic findings**

All 16 neoplasms fulfilled the basic histological features of BSC<sup>[1]</sup>. The basaloid cells arranged mainly in the form of solid, smooth-contoured lobules, some cases also in the form of solid sheets, anastomosing trabeculae, or microcystic structures (Figure 1). In fourteen cases, the basaloid component was found to represent between 60% to 95% of the tumor examined. In the remaining two cases (case 6 and case 7), it accounted for less than 20% and 30% respectively. In all 16 cases, the intertrabecular spaces and stroma of the tumors had eosinophilic hyaline materials. These hyaline materials, extending between and replacing the tumor cells, were PAS positive both before and after diastase treatment (Figure 2). In 8 cases, the microcystic spaces, some of which lined by PAS-positive lamina material, contained basophilic mucoid matrix which was Alcian blue positive but PAS negative. The basaloid cells were round to oval in shape, with scant, amphophilic cytoplasm, but sometimes it was abundant and clear. The nuclei showed either dark, hyperchromatin without nucleoli or dusty chromatin, vacuolated nucleoplasm with 1 to 3 small distinct nucleoli. The nuclear pleomorphism was frequently observed in all cases. The number of mitotic figures (including atypical ones) was extremely high, ranging from 45 to 198 mitoses per 10 high-power fields. The cells at the edges of the basaloid islands tended to show peripheral nuclear palisading. Comedo necrosis was found within the basaloid lobules in all cases (Figure 3).

An intimately associated squamous cell component was another major histopathologic feature of the tumor. In seven cases, invasive, keratinizing-squamous cell carcinoma covered 5%-80% and merged with the basaloid component. Two cases (cases 1 and 7) showed conventional squamous cell carcinoma as well as spindle cell component, the latter infiltrated between the basaloid lobules (Figure 4). In case 5 and 13 carcinomas *in situ* were found in the overlying epithelium. Four of the 16 (case 3, 11, 15 and 16) cases only had focal squamous differentiation and keratinization in the basaloid lobules (Figure 3). In one case (case 14), squamous cell dysplasia, squamous cell carcinoma *in situ* in the overlying epithelium as well as invasive squamous cell carcinoma and small cell carcinoma were the associated components of basaloid cell

carcinoma with areas of ductular or glandular differentiation.

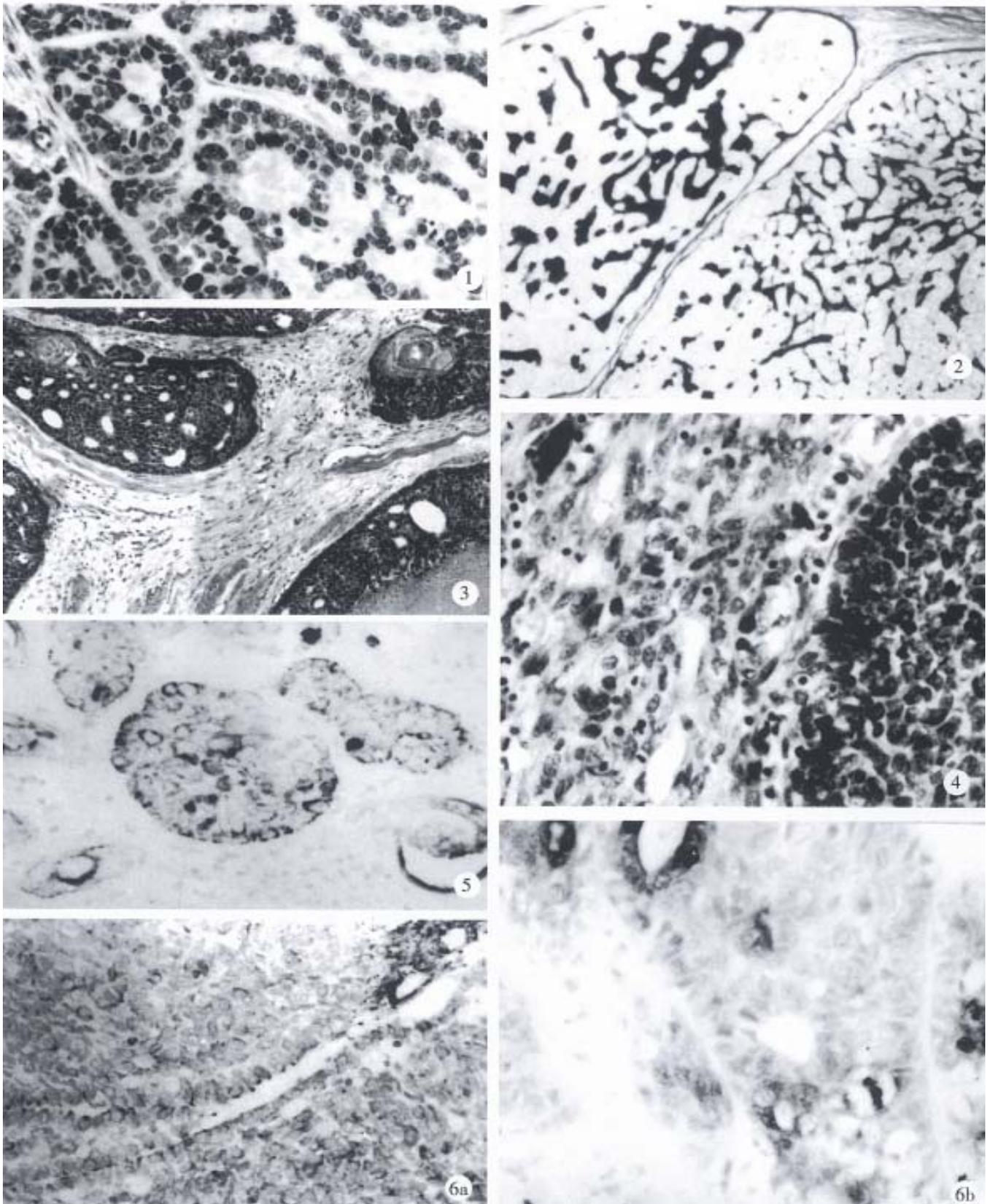
**Table 2 Histochemical and immunohistochemical findings**

Case	Component	CK	EMA	CEA	Vim	S-100	SMA	Stroma		
								ABP	AS	D-PAS
1	B	+	+	-	-	-	-	+	+	+
	S	++	++	-	-	-	-			
	SP	-	-	++	-	-	-			
2	B	+++	+	-	+	-	-	-	+	+
	S	+++	+++	-	-	-	-			
3	B	++	+	-	-	-	-	+	+	+
	S	+++	+	-	-	-	-			
4	B	++	+	-	-	-	-	-	+	+
	S	+++	+++	-	-	-	-			
5	B	+	+	-	-	-	-	+	+	+
	S	+	+++	+	-	-	-			
6	B	+++	+	-	++	-	-	-	+	+
	S	+++	+++	+	-	-	-			
7	B	+++	-	-	-	++	-	+	+	+
	S	+++	+++	-	-	-	-			
	SP	-	-	-	++	-	+			
8	B	++	+	-	-	-	-	+	+	+
	S	++	++	-	-	-	-			
9	B	+++	++	-	-	+	-	-	+	+
	S	+++	++	-	-	-	-			
10	B	+	+	-	-	+	-	-	+	+
	S	++	+++	-	-	-	-			
11	B	-	++	-	-	-	-	-	+	+
	S	+++	++	-	-	-	-			
12	B	++	++	-	-	+	-	+	+	+
	S	+++	++	-	-	-	-			
13	B	-	+	-	-	+	-	-	+	+
	S	++	++	-	-	-	-			
14	B	+++	++	-	-	-	-	+	+	+
	S	+++	++	-	-	-	-			
	SM	++	++	-	-	-	-			
15	B	++	+	-	+++	+++	-	+	+	+
	S	+++	+++	++	-	-	-			
16	B	+	+	-	++	+	-	-	+	+
	S	+++	++	-	-	-	-			

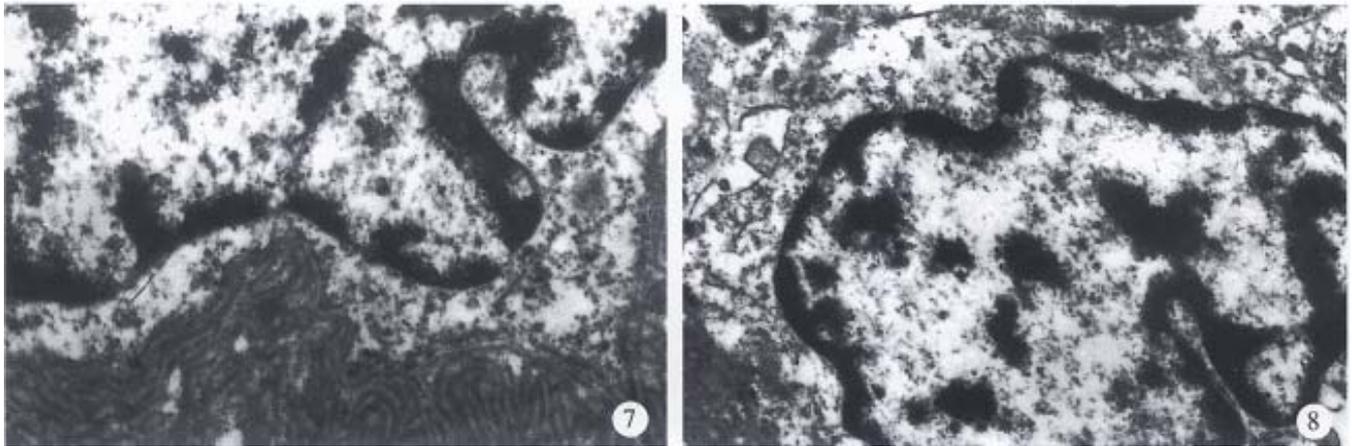
B: basaloid cell; S: squamous cell; SP: spindle cell; SM: small cell; D-PAS: diastase treating PSA stain

**Immunohistochemical findings**

Table 2 gives the immunohistochemical staining pattern of the 16 neoplasms studied. All squamous cell component, and basaloid cell component of 14 cases showed variable intracytoplasmic staining for cytokeratin (Pan) (Figure 5). But the spindle cells in case 1 and case 7 had negative staining for CK. EMA-positivity was also demonstrated in nearly all the cases, nevertheless, the reactivity was focal and faint in the basaloid component, and in some cases, CK and EMA staining highlighted the glandular spaces focally. CEA was weakly expressed in squamous area of three cases. Vimentin immunoreactivity was found focally in the basaloid component of three cases and in the spindle cells in case 1 and case 7. In six cases, S-100 protein positive reaction was focally found within the lobules of basaloid component. However, the basaloid component in case 15 had diffusely and strongly positive staining for vimentin and S-100 protein (Figure 6). SM-actin immunoreactivity was absent in all cases except the spindle cell component in case 7.



- Figure 1** The basaloid cells arranged in the form of anastomosing trabeculae and microcystic structures. H&E,  $\times 200$
- Figure 2** The intertrabecular and microcystic spaces filled with eosinophilic hyaline material which were PAS positive.  $\times 100$
- Figure 3** Focal squamous differentiation and keratinization and comedo necrosis were found in the basaloid lobules. H&E,  $\times 100$
- Figure 4** Basaloid cell carcinoma with spindle cell component. H&E,  $\times 200$
- Figure 5** Immunohistochemical studies (S-P method) show positivity in the basaloid components for cytokeratin (Pan).  $\times 200$
- Figure 6** The basaloid components in case 15 are diffusely and strongly positive staining for (a) vimentin and (b) S-100 protein.  $\times 200$



**Figure 7** Electron microscopic photograph of esophageal BSC demonstrating replicated basal lamina in fingerprint-like pattern filled in the intertrabecular and intercellular spaces.  $\times 20000$

**Figure 8** Electron microscopic photograph of esophageal BSC demonstrating well-formed intercellular desmosomes.  $\times 16000$

### Electron microscopic features

Seven cases were examined under electron microscopy (case 9, 10, 11, 12, 14, 15, 16). Lobules of basaloid cells were separated from the stroma by an external lamina. The cells within the lobules had widened intercellular spaces. The microcystic and intertrabecular spaces identified by light microscopy were lined by basal membranes and filled with either loose reduplicated or compact globoid basal lamina, showing fingerprint-like pattern (Figure 7). The basaloid cells were polygonal. The nuclei had oval profiles and there were irregular indentations, containing finely dispersed chromatin and small clumps of heterochromatin. One to three compact nucleoli were present in some nuclei. Within the cytoplasm were numerous free and polyribosomes, a few desmosomes, tonofilaments and mitochondria (Figure 8), but rare other organelles, and absence of neuro secretory granules.

### DISCUSSION

Before the term basaloid squamous carcinoma was introduced by Wain<sup>[1]</sup> in 1986, most malignancies of the esophagus with similar histopathologic pattern to BSC were diagnosed as adenoid cystic carcinoma (ACC). But scrutiny of the published reports showed that most cases were histologically identical to BSC<sup>[16,17]</sup>, and behaved more aggressively than ACC. Tumors with identical morphology and similar clinical behavior have also been noted in other areas such as the uterine cervix<sup>[18]</sup>, anal canal<sup>[19]</sup> and lung<sup>[9]</sup>. Other terms had also been used to describe this tumor such as adenosquamous carcinoma, poorly-differentiated squamous cell carcinoma, and small undifferentiated cell carcinoma. In the recent World Health Organization classification of esophageal tumors,

there is no mention of this newly recognized type of basaloid squamous carcinoma<sup>[20]</sup>. But the presence of lobules or cords of small, closely packed basaloid cells with scant cytoplasm, with or without small cystic spaces and hyalinized stroma, as well as an associated abnormal squamous cell component makes this tumor different from other common esophageal carcinomas. They have identical clinical and histopathologic features to that of BSC occurring in the larynx, pharynx and base of tongue described first by Wain *et al.*, and belong to the same entity of malignant carcinoma.

In this series, sixteen cases of BSC of esophagus were found, accounting for 2.1% of 763 esophageal carcinomas reviewed. The incidence is higher than that previously reported<sup>[2,21]</sup>, but similar to the incidence by Abe *et al.*<sup>[22]</sup>. This may be due to the difference of case selection. In our series, all cases were confirmed by esophagectomy. Moreover, the main portion of each tumor is composed of basaloid carcinoma in 14 of 16 BSC cases as described in the literature<sup>[1]</sup>, but in the remaining two, the major portion of the tumor is composed of squamous cell carcinoma, the basaloid component accounting for less than 30%. The most common location of the tumor is the middle third of the esophagus (11/16). The gross appearance of BSC is similar to that of other squamous cell carcinoma, only the cut surface in the former is more delicate.

BSC of esophagus should be distinguished from adenoid cystic carcinoma (ACC). The latter occurs more commonly in females, usually in women aged 40 to 60 years, with a mean age of 52 years<sup>[23]</sup>, and with a more protracted clinical course. Histologically, the focal continuity with the abnormal surface epithelium or carcinoma *in situ*, an associated invasive squamous cell carcinoma, and focal squamous differentiation in the islands of

basaloid cells are not features of ACC, while alone or in combination, they have been found in all cases in the present series. The cells in ACC seem bland with mild pleomorphic nuclei and infrequent mitosis, and often exhibits identifiable two-cell-type differentiation (pale ductal epithelium and darker basaloid cells) and distinctive cribriform structures in nerve invaded areas<sup>[24]</sup>. Under electron microscopic examination, four cell types were revealed in ACC: the intercalated duct cells; the secretory cells; the myoepithelium; and the pluripotential reserve/stem cells. These cells, especially the secretory cells and myoepithelium, were not noted by either immunohistochemical staining or electron microscopic examination in current series. The correct differential diagnosis between BSC and ACC is of important prognostic value. Basaloid squamous carcinoma of the esophagus is associated with poor outcome. The overall 3-year survival rate of BSC has been estimated at 28.5%<sup>[25]</sup>. In our series, most patients had developed into advanced stages at their presentation. Of the 11 patients with adequate follow-up, 8 died within 2 years from diagnosis, the survival rate being 60% at 12 month and 20% at 24 month. While the cumulative survival rates for patients with grade I, II and III of ACC were 92%, 65% and 14% at 5 years<sup>[24]</sup>. Epstein and coworkers<sup>[17]</sup> have proposed labelling tumors in the esophagus with histological features of BSC as “carcinoma with adenoid cystic differentiation” instead of “adenoid cystic carcinoma”. But we think this descriptive term is too long and inconvenient to use, and the prognosis is apt to be overestimated by clinicians. In contrast, the term basaloid squamous carcinoma is shorter and more convenient to use. Furthermore, the two tumor elements, basaloid and squamous, of the term make it unique and differ from other kinds of tumors.

Cytoplasmic staining of BSC for a variety of antibodies to cytokeratins of different molecular weight has demonstrated variable staining with these antibodies<sup>[2,8,26,27]</sup>. In this study, the faint immunoreactivity pattern for EMA in basaloid component, and focal positivity for CK in some cases, together with intercellular desmosomes and cytoplasmic organelles and fibrils revealed by electron microscope provides an evidence that the basaloid cells in BSC may be largely undifferentiated, sometimes exhibiting focal tubular or squamous differentiation. Although Klijanienko and associates<sup>[27]</sup> suggested that the immunophenotype of S-100 protein-positive and vimentin-positive cells would indicate diagnosis of adenoid cystic carcinoma, our seven S-100 protein-positive cases and four vimentin-positive cases are typical BSC rather than ACC. The S-100 protein-positive cells are not dendritic Langerhan's cells as

described by the same authors, but basaloid carcinoma cells.

The exact line of differentiation or pathogenesis of basaloid squamous carcinoma is still unknown. Some reported cases of BSC were associated with smoking and heavy alcohol consumption<sup>[4,16]</sup>, with a second primary tumor<sup>[28]</sup>, and rarely, with previous irradiation<sup>[3]</sup>. The relationship of this tumor with viral agents, i. e., Epstein Barr viruses vs BSC of the nasopharynx<sup>[6]</sup>, human papillomavirus vs BSC of the external genitalia, perineum, and anus<sup>[11-13]</sup>, has also been suggested by some authors. Based on the evidence provided by the present series and those reported in the literature, we agree with the theory of Ho *et al*<sup>[29]</sup> that a totipotent primitive cell is the common precursor of all epithelium neoplasms of the esophagus. With certain carcinogenic stimulation, the totipotent cells are activated and transformed into malignant cells. These transformed cells may differentiate into neoplastic squamous cells, adenocarcinoma, basaloid cell carcinoma (reserve cell carcinoma) and small cell carcinoma. These basaloid and small cells are rather primitive and retain their potential for further differentiation into keratin-forming cells, spindle-cell carcinoma, mucous-producing cells, and so on. We believe that only those tumors bearing a biphasic cellular pattern of basaloid and squamous components in an intimate relationship should be considered true BSC, if not, as some cases reported by Brambilla *et al*<sup>[8]</sup>, should be considered basaloid (or reserve) cell carcinoma.

The cellular differentiation and biologic behavior of BSC were assumed to occupy a station intermediate between that of conventional squamous cell carcinoma and small cell carcinoma. This assumptive placement into an intermediate position was based on the following. First, the clinical behavior of the tumors, which was less aggressive than that of small cell carcinoma (most of the patients died within 6 months from the time of diagnosis)<sup>[30]</sup>, but more aggressive than that of conventional squamous cell carcinoma. In their 170 radical resected esophageal squamous cell carcinoma, Zheng *et al*<sup>[31]</sup> reported that the 5-year survival rate was 47.3% for stage II A cases, 22.2% for stage II B, and 16.1% for stage III; while in our patients whose follow-up data were obtained, no stage II, III, or IV cases survived beyond 5 years. Second, the histopathologic feature of this tumor, i. e., the combination of basaloid and squamous carcinoma in most cases in our series and differentiation pattern in some cases. And third, numerous mitotic figures which were observed throughout tumor tissues in all our cases.

In conclusion, the BSC of the esophagus represents a specific and unique clinico pathological entity with a highly aggressive behavior and a poor

outcome. Identification of BSC is important because this lesion may be confused with less aggressive lesions, such as adenoid cystic carcinoma.

## REFERENCES

- 1 Wain SL, Kier R, Vollmer RT, Bossen EH. Basaloid-squamous carcinoma of the tongue, hypopharynx, and larynx: report of 10 cases. *Hum Pathol*, 1986; 17(11):1158-1166
- 2 Tsang WYW, Chan JKC, Lee KC, Leung AKF, Fu YT. Basaloid-squamous carcinoma of the upper aerodigestive tract and so-called adenoid cystic carcinoma of the oesophagus: the same tumor type. *Histopathology*, 1991;19(1):35-46
- 3 Wan SK, Chan JK, Tse KC. Basaloid-squamous carcinoma of the nasal cavity. *J Laryngol Otol*, 1992;106(4):370-371
- 4 Coppola D, Catalano E, Tang CK, Elfenbein IB, Harwick R, Mohr R. Basaloid squamous cell carcinoma of floor of mouth. *Cancer*, 1993;72(8):2299-2305
- 5 Banks ER, Frierson HF Jr, Ovell JL. Fine needle aspiration cytologic findings in metastatic basaloid squamous carcinoma of the head and neck. *Acta Cytol*, 1992;36(2):126-131
- 6 Wan SK, Chan JKC, Lau WH, Yip TTC. Basaloid-squamous carcinoma of the naso-pharynx: an Epstein Barr virus-associated neoplasm compared with morphologically identical tumors occurring in other sites. *Cancer*, 1995; 76(10): 1689-1693
- 7 Saltarelli MG, Fleming MV, Wenig BM, Gal AA, Mansour KA, Travis WD. Primary basaloid squamous cell carcinoma of the trachea. *Am J Clin Pathol*, 1995;104(5):594-598
- 8 Lin O, Harkin TJ, Jagirdar J. Basaloid-squamous cell carcinoma of the bronchus: report of a case with review of the literature. *Arch Pathol Lab Med*, 1995;119(12): 1167-1170
- 9 Brambilla E, Moro D, Veale D, Brichon PY, Stoeber P, Paramelle B *et al.* Basal cell (basaloid) carcinoma of the lung: a new morphologic and phenotypic entity with separate prognostic significance. *Hum Pathol*,1992;23(9): 993-1003
- 10 Croce A, Bianchedi M, Neri G, Moretti A, Angelucci D. Basal cell and basosquamous carcinoma of the external ear. Immunohistochemical study. *Acta Otorhinolaryngol Ital*, 1994;14(4):367-375
- 11 Shroyer KR, Brookes CG, Markham NE, Shroyer AL. Detection of human papilloma virus in anorectal squamous cell carcinoma. Correlation with basaloid pattern of differentiation. *Am J Clin Pathol*, 1995;104(3):299-305
- 12 Kurman RJ, Toki T, Schiffman MH. Basaloid and warty carcinomas of vulva. Distinctive types of squamous cell carcinoma frequently associated with human papillomaviruses. *Am J Surg Pathol*, 1993;17(2):133-145
- 13 Gregoire L, Cubilla AJ, Reuter VE, Haas GP, Lancaster WD. Preferential association of human papillomavirus with highgrade histologic variants of penile-invasive squamous cell carcinoma. *J Natl Cancer Inst*, 1995;87(22): 1705-1709
- 14 Shi CR, Itzkowitz SH, Kim YS. A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. *J Histochem Cytochem*, 1988;36(3):317-322
- 15 Hermanek P, Sobin LH, eds. UICC TNM classification of malignant tumors. 4th ed. Berlin: Springer-Verlag, 1987:40-42
- 16 Sweeney EC, Cooney T. Adenoid cystic carcinoma of the esophagus. A light and electron microscopic study. *Cancer*, 1980;45(6):1516-1525
- 17 Epstein JI, Sears DL, Tucker RS, Eagan JW Jr. Carcinoma of the esophagus with adenoid cystic differentiation. *Cancer*, 1984;53(5):1131-1136
- 18 Ferry JA, Scully RE. "Adenoid cystic" carcinoma and adenoid basal carcinoma of the uterine cervix: a study of 28 cases. *Am J Surg Pathol*, 1988;12(2): 134-144
- 19 Dougherty BG, Evans HL. Carcinoma of the anal canal: a study of 79 cases. *Am J Clin Pathol*,1985;83(2):159-164
- 20 Watanabe H, Jass JR, Sobin LH. Histological typing of esophagus and gastric tumors. World Health Organization International Histological Classification of Tumors. Berlin: Springer-Verlag, 1990;13-14:53-54
- 21 Huang ZZ, Liang YR, Wu XY. Basaloid squamous carcinoma of the esophagus: a distinctive clinicopathological entity. *Clin J Pathol*, 1995;24 (2):90-92 (in Chinese with English abstract)
- 22 Abe K, Sasano H, Itakura Y, Nishira T, Mori S, Nagura H. Basaloid squamous carcinoma of the esophagus: a clinicopathologic, DNA ploidy, and immunohistochemical study of seven cases. *Am J Surg Pathol*, 1996;20(4):453-461
- 23 Spiro RH, Huvos AG, Strong EW. Adenoid cystic carcinoma: factors influencing survival. *Am J Surg*,1977;138(5):579-583
- 24 Szanto PA, Luna MA, Tortoledo ME, White RA. Histologic grading of adenoid cystic carcinoma of the salivary glands. *Cancer*, 1984;54(6):1062-1069
- 25 Ereno C, Lopez JI, Sanchez JM, Toledo JD. Basaloid-squamous cell carcinoma of the larynx and hypopharynx: a clinicopathologic study of 7 cases. *Pathol Res Pract*, 1994;190(2):186-193
- 26 Banks ER, Frierson HF Jr, Mills SE, George E, Zarbo RJ, Swanson PE. Basaloid squamous cell carcinoma of the head and neck: a clinicopathologic and immunohistochemical study of 40 cases. *Am J Surg Pathol*, 1992;16 (10):939-946
- 27 Klijanienko J, El-Naggar A, Ponzio-Prion A, Marandas P, Micheau C, Caillaud JM. Basaloid squamous carcinoma of the head and neck: immunohistochemical comparison with adenoid cystic carcinoma and squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg*, 1993;119(8):887-890
- 28 Seidman JD, Berman JJ, Yost BA, Iseri OA. Basaloid squamous carcinoma of the hypopharynx and larynx associated with second primary tumors. *Cancer*, 1991; 68(7):1545-1549
- 29 Ho KJ, Herrera GA, Jones MJ, Alexander CB. Small cell carcinoma of the esophagus: evidence for a unified histogenesis. *Hum Pathol*, 1984;15(5): 460-468
- 30 Ibrahim NBN, Briggs JC, and Corbishley CM. Extrapulmonary oat cell carcinoma. *Cancer*, 1984;54(8):1645-1661
- 31 Cheng GY, Zhang DW, Zhang LG, Wang GQ, Zhang DC, Lui XY *et al.* Evaluation of the new international TNM staging system for carcinoma of the esophagus as compared with the Chinese trial clinicopathological staging system. An analysis of 224 cases. *Chin J Oncol*, 1993;15(5):358-361 (in Chinese with English abstract)

# Immuno-protective effect of tumor cell vaccine on Kunming mice bearing Ehrlich ascites tumor \*

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**Subject headings** Ehrlich ascites tumor; tumor cell vaccine; kunming mice; antigen, neoplasm

## Abstract

**AIM** To evaluate the immunity of chemically modified tumor cell vaccine.

**METHODS** Tumor cell vaccines (TCV) were prepared by incubating the live Ehrlich ascites tumor cells with concanavalin A-mitomycin C (ConA-MMC), mitomycin C (MMC), concanavalin A-glutaraldehyde (ConA-Glu), glutaraldehyde (Glu), or paraformaldehyde (Para), respectively. The whole cell or soluble forms of the vaccines were administered intraperitoneally into Kunming mice once a week for three times prior to the intraperitoneal inoculation of a lethal dose of live tumor cells. A second challenge with live tumor cells was given four weeks later. Survival and antibody production of the mice were analyzed.

**RESULTS** After the first challenge, the mice, received whole TCV of ConA-MMC, MMC ( $P < 0.01$ ) and Glu ( $P < 0.05$ ) promoted survival incidence than the controls. All the treated mice had the survival time prolonged. ConA-MMC vaccine treated mice had longer survival days than that of ConA-Glu ones ( $P < 0.05$ ). For the soluble TCV immunized mice, those treated with vaccines of Para ( $P < 0.01$ ), ConA-Para and ConA-Glu ( $P < 0.05$ ) had longer survival periods compared with that of the controls. Following the second challenge, survival incidence of the mice received vaccines of ConA-MMC, MMC, ConA-Glu or Glu was significantly increased ( $P < 0.01$ ). Moreover, all the treated mice had the survival time prolonged, and ConA-MMC vaccine

treated mice had longer survival days than that of Para treated ones ( $P < 0.05$ ). Antibodies against Ehrlich ascites tumor cells were found to be positive in sera of the mice treated with whole TCV of ConA-MMC.

**CONCLUSION** Ehrlich ascites tumor cells are immunogenic when treated with ConA-MMC, MMC, ConA-Glu, Glu or Para, which might act as safe and effective tumor vaccines with safety and effectiveness.

## INTRODUCTION

Biotherapy represents an alternative option after surgery, radiotherapy and chemotherapy for cancer. Tumor vaccine and the induction of active specific immunity are of great interest in this field. However few tumor antigen isolated from tumor cells has been identified as the targets for immune system, except for some from the melanoma. Early animal experiments indicated that tumor cell antigens should be presented as a certain form to induce the protective immune response. Modification of tumor cells by different methods as tumor vaccines applies to all kinds of tumor types under the condition of unidentified tumor antigen. This method is simple, cost-effective and useful in clinic practice<sup>[1]</sup>. The aim of this study is to investigate the possibility and feasibility of this kind of modified tumor vaccines.

## MATERIALS AND METHODS

### *Preparation of tumor cell vaccine (TCV)*

Ehrlich tumor cells were obtained from the Animal Center of the Fourth Military Medical University, which passed on by inoculating up to  $2 \times 10^6$  cells in 0.1 ml suspension intraperitoneally into naive mice. Tumor cells were collected from ascites and incubated with mitomycin C (MMC) at a concentration of 20 pg/cell at 37°C for one hour, or with glutaraldehyde (Glu) at concentrations of 0.12 mL/L-0.25 mL/L on ice for 30 minutes, or with 40 mg/L Para (the final concentration was 20 mg/L) on ice for one hour, then followed by washing cells for 3 times by centrifugation at  $800 \times g$  with normal saline (NS). The modified tumor cell sample is termed as MMC-Tu, Glu-Tu and Para-Tu

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\*Supported by the Outstanding Youth Fund from National Natural Sciences Foundation of China, No.39525020.

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Received 1998-05-20

vaccines, respectively. MMC-Tu/Glu-Tu/Para-Tu cell vaccines were mixed with 1g/L Concanavalin A (ConA, the final concentration was 200 mg/L) on ice for one hour, washed for 3 times, and then referred to ConA-MMC Tu, ConA-Glu Tu and ConA-Para Tu vaccines, respectively. For preparation of soluble TCv, the whole TCv cells were suspended in antigen extract buffer (0.01 mol/L Tris-HCl containing 0.5 mol/L NaCl, 1 mmol/L EDTA, 2.5 mL/L NP-40). After being ultrasonicated with 20Hz at 4°C, for 10min × 10 times, cells were centrifuged at 12000×g for 60min at 4°C. The supernatant was passed through a 0.22μm filter. The soluble antigens amounting to 10<sup>7</sup> cells/mouse were emulsified with Freud's adjuvant of the same volume.

### Immunization protocols

Female Kunming mice aged 8 weeks were provided by the Animal Center of the Fourth Military Medical University, divided into MMC-Tu, ConA-MMC-Tu, Glu-Tu, ConA-Glu-Tu, Para-Tu and ConA-Para groups (for soluble TCv only) randomly. Each mouse was inoculated intraperitoneally with 10<sup>7</sup> of whole TCv cells in 0.2mL of suspension at day 1, 12 and 19. The controls were given 0.2mL of NS. Two weeks after the last inoculation, each mouse was challenged intraperitoneally by injection of 10<sup>7</sup> (lethal dose) of live tumor cells. The survivals of free tumor one month after the 1st challenge were repeatedly inoculated with 1.44×10<sup>7</sup> of live tumor cells. The mice were observed daily within 2 months. The soluble TCv were inoculated intraperitoneally into mice at day 1, 21 and 28. After 2 weeks of the last vaccination, all the mice were challenged with 10<sup>7</sup> of live tumor cells. The mice were observed daily within one and a half months. Therapeutic response was evaluated by promotion of incidence of survivals and by prolongation of life span of each mouse. The survival days were expressed as  $\bar{x} \pm s$ .

### Statistics

The results were analyzed for significance using the ANOVA in multiple experiments for prolongation of life span and Fisher exact test with SPLM software for survival incidence.

### Antibody detection

The tumor cell smears were prepared and fixed in acetone, followed by treating in methanol containing 0.3 mL/L H<sub>2</sub>O<sub>2</sub> at room temperature for 30min. After washing with PBS for 3 times, the cells were blocked with 100 mL/L FCS at 37°C for 30min, then incubated with the serum collected from the mice immunized with the whole ConA-MMC-Tu TCv as primary antibodies overnight at

4°C, washed 3 times with PBS and covered with a biotin-conjugated secondary antibody for 30min at 37°C. For the staining, an avidin-biotin-complex HRP was used (vector). For the controls, normal mouse sera were used instead of the primary antibodies.

## RESULTS

### Response to the whole TCv after the first challenge

There was no evidence of tumorigenesis such as ascites or palpable tumor node subcutaneously when the modified TCv was injected into the peritoneal cavity of mice. By the end of day 30, 8/10 ConA-MMC mice, 7/10 MMC mice, 6/10 Glu mice, 3/9 ConA Glu mice and 4/10 Para mice survived from the first tumor challenge, whereas none of the controls did. The survival of the mice in ConA-MMC, MMC groups ( $P < 0.01$ ) and Glu group ( $P < 0.05$ ) has been prolonged significantly as compared with the controls (Table 1). At the same time, there was significant difference in the survival days of the mice in ConA-MMC group (28 d ± 3 d), MMC group (27 d ± 5 d) (both  $P < 0.01$ ), ConA-Glu group (23 d ± 7 d), Glu group (25 d ± 8 d) and Para group (24 d ± 7 d) (all  $P < 0.05$ ) as compared with 17 d ± 3 d of the controls ( $F = 4.55$ ,  $P = 0.00164$ , Figure 1). Among all the treated mice, the ConA-MMC group appeared to have longer survival time than ConA-Glu group ( $t = 2.49$ ,  $P = 0.02$ ). Antibodies against the cellular membrane of Ehrlich ascites tumor cells were positive in the sera of the mice immunized with the whole TCv of ConA-MMC for three times about 35 days (Figure 4).

**Table 1** Survival rate of whole TCv treated mice after the first challenge

Group	Survival/total	$\chi^2$	$P$ value
ConA-MMC	8/10	9.37	0.00 <sup>b</sup>
MMC	7/10	7.19	0.00 <sup>b</sup>
ConA-Glu	3/9	1.60	0.21
Glu	6/10	5.36	0.01 <sup>a</sup>
Para	4/10	2.47	0.09
Control	0/9		

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , survival vs total Fisher exact value.

### Response to soluble TCv after the first challenge

The survival days and rates of the mice in groups of MMC, Glu ( $P < 0.05$ ), ConA-Para and Para ( $P < 0.01$ ) were obviously different from that of the control (Table 2,  $F = 3.23$ ,  $P = 0.00653$ ,

Figure 2). Among them, ConA-Glu, ConA-Para (both  $P < 0.05$ ) and Para ( $P < 0.01$ ) treatment conferred the immunity to prolong the life span of the immunized mice, particularly the Para TCV treated mice (longer than those treated with ConA-MMC, MMC and ConA-Glu, all  $P < 0.05$ ).

**Table 2** Survival rate of soluble TCV treated mice after the first challenge

Group	Survival/total	P value
ConA-MMC	6/18	0.23
MMC	8/16	0.03 <sup>a</sup>
ConA-Glu	6/14	0.09
Glu	5/9	0.03 <sup>a</sup>
ConA-Para	5/7	0.00 <sup>b</sup>
Para	12/13	0.00 <sup>b</sup>
Control	2/17	

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , survival vs total Fisher exact value.

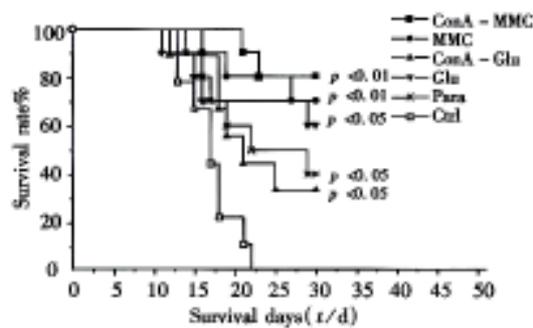
**Response to whole TCV after the second challenge**

The survivors of the whole TCV immunized individuals after the first challenge free of tumor signs (i.e., increase of ascites fluid and/or delayed palpable S.C. tumor growth) received the second challenge with live tumor cells. ConA-MMC mice 7/8, MMC mice 6/7, Glu mice 4/6, ConA-Glumice 3/3 and Para mice 1/4 survived as long as 12 weeks, whereas all the controls died within 1 month (Table 3). The survival rate of all the treated groups, except Para, was significantly different from the control ( $P < 0.01$ ). All the vaccines prolonged the survival periods except the control (Para group  $P < 0.05$ , other groups  $P < 0.01$ , Figure 3). The survival periods of the mice in groups of ConA-MMC, MMC, ConA-Glu, Glu and Para were  $77 \text{ d} \pm 20 \text{ d}$ ,  $75 \text{ d} \pm 25 \text{ d}$ ,  $84 \text{ d} \pm 0 \text{ d}$ ,  $62 \text{ d} \pm 34 \text{ d}$  and  $41 \text{ d} \pm 33 \text{ d}$ , which were significantly different from  $18 \text{ d} \pm 3 \text{ d}$  of the controls ( $F = 9.83$ ,  $P = 9.2 \pm 10^{-6}$ ). ConA-MMC group showed a stronger response than Para group ( $t = 2.41$ ,  $P = 0.04$ ).

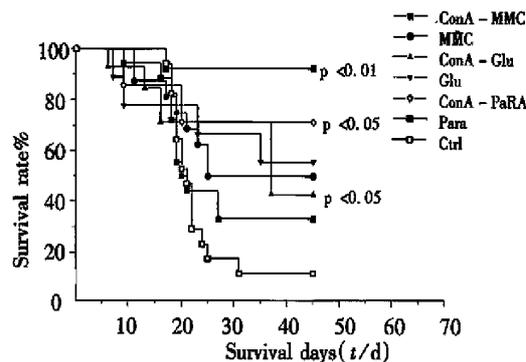
**Table 3** The survival rate of whole TCV treated mice after the second challenge

Group	Survival/total	$\chi^2$	P value
ConA-MMC	7/8	10.87	0.00 <sup>b</sup>
MMC	6/7	9.76	0.00 <sup>b</sup>
ConA-Glu	3/3	7.98	0.00 <sup>b</sup>
Glu	4/6	5.69	0.01 <sup>b</sup>
Para	1/4	0.24	0.29
Control	0/10		

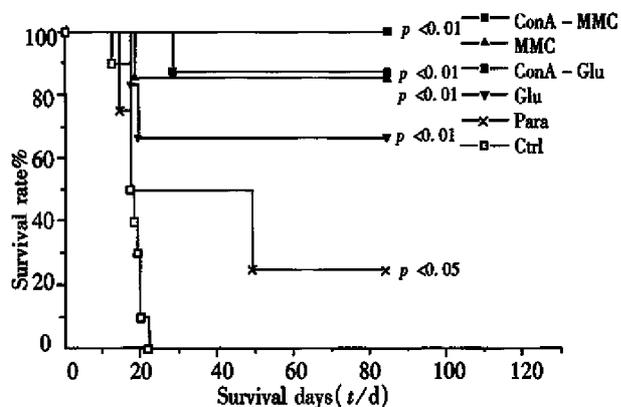
<sup>b</sup> $P < 0.01$ , survival vs total Fisher exact value.



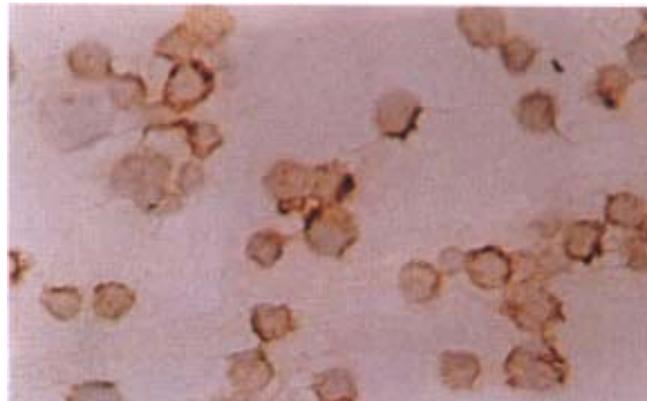
**Figure 1** Survival days of whole tumor cell vaccine-immunized mice after the first tumor challenge



**Figure 2** Survival days of soluble tumor cell vaccine-immunized mice after the first tumor challenge



**Figure 3** Survival days of whole tumor cell vaccine-immunized mice after the second tumor challenge



**Figure 4** Ehrlich ascites tumor cells were stained with the sera of the mice immunized with whole TCV of ConA-MMC.  $\times 100$

## DISCUSSION

### *The immunity to the whole TCV*

The results have provided important evidence that chemicals have exerted some influences on tumor cells and manifested in the following aspects. All the treatment could make tumor cells lose their tumorigenesis. No evidence of ascites was observed in mice inoculated with the treated tumor cells. Combination analysis of the results after 2 times of challenge revealed that ConA-MMC, MMC or Glu treatment has a consistent tendency of prolonging the life span of immunized mice and increasing the incidence of survival. And administration of such vaccines could protect for as long as 12 weeks, in which memory cells may be involved. Although mice with ConA-Glu treatment for twice both had longer span than the controls, the survival rate was improved only after the second challenge ( $P = 0.00035$ ). Para treatment could prolong the survival period but not increase the rate after two times of tumor challenge. The whole TCV, therefore, was able to promote the immunized mice with a higher threshold of resistance to lethal dose of live tumor cells.

Previous studies indicated that both ConA-MMC and ConA-Glu treated L1210 leukemia cells could resist the challenge of tumor cells, and ConA-MMC-Tu vaccine was more potent immunoprophylactics in inducing immune resistance of mice than ConA-Glu vaccine<sup>[2]</sup>. Our study showed that both types of vaccines with Ehrlich ascites tumor could prolong the life span of survivors, but as for short-term immune response, ConA-MMC had longer survival days than ConA-Glu ( $P < 0.05$ ), and as for long-term one, there was no difference between the two, possibly because of the different tumor models.

We found that the immunized mice contained antibody against cellular membrane of the tumor cells, but whether it is a specific antibody against a tumor antigen, H-2 allele or a common cellular component remains to be elucidated. Intravenous injection of paraformaldehyde-fixed autologous cells infected *in vitro* with recombinant vaccinia virus expressing HIV-1 protein gave high levels of neutralization antibody, cell-mediated cytotoxicity and DTH response<sup>[3]</sup>. Recently Shroyer *et al.*<sup>[4,5]</sup> reported that the immunized mice could acquire cytotoxic antibodies when injected with paraformaldehyde or glutaraldehyde treated tumor vaccines. The antibodies not only inhibited the proliferation of the tumor cells, but also eradicated the tumor cells and exerted the protective immune response by the mechanisms of ADCC and CDC. Similar results were obtained with Lewis lung

carcinoma<sup>[6]</sup>. Further studies to determine whether the antibodies are cytotoxic are being carried out.

### *The immunity to soluble TCV*

The soluble TCV showed different results from those of the whole TCV. Although ConA-Para treated whole cells had a marginal vaccine effect, the soluble fragments showed the most obvious one. On the contrary, the soluble ConA-MMC TCV did not show the expected vaccine effect as the whole one did. Whether the procedure of sonication released some “new” immunogenic determinants that existed originally in the cytoplasm or nucleus, or whether it destroyed some antigens remains to be investigated in detail.

### *The possible mechanisms of TCV*

Conventional wisdom holds that the MMC could bind the DNA of tumor cell and totally block DNA synthesis especially targeted G1 and S phases, which resulted in losing the tumorigenesis and exposing “new” TAA determinants. Formalin treatment not only stopped cell division, but also kept the integrity and preserved the antigenicity of the tumor cells. Though less liable in solubilization procedures, formalin-stabilized surface antigens could be solubilized through sonication. Other studies assumed that the increased efficacy of formalin was due to exposed aldehyde groups, resistance of aldehyde cross-links to hydrolysis at low pH, and the nature of cross-linking of antigen monomers<sup>[4]</sup>. The treatment of further modification by ConA could enhance the interaction of tumor cells and immune cells<sup>[7]</sup>.

### *Advantage and disadvantage of mice chosen as the model*

Kunming mice were developed in China and obtained the acknowledgement of the world. But till now their genetic background was not known. There might exist slight allelic difference between individuals. Actually such treated tumor cells as vaccines is a kind of incomplete allogeneic tumor cell vaccines when injected into the individuals who had haplotype difference between the recipients and donors. Allogeneic tumor cell vaccines, either sharing at least one MHC class I restricting element (incompletely) or not matching for any MHC molecule (completely), can induce syngeneic protective T cell-mediated antitumor response, instead of deducing the responses<sup>[8]</sup>. The phenomenon is explained by the theory of cross priming.

In our study, the immune response to the vaccines may represent a combination of the

immunity to the tumor antigens as well as the allogeneic antigens. In fact, the present study showed that there existed the discrepancy of individual reaction to the same tumor vaccines. On the basis of the common Ehrlich ascites tumor cells vaccine, the greater the difference is, the stronger the immunity is<sup>[9]</sup>.

#### *Tumor model*

Ehrlich ascites tumor cell belongs to undifferentiated type. It was established in 1932 by injection of saline containing spontaneous breast adenocarcinoma into peritoneum. The characteristics of this tumor cell line are low immunity, being transplantable and spontaneous origin, similar to that of the non-virus originated human tumor<sup>[10]</sup>.

In conclusion, tumor cells treated with chemicals or antitumor drugs can be used as tumor vaccines as indicated by production of many cured animals and prolongation of life span of immunized

mice. However the cellular or humoral mechanism awaits further studies.

#### REFERENCES

- 1 Mallmann P. Tumor vaccination. *Hybridoma*, 1995;14(2):187-190
- 2 Kataoka T, Ogihara K, Sakurai Y. Immunoprophylactic and immunotherapeutic response by concanavalin A-bound tumor vaccine enhanced by chemotherapeutic agents eliminating possible suppressors. *Cancer Res*, 1980;40(10):3839-3845
- 3 Kataoka T, Oh-hashi F, Tsukagoshi S, Sakurai Y. Induction of resistance to L1210 leukemia in BALB/c×DBA/2Cr F1 mice, with L1210 cells treated with glutaraldehyde and concanavalin A. *Cancer Res*, 1977;37(4):964-968
- 4 Shraye D, Kouttab N, Maizel A, Wanebo H, Hearing VJ, Gersten DM. Generation of cytotoxic antibodies to the B16 murine melanoma using a formalinized vaccine. *Int J Cancer*, 1993;53(4):696-702
- 5 Shraye D, Bogaars H, Hearing VJ. Further characterization of a clinically relevant model of melanoma metastasis and an effective vaccine. *Cancer Immunol Immunother*, 1995;40(5):277-282
- 6 Veltri RW, McKolanis JR, Rookoff SD, McIntire KR. Protein A antibody-binding (TPAAB) technique for detection of tumor-associated membrane antigen (TAMA) of Lewis lung carcinoma. *Int J Cancer*, 1980;25(1):141-145
- 7 Toes REM, Blom RJJ, van der Voort E, Offringa R, Melief CJM, Kast WM. Protective antitumor immunity induced by immunization with completely allogeneic tumor cells. *Cancer Res*, 1996;56(16):3782-3787
- 8 Bevan MJ. Antigen presentation to cytotoxic T lymphocytes *in vivo*. *J Exp Med*, 1995;182(2):639-641
- 9 Zagury D, Bernard J, Cheynier R, Desportes I, Leonard A. A group specific anamnestic immune reaction against HIV-1 induced by a candidate vaccine against AIDS. *Nature*, 1988;332(6166):728-731
- 10 Shi XY. Animal medical experimental methods (in Chinese). Beijing: People's Medical Publishing House, 1980:227-232

# Liver metastasis models of human colorectal carcinoma established in nude mice by orthotopic transplantation and their biologic characteristic \*

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**Subject headings** colorectal neoplasms; liver neoplasms/secondary; neoplasms transplantation; neoplasms metastasis; disease models, animal

## Abstract

**AIM** To establish a liver metastasis model of human colorectal carcinoma in nude mice.

**METHODS** Orthotopic transplantation of histologically intact colorectal tissues from patients into colorectal mucosa of nude mice. Tumorigenicity, invasion, metastasis and morphological characteristics of the transplanted tumors were studied by light microscopy, electron microscopy and immunohistochemistry.

**RESULTS** Liver metastasis models of human colon carcinoma (HCA-HMN-1) and human rectal carcinoma (HRA-HMN-2) were established after seeding from 34 colorectal carcinomas. They had been passaged *in vivo* for 18 and 21 generations respectively. There were lymphatic, hemotogenous and implanting metastases. CEA secretion was maintained after transplantation. The primary and liver metastatic tumors were similar to the original human carcinoma in histopathological and ultrastructural features, DNA content and chromosomal karyotype.

**CONCLUSION** The liver metastasis models provide useful tools for the study of mechanism of metastasis and its treatment of human colorectal cancer.

## INTRUDUCTION

Some models of nude mice that fresh human colorectal carcinoma tissue or cells were successfully transplanted subcutaneously have been reported at home and abroad<sup>[1,2]</sup>. But until now there has been no report on a liver metastasis model of human colorectal carcinoma established by orthotopic transplantation in nude mice in China. Based on our previous models of human liver and pancreas carcinoma by orthotopic transplantation<sup>[3,4]</sup>, we established liver metastasis models of colon and rectum carcinoma with a spontaneous metastasis rate of 100%.

## MATERIALS AND METHODS

### Animals

BALB/C-nu/nu nude mice were provided by Institute of Oncology, Chinese Academy of Medical Sciences. aged 3-5 weeks, weighing 17 g - 20 g, fed under the SPF (specific-pathogen-free) environment.

### Specimen

Specimens were obtained from resections of patients with colorectal carcinoma (16 cases of poorly differentiated infiltrating colon adenocarcinoma and 14 cases of poorly differentiated infiltrating rectum adenocarcinoma). The tissues were cut by aseptic manipulation and put into culture liquid RPMI 1640 and cut into pieces of 1 mm×1 mm×1mm for transplantation.

### Orthotopic transplantation

Nude mice were anaesthetized with pentobarbital injection via peritoneal cavity and through the middle incision the colon or rectum were identified. Two tissue masses were sewn into the submucosa from outside the lumen with 0/10 no-injury thread. The growth of the tumor was observed every day. One nude mice who was about to die was killed by cervical vertebra dislocation and the tumor tissues were taken out. A part of tumor tissue was transplanted in situ to other nude mice (5-16 nude mice once) and passaged by the method of primary-generation transplantation continuously the remaining part was frozen in liquid nitrogen. Other animals were reserved to observe the tumor metastasis.

### Detection and measurement

**Autopsy and histology** All the nude mice were

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\*Project supported by the Military Science Foundation, No.97-0056.

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Received 1998-06-06

examined carefully, the volume of the tumor in situ, and the liver or the other organs and all the abdominal lymph nodes were detected. Specimens were fixed in 10% formalin and hemotoxylineosin stained. Some sections underwent CEA histochemistry study (ABC procedure). The whole liver was insected into 10-20 pieces of slices at different levels to observe the liver metastasis focus.

Tumor in situ and liver metastatic carcinoma were double fixed in 2.5% glutaraldehyde and 1% osmium acid and embraced in Epon-812. After ultramicrotomy, the section was uranium-lead double stained and observed under the TEM (Philips-CM10).

CEA and CA-50 in serum and tumor tissues in situ were measured by radio-immunologic method.

Human tumors, tumor in situ, liver metastatic carcinoma of the 1st, 5th, 10th and 15th generation were analyzed according to the method of reference 5.

Tumor in situ and metastatic liver carcinoma were studied following the methods of Deaven's and Peterson's.

## RESULTS

After 34 specimens of fresh human colo-rectal carcinoma were orthotopic transplanted to the nude mice, 21 cases were successful one strain of liver metastatic carcinoma model from colon carcinoma was screened, which is named HCA-HMN-1 and another strain of liver metastatic carcinoma model from rectum carcinoma is named HRA-HMN-2.

### *Establishment of HCA-HMN-1 and HRA-HMN-2 and their continuative passage*

HCA-HMN-1 was derived from a 37-year-old male, patient with poorly differentiated infiltrating adenocarcinoma accompanied with the metastasis of lymph nodes. This tumor tissue was transplanted orthotopically to 5 nude mice and had passed the 18th generation. The mean latent period was 10 days. A total of 102 nude mice received the grafting and 30 days from a generation. Both passing survival rate and resuscitation rate were 100% (17/17). Necropsy found that transplanted tumor grew locally infiltrating in large scale. Liver metastatic tumor, abdominal lymph node metastasis and peritonealcavity metastasis could be seen. Pathological evidence suggested that 102 nude mice had liver and lymph node metastasis and 27 had metastatic lung carcinoma.

HRA-HMN-2 was derived from the tumor of a 41-year-old female, patient with poorly differentiated infiltrating adenocarcinoma complicated with the lymph node and liver metastasis. The primary tumor tissue and metastatic liver tumor were transplanted to 8 nude mice successfully and have passed the 21st generation. The mean latent period was 7 days, and 20 days for one generation. A total of 126 nude mice received

the grafting. Both passing survival rate and resuscitation rate were 100% (17/17). Necropsy found that both the primary tumor in situ and liver metastatic tumor by orthotopic transplantation grew intensively in mucosa of the rectum in situ accompanying the metastasis of liver and local lymph nodes. The nude mice transplanted with liver metastatic tumor had bloody ascites and extensive tumor seeding in peritoneum. Pathological evidence suggested that the 126 nude mice all had liver and lymph node metastasis, and 31 had lung metastatic and 23 ovarian metastasis.

### *Infiltration and metastasis of HCA-HMN-1 and HRA-HMN-2*

Liver metastasis appeared at the end of 3 weeks after transplantation. The most of metastatic foci were located in the right lobe of the liver, which were of mononoeud type. A few had polynoeud in both right and left lobes. The diameter of tumor ranged from 0.4mm to 2.5cm. Some liver tissues of nude mice could be replaced by metastatic tumor (Figure 1). Pathohistological evidence documented that liver metastatic carcinoma was poorly differentiated colorectal adenocarcinoma.



Figure 1 HRA-HMN-2 the 19th generation liver metastasis carcinoma.

Lymph node metastasis was limited in the abdominal cavity. Metastasis of lymph nodes of colon, rectum, inguinal, mesentery and a few hilar hepatis could be seen, which can be divided into three stages: in early stage, carcinoma cell mass only appeared in afferent lymphatic and marginal sinus; in middle stage, cancer cell progressively invaded the paracortical zone and medulla; and in late stage, the whole lymph node was nearly occupied by the cancer cells except for the residual margins.

### *Histopathology and TEM*

Under light microscopy, the distribution of HCA-HMN-1 cancer cells was characterized by blocks and mass with small adenoid structure which had plenty of plasma, irregular giant nuclear, obvious nucleoli and polynuclear tumor giant cells. The HRA-HMN-

2 cells appeared circle and ellipsoid in shape with, abundant plasma, heavily stained giant nuclear, obvious nucleoli, rough and large karyotin granulae, and tumor giant and pomynuclear tumor cells appeared frequently. TEM showed that HCA-HMN-1 cancer cells had irregular shape, heteromorphic giant nucleus and polynucleoli. The heterochromatin was distributed around the nucleus. In cytoplasm there were mitochondria and endoplasmic reticulum, occasionally microvilli. Intracellular junction was complex or desmosome. Liver metastatic tumors showed the typical ultrastructure of the poorly differentiated colon adenocarcinoma. HRA-HMN-2 cells were circled as adenoid lumen in which the surface had a little microvilli in which the direction, length and diameter were highly different. Permutation of peripheral cancer cells was irregular, with large nucleus, obvious karyotin and nucleoli, and wrapped nuclear membrane. In cytoplasm, there was a moderate quantity of rough endoplasmic reticulum and a small amount of edema mitochondria. The maldevelopment desmosome-like intracellular junction was seen in a few cells. Liver metastatic tumor presented the ultrastructural features of poorly differentiated rectal adenocarcinoma.

#### **The quantities of CEA and CA-50**

The mean value of the CEA in tumor tissues was 79.8 mg/L, and CA-50 was 57.6U/mL; and in peripheral blood, 24.3mg/L and 33.6U/mL respectively.

#### **CEA immunohistochemical study**

Whole-plasma-positive cells could be seen in the transplanted tumor cells and liver metastatic carcinoma cells of nude mice.

#### **Flow cytometry**

The DNA ploidy of the original human tumor specimen, implanted tumor of nude mice and liver metastatic tumor tissue were all nearly tetraploid.

#### **Chromosomal detection**

Transplanted tumor and liver metastatic carcinoma of nude mice had the similar karyotype to human tumor cells. The number of chromosome ranged from 77 to 86, which demoustrcted as supertriploid or subtetraploid.

### **DISCUSSION**

Liver is the commonest metastatic locus of colorectal carcinoma<sup>[6]</sup>. The incidence of liver metastasis might be 20% -40% when the diagnosis of colo-rectal carcinoma was documented and 40%-50% after radical resection of colo-rectal carcinoma. About 67% of the patients who died of metastasis had liver metastasis carcinoma<sup>[7]</sup>. The deep invasion and metastasis did not occur in the subcutaneous tumor models by implanting fresh human colorectal

carcinoma tissue or cells into nude mice<sup>[1,2]</sup>. The liver metastatic tumor produced by endosplenic grafting of colon carcinoma cell line was experimental but not spontaneous<sup>[8]</sup>, and was not ideal model to be used for studying the metastatic liver carcinoma.

To study the mechanism and therapy of liver metastasis, a model of ortotopic transplantation, which was similar to human being and non-cell-line, should be established. In this study two strains of models, called HCA-HMN-1 and HRA-HMN-2 were established, which perfectly demonstrated the clinical metastatic process of human colo-rectal carcinoma not only in the aspect of the invasive growth and tumor formation in the colo-rectum of nude mice but also in keeping 100% of the liver and lymph node metastatic rate. So our models subjectly simulated the clinical features of patients with liver metastasis.

The results showed that in colon carcinoma the two models, after they continuously passaged for two years, the feature of chromosome and histological ultrastructure were totally the same as that of the original tumors which can secrete the CEA and CA-50. That liquid nitrogen frozen tissue of transplanted tumor can still be reimplanted successfully suggested that the models had stable biologic features.

There were two causes of liver metastasis. Tumor cells itself had strong potential ability of invasion. This study successfully used two specimens of Duck's D stage of colorectal carcinoma; The microcirculation (the colo rectum has special structures, and plenty of lymph and blood supply. It is the colo-rectal microcirculation of nude mice which was so similar to the that of human being that the transplanted tumor of nude mice has the similar clinical process of liver metastasis from human colo-rectal carcinoma. We consider that only the orthotopic transplantation method of nude mice can produce metastasis similar to patients with higher spontaneous metastasis rate than that of other models.

Our results show that HCA-HMN-1 and HRA-HMN-2, have obtained the biologic features in aspects of morphology, biochemistry, immunology and genetics, and are ideal models to study the mechanism of human colo-rectal carcinoma metastasis and anti-metastasis therapy.

### **REFERENCES**

- 1 Rygadr J, Povlsen Co. Heterotransplantation of a human malignant tumour to nude mice. *Acta Pathol Microbiol Scand (A)*, 1969;77:758-760
- 2 Wu BQ, Sun YK, Zhen J. The study on the transplanted tumor of human mucus adenocarcinoma in nude mice and its biologic characteristics. *J B Med U*, 1982; 14:205-208
- 3 Tuo CW, Liu QZ, Wu BQ. The models of human liver cancer transplanted orthotopically into nude mice and its biologic characteristics. *Chin J Experim Surg*, 1992;9:28-29
- 4 Liu QZ, Tuo CW, Wu BQ. The models of human pancreas cancer transplanted orthotopically into nude mice and its biologic characteristics. *Chin J Oncol*, 1992;14:403-406
- 5 Vindeolow LI, Christensen JJ, Nissen NI. A detergent trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry*, 1983;3:323-327
- 6 Nordlinger B, Panis Y, Puts JP. Experimental model of colon cancer: recurrences after surgery alone or associated with intraperitoneal 5-fluorouracil chemotherapy. *Dis Colon Rectum*, 1991;34:658-663
- 7 Cady B, Stone MD. The role of surgical resection of liver metastases in colorectal carcinoma. *Seminars Oncol*, 1991;18:399-406
- 8 Morikawa K, Walker SM, Jessup JM. In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinoma implanted into nude mice. *Cancer Res*, 1988;48:1943-1948

# Relationship between expression of CD44v6 and nm23-H1 and tumor invasion and metastasis in hepatocellular carcinoma \*

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**Subject headings** liver neoplasms; carcinoma, hepatocellular; neoplasm invasion; neoplasm metastasis; CD44v6; nm23-H1; RNA, messenger

## Abstract

**AIM** To detect the expression of CD44v6 mRNA and nm23-H1 mRNA in hepatocellular carcinoma (HCC) by *in situ* hybridization, and to evaluate the relationship between their expression and also relationship between their expressions and tumor invasion and metastasis.

**METHODS** CD44v6 cDNA probe was synthesized with PCR technique and the nm23-H1 cRNA probe by *in vitro* transcription. The expression of CD44v6 mRNA and nm23-H1 mRNA was detected by *in situ* hybridization.

**RESULTS** In group with high invasion and metastasis potential, the positive rates of CD44v6 mRNA and nm23-H1 mRNA were 80% (8/10) and 40% (4/10), in group with poor invasion and metastasis potential, they were 21.7% (5/23) and 91.3% (21/23). There was a positive correlation between the expression of CD44v6 mRNA and tumor invasion and metastasis potential in HCC ( $P < 0.01$ ), and a reverse correlation between the expression of nm23-H1 mRNA and tumor invasion and metastasis potential ( $P < 0.01$ ) and a reverse correlation in the expression between CD44v6 mRNA and nm23-H1 mRNA in HCC ( $P < 0.01$ ).

**CONCLUSION** Detection of CD44v6 mRNA and nm23-H1 mRNA may be useful for tumor invasion and metastasis in HCC.

## INTRODUCTION

CD44 is a cell surface transmembrane glycoprotein. As a kind of adhesive molecule, it participates in cell-cell and cell-matrix adhesion and interactions. Many studies revealed a correlation between high-level expression of CD44, especially CD44v and tumor invasion, metastasis and prognosis. The exon 6v containing isoforms may be an independent diagnostic parameter<sup>[1,2]</sup>. Some other studies, however, had different results<sup>[3,4]</sup>. Some researches showed a reverse correlation between the expression of nm23-H1 mRNA and tumor metastasis<sup>[5,6]</sup>. In order to evaluate the relationship between the expression of CD44v6 mRNA and nm23-H1 mRNA and tumor invasive and metastatic potential in HCC and to evaluate the relationship in the expression between CD44v6 mRNA and nm23-H1 mRNA, we detected their expression in HCC by *in situ* hybridization.

## MATERIAL AND METHODS

### Tissue samples

Thirty-three cases of surgically resected HCC specimens were studied, including 24 males and 9 females, aged from 28 - 61 years (average 45.2 years). According to the clinicopathological data, the specimens were divided into two groups: high invasion and metastasis potential group (high-risk group) and poor invasion and metastasis potential group (low-risk group). The specimens meeting with the following standards were assigned to the high-risk group: ① presence of metastasis in hepatic portal lymph nodes; ② tumor embolus in the portal vein; and tumor satellite nodules near the main mass or tumor capsule deficiency or capsule infiltrated or even penetrated. Ten of 33 cases of HCC tended to belong to the high-risk group, 23 to the low-risk group.

### CD44v6 cDNA probe preparation by PCR

CD44-cDNA-containing plasmid PGEX-2T was obtained from Dr. Ursula Gunthert (Basel Institute for Immunology, Switzerland). CD44v6 primers: 5' TCCAGGCAACTCCTAGTAGT and 5' CAGCTGTCCCTGTTGTCGAA. The conditions of PCR were 94°C for 30 seconds, 60°C for 30 second, 72°C for one minute, repeated for 30 cycles. The probes were labeled with Bio-11-dUTP (Boehringer

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\*Project supported by the National Natural Science Foundation of China, No.39370294

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Received 1998-07-06

Mannheim).

#### ***nm23-H1 cRNA probe preparation by in vitro transcription***

PGEM-4Z constructed with the nm23-H1 cDNA insert was linearized with Hind III and transcribed *in vitro* from the SP6 polymerase promoter to yield the antisense RNA probe and from the T7 polymerase promoter to yield the sense RNA probe (control) with Dig-RNA labeling Kit (Boehringer Mannheim).

#### ***In situ hybridization***

Frozen sections were cut at 8  $\mu$ m, washed in 0.1 mol/L PBS, glycine/ PBS and Triton X-100/ PBS, and were then treated with 0.1% proteinase K, and fixed in 4% paraformaldehyde, immersed in freshly prepared 0.25% acetic anhydride, and in 0.1 mol/L triethanolamine and washed with 2  $\times$  SSC. Each slide was covered with 20  $\mu$ l - 40  $\mu$ l hybridization solution containing CD44v6 cDNA probe (denatured before hybridization) or nm23-H1 cRNA probe (digested with proteinase K after hybridization). Hybridization was performed at 50°C - 55°C for 12-16hrs in a moisture chamber, washed with 4  $\times$  SSC, 2  $\times$  SSC and 1  $\times$  SSC, incubated with avidin-AP or anti-Dig-Fab-AP, and stained with freshly prepared NBT-BCIP.

#### ***Control experiment***

The sections were pretreated with RNase A (0.05 g/L) for 30 minutes at 37°C. The procedures were the same as above but without probe in hybridizationsolution.

#### ***Statistical analysis***

The significance of differences was tested by  $\chi^2$  test.

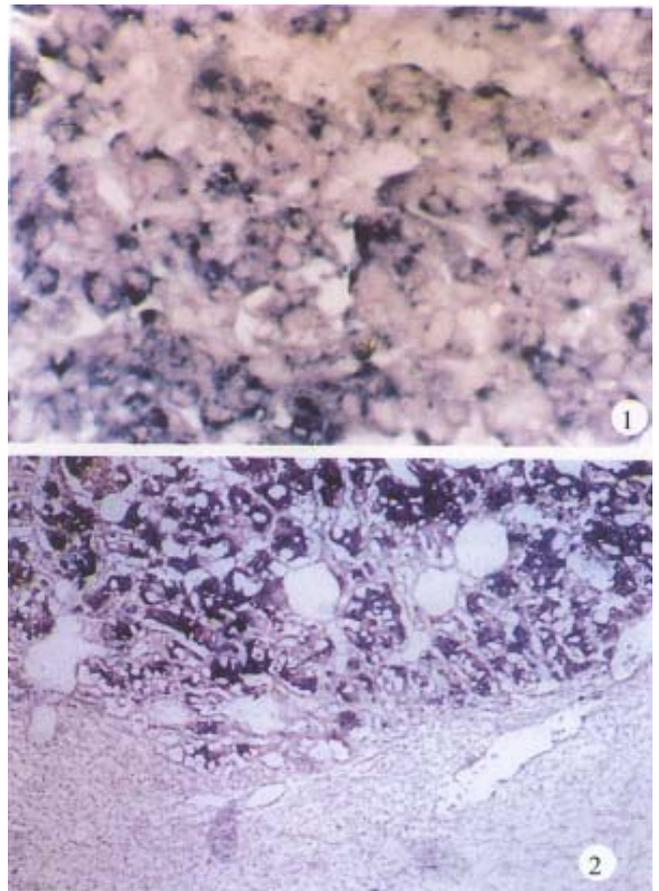
## **RESULTS**

#### ***In situ hybridization results of CD44v6 mRNA***

Positive granules for hybridization signal were mainly distributed in cytoplasm of HCC cells (Figure 1). Eight of 10 cases in high-risk group and 5 of 23 cases in low-risk group were positive. And difference between the two groups was significant ( $P < 0.01$ ). There was a positive correlation between the expressions of CD44v6 mRNA and tumor invasive and metastatic potential.

#### ***In situ hybridization results of nm23-H1 mRNA***

Positive granules for hybridization signal were mainly distributed in cytoplasm of HCC cells (Figure 2), 4 of 10 cases in high-risk group and 21 of 23 cases in low-risk group were positive, the difference being significant ( $P < 0.01$ ). There was a reverse correlation between the expressions of nm23-H1 mRNA and tumor invasive and metastatic potential.



**Figure 1** CD44v6 mRNA positive in cytoplasm of HCC cells. ISH $\times$ 200

**Figure 2** nm23-H1 mRNA positive in cytoplasm of HCC cells. ISH $\times$ 200

#### ***Relationship between the expressions of CD44v6 mRNA and nm23-H1 mRNA***

In situ hybridization showed that nm23-H1 mRNA was present in 20 cases, in which CD44v6 mRNA expression was all negative, and nm23-H1 mRNA was negative in 8 cases and positive in 5 cases of 13 cases with CD44v6 positive expression. There was a reverse correlation between the expressions of CD44v6 mRNA and nm23-H1 mRNA ( $P < 0.01$ ).

## **DISCUSSION**

CD44 is a cell surface transmembrane glycoprotein. As a kind of adhesion molecule, it mainly participates in cell-cell and cell-matrix interactions. In human CD44 gene is located at chromosome 11p13. Many studies showed that high-level expression of CD44 especially CD44v was correlated with tumor invasion and metastasis, the exon 6v containing isoforms may be an independent diagnostic parameter<sup>[1,2]</sup>. Some studies have demonstrated that CD44 expression was positively correlated with the metastasis of hepatocellular carcinoma<sup>[7,8]</sup>. The following methods were mainly

adopted: detection of the CD44 proteins by immunohistochemistry or detection of CD44 mRNA by RT-PCR. CD44 mRNA was detected by *in situ* hybridization only in colorectal carcinomas<sup>[9]</sup>. Because the formation of tumor invasion and metastasis was a multi-step process regulated by multi-gene and multi-factors, and the function of CD44 in tumor invasion and metastasis must be regulated by other genes. Some studies showed that the expression of nm23-H1 mRNA was closely related to tumor metastasis, and was regarded as the candidate gene of tumor metastasis inhibitory gene<sup>[5,6]</sup>. Due to the functional difference between CD44 gene and nm23 gene, it is significant to evaluate the relationship between their expressions, so as to understand and explain the mechanism of tumor metastasis. We synthesized CD44v6 cDNA probes by PCR, and detected the expression of CD44v6 mRNA by *in situ* hybridization in 33 cases of HCC. The results showed that CD44v6 mRNA expression was positively related to tumor invasive and metastatic potential. We detected the expression of nm23-H1 mRNA in these specimens, and found that there was a reverse correlation between the expression of nm23-H1 mRNA and CD44v6 mRNA. However, whether such correlation is of certain significance, which factor is a decisive one, and whether other exons of CD44 participate in tumor metastasis, still await farther studies.

As CD44 molecule is highly variable, the molecular structure of CD44 varies in different tissues or cells. But the probes used in current studies were produced with multi-exon continent. This kind of probes showed different hybridization abilities with different CD44 molecules in different

tissues or cells. This might affect the accuracy of hybridization. If the different primers are designed to produce the cDNA probes formed from one of the exons in the probe synthesis by PCR, and if SS-cDNA probe is obtained by non-symmetry PCR, the sensitivity and stability of hybridization can be improved and non-radiation labeling can be completed at the same time. Thus, the complicated techniques and equipment in RNA probe preparation and *in situ* hybridization can be avoided. A plenty of probes can be obtained in a short time with PCR technique and can meet the needs of large numbers of detection.

In conclusion, the detection of CD44v6 mRNA and nm23-H1 mRNA may be useful for diagnosis and prediction of tumor invasion and metastasis in HCC.

## REFERENCES

- 1 Seiter S, Arch R, Reber S, Komitowski D, Hofmann M, Ponta H *et al*. Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med*, 1993;177(2):443-455
- 2 Harn HJ. The variant mRNA isoform of human metastasis gene (CD44) detected in the cell lines of human hepatocellular carcinoma. *Biochem Mol Biol Int*, 1994;32(2):233-238
- 3 Fox SB, Fancett J, Jackson DG, Collins I, Gatter KC, Harris AL *et al*. Nomal human tissues, in addition to some tumors, express multiple different CD44 isoforms. *Cancer Res*, 1994;54(16):4539-4546
- 4 Terpe HJ, Christriansen H, Gonzales M, Berthold F, Lampert F. Differentiation and prognosis of neuroblastoma in correlation to the expression of CD44s. *Eur J Cancer*, 1995;31A(4):549-552
- 5 Lizuka N, Nasaaki O, Nama J *et al*. nm23-H1 and nm23-H2 messenger RNA abundance in HCC. *Cancer Res*, 1995;55:652-655
- 6 Chen XD, Dai YM, Yang JM, Bao JZ, Wang JJ, Cong WM. Liver tumor metastasis inhibitory gene nm23 expression in hepatocellular carcinoma. *Chin J Pathol*, 1996;25(2):76-78
- 7 Joseph M, Julie E, John O, Andrew W, Kieran T and Alastair D. CD44 is expressed in hepatocellular carcinomas showing vascular invasion. *J Pathol*, 1996;179:74-79
- 8 Washington K, Telen MJ, Gottfried MR. Expression of cell adhesion molecule CD44 in primary tumors of the liver: an immunohistochemical study. *Liver*, 1997;173:17-23
- 9 Gorham H, Sugino T, Woodman AC, Tarin D. Cellular distribution of CD44 gene transcripts in colorectal carcinomas and in normal colonic mucosa. *J Clin Pathol*, 1996;49:482-488

# study of portal vein embolization with absolute ethanol injection in cirrhotic rats

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**Subject headings** liver cirrhosis/therapy; embolization, therapeutic; absolute ethanol; portal vein; hemodynamics

## Abstract

**AIM** To investigate the effects of portal vein embolization (PVE) with absolute ethanol injection on the cirrhotic livers.

**METHODS** Absolute ethanol was injected intraportally into normal and cirrhotic SD rats and the changes of the animals in anatomy, pathology, liver function as well as portal hemodynamics were observed.

**RESULTS** At a dose of 0.05mL/ 100g of ethanol, the survival rate was 100% in normal rats compared with 40.9% in cirrhotic rats. PVE in the cirrhotic rats with 0.03mL/100g of ethanol, caused significant hypertrophy in non-embolized lobes, mild or moderate damage to the hepatic parenchyma, slight and transient alterations in liver function, portal pressure and portal flow.

**CONCLUSION** PVE with absolute ethanol injection in the setting of liver cirrhosis could be safe at an appropriate dose, and precautions aimed at preserving liver function were preferable.

## INTRODUCTION

Portal vein embolization (PVE) plays an important role in the management of hepatocellular carcinoma (HCC). We modified the conventional method of transcatheter embolization and developed a new PVE technique with ethanol injection via a fine needle in experimental study<sup>[1]</sup> and subsequent clinical application under guidance of portoechography<sup>[2]</sup>. To further elucidate the therapeutic basis of this technique, particularly its effects on the cirrhotic liver, we observed the alterations in liver anatomy, pathology, biochemistry and portal hemodynamics in cirrhotic rats undergoing PVE with ethanol injection.

## MATERIAL AND METHODS

### *Reproduction of cirrhotic rat model*

Normal Sprague-Dawley (SD) rats with a body weight between 200 g - 250 g obtained from The Laboratory Animal Center of our university were used. Based on our previous method of producing cirrhotic dog model<sup>[3]</sup>, a dose of 0.3 mL/ 100 g of 60% CCl<sub>4</sub> solution was injected subcutaneously at abdominal wall of rats once every 4 days. Throughout the period, the rats were fed with ordinary food and 5% ethanol as drinking water. Pathohistological examination confirmed the development of cirrhosis at 60 days after initial administration of CCl<sub>4</sub> solution.

### *Measurement of weight ratio of rat liver lobes*

Livers of 20 SD normal rats were resected under anaesthesia and weighed, and then the right, middle and left lobes of each liver were weighed individually. The mean weight ratios of right, middle and left lobes to the whole liver were 40.5%, 36.5% and 23.0%, respectively.

### *Test of rat tolerance to ethanol*

Laparotomy was performed in normal rats ( $n = 10$ ) and cirrhotic rats ( $n = 22$ ) under intraperitoneal anaesthesia with penobarbitol. After exposure of the hepatic hilum, portal vein was punctured with a 3-gauge needle and a dose of 0.05 mL/ 100g of absolute ethanol was injected into the vessel. The test showed that all rats in the normal group kept alive and only 9 (40.9%) of 22 in the cirrhotic group were alive 4 days after operation.

## PVE

According to the method of PVE described above

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\*Supported by the National Natural Scientific Foundation of China, No.393706697.

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Received 1998-05-06 Revised 1998-06-23

and the results of tolerant test, a dose of 0.05 mL/100g of absolute ethanol was used to embolize the portal branches of left and middle lobes in normal rats (NE group,  $n = 24$ ), in which the embolized tissue accounted for 77% of the whole liver. In cirrhotic rats (ME group,  $n = 24$ ), the portal branch of middle lobe (accounting for 36.5% of the liver) was embolized with a dose of 0.03mL/100g of absolute ethanol. In addition, the same quantity of normal saline was injected into portal vein in 10 normal rats (NC group) and 10 cirrhotic rats (MC group).

### Investigations after PVE

The following aspects were investigated at day 1, 3, 7 and 14 after PVE, respectively: patency of the portal system by X-ray portography; the weight ratio of hepatic lobes; blood biochemical profile (ALT, TBIL, ALP, ALB, A/G); pathohistological changes of liver tissue microscopically; and portal blood flow (Qpv) and portal pressure (Ppv) measured by MRF-1200 electromagnetic flowmetry (NIKON, Japan).

### RESULTS

The survival rate after PVE was 95.8% in both NE and ME groups. Postoperative X-ray portographies demonstrated filling defects at injection sites and the corresponding portal branches were embolized. Dissection of the intrahepatic portal system revealed that in the sites of filling defects on portographies, there were intraluminal red thrombi, which varied from 10 mm to 20 mm in length. The thrombi were soft in consistency and easily separated from the vascular wall at day 1 and 3 after PVE, became less bright at day 7, and then brightless, fragile and tightly adhered to the wall at day 14.

The embolized hepatic lobes gradually atrophied and the non-embolized lobes became hypertrophical with the time after PVE. The weight ratio of the embolized lobes to the non-embolized lobes at the 14th post-PVE day was significantly higher than the physiological value (Table 1) statistically.

Pathohistological examination revealed focal intimitis with development of thrombi at the site of ethanol injection, degeneration of hepatocytes, infiltration of inflammatory cells and focal coagulation necrosis at the regions surrounding the portal triads of the embolized lobes. With respect to the ratio of total necrosis area to embolized area, ME group (30% - 40%) was more severe than NE group (10% - 20%). One week after PVE, organization or calcification of thrombi with partial recanalization, as well as hyperplasia of fibrotic tissue in the embolized lobes, was noted. In addition, hepatocytes of the non-embolized lobes became hypertrophic and proliferative, which was more remarkable in ME group than in NE group.

Blood biochemical investigations showed that hepatic functional parameters were significantly higher in MC group than in NC group (Table 2). ALT, TBIL and ALP in both NE and ME groups were elevated from their own baseline values in small amplitude after PVE, began to fall at the day 3, and returned to the baseline values at week 1 after injection. In contrast, there was no obvious change of ALB and A/G in both groups after PVE. ME group had significantly higher portal blood flow and portal pressure than NE group before PVE. At the day 1 after PVE portal blood flow and portal pressure declined slightly in both groups, thereafter elevated in a limited degree, and returned to the baseline values at 1 week after PVE.

**Table 1 The weight ratio of embolized lobe to the whole liver**

		Time after PVE (days)				Baseline value( $n = 20$ )
		1( $n=6$ )	3( $n = 6$ )	7( $n = 6$ )	14( $n = 6$ )	
A. nonembolized lobe (g)	NE	3.04±0.29	2.75±0.20	4.04±0.42	5.25±0.38	1.86±0.42
	ME	8.04±0.22	7.71±0.17	8.63±0.24	9.58±1.10	5.13±0.53
B. whole liver (g)	NE	10.62±0.32	8.63±0.28	8.12±0.33	7.63±0.14	8.08±0.51
	ME	12.29±0.31	12.09±0.21	12.13±0.21	11.04±0.72	8.08±0.51
A/B (%)	NE	30.0	31.9	49.8 <sup>a</sup>	68.8 <sup>a</sup>	23.0 <sup>a</sup>
	ME	65.5	63.8	71.7	86.8 <sup>b</sup>	63.5 <sup>b</sup>

<sup>a, b</sup> $P < 0.01$ , as compared with each own baseline value by Student's  $t$  test.

**Table 2 Changes in portal pressure and portal blood flow after PVE**

		Time after PVE (days)				Baseline value( $n = 20$ )
		1( $n = 6$ )	3( $n = 6$ )	7( $n = 6$ )	14( $n = 6$ )	
FPP (kPa)	NE	1.27±0.23	1.82±0.20	1.82±0.32	1.52±0.59	1.67±0.48 <sup>a</sup>
	ME	2.82±0.50	3.67±0.37	3.96±1.65	3.17±0.50	2.97±0.33 <sup>a</sup>
PVI (mL/min)	NE	13.30±2.16	14.50±2.07	14.30±1.75	13.40±2.20	13.50±3.03 <sup>b</sup>
	ME	14.50±4.04	16.80±2.48	18.30±9.05	17.17±7.49	18.20±4.16 <sup>b</sup>

<sup>a, b</sup> $P < 0.01$ , as compared with each own baseline value by Student's  $t$  test.

## DISCUSSION

In comparison with transcatheter embolization, the technique of PVE with absolute ethanol injection via fine needle has the advantages of easy attainment of hyperselective embolization, simple manipulation and avoidance of radiation exposure. Studies of PVE with absolute ethanol injection in normal dogs have been reported<sup>[1,4]</sup>. However, the fact is that 80% of patients with HCC in our country have underlying cirrhosis, therefore it is necessary to research into its effects on cirrhotic liver so as to better orientate its clinical application. The present study demonstrated that the cirrhotic rats became much less tolerant to ethanol than the normal rats, so the dose of ethanol used for PVE should be strictly controlled. On the other hand, the results showed that once PVE is undertaken with a tolerant dose of ethanol, the changes in hepatic histology and anatomy, liver function and portal hemodynamics in cirrhotic rats were no more severe and persistent than those of normal rats, even though these aspects were significantly different between the two groups before PVE. It indicated that a good postoperative course could also be achieved in cirrhotic rats with an appropriate dose of ethanol.

Like other methods of portal vein (PV) blockade, PVE with ethanol injection could effectively lead to proliferation and hypertrophy of non-embolized lobes. It was due to the fact that nourishing factors carried by PV blood were increasingly drained to the non-embolized lobes after PVE, which caused proliferation of endoplasmic reticulum, increase in number of mitochondria and synthesis of ATP, speeding up synthesis of DNA and RNA<sup>[5]</sup>, and thus improving the liver function. This may not only account for that abnormality of hepatic function after PVE in cirrhotic rats could recover over a short period of time, but suggest that indications of hepatectomy for HCC could be broadened as well. In addition, it was found that PV thrombus induced by ethanol injection was often subject to organization or calcification with partial recanalization 2 weeks after PVE, which suggested that resection of HCC

should not be delayed too long in clinical practice.

After being injected into PV, ethanol mainly mixed with blood and gradually led to formation of thrombus. The part entering into the hepatic parenchyma was usually diluted and induced a relatively mild damage to the tissue. It was advantageous to the patients with liver cirrhosis. Meanwhile, our results showed that PVE caused more severe hepatic necrosis in cirrhotic rats than normal rats, which may be related to a reduced resistance of cirrhotic liver to injury. Prophylactic administration of antibiotics was necessary to prevent development of postnecrotic hepatic abscess.

In addition to the mechanic obstruction, some other factors could affect the portal pressure. Ligation of PV led to an elevated portal pressure and reduced portal blood flow, which returned to the normal values within 1-4 weeks<sup>[5]</sup>. In contrast, PVE with ethanol injection caused a slightly different pattern of portal hemodynamic changes, in which portal pressure and blood flow rose after an transient initial decline, and returned to the baseline values within 2 weeks. Two reasons were presumed to contribute to the difference. One was that blood flow was not blocked completely at the beginning after ethanol injection. The other was due to the instant injury of ethanol to liver tissue and accumulation of acid metabolites, which induced a lowered PV pressure. With reference to this regularity, for those patients with remarkable portal hypertension undergoing PVE, it is advisable to administer  $\beta$ -blocker in short term for lowering PV pressure from the 3rd day after PVE.

## REFERENCES

- 1 Lu MD, Liang LJ, Huang JF, Ye WJ, Yang QS, Peng BG *et al.* Portal vein embolization with ethanol injection via a fine needle in dogs. *Jpn J Surg*, 1995; 25(1):416-420
- 2 Lu MD, Huang JF, Liang LJ, Xie XY, Peng BG, Cao XH. Portal vein embolization via a fine needle guided by portochography. *Ultrasound International*, 1995;1(4):212-216
- 3 Lu MD, Huang JF. A model of liver cirrhosis induced by intraperitoneal injection of CCl<sub>4</sub> combined with nutritional adjustment. *Chin J Exp Surg*, 1993;10(1): 56-57
- 4 Kokudo N, Nomura Y, Koyama H, Otomo Y, Ohashi K, Sanjo K *et al.* Experimental transcatheter hepatic subsegmentectomy using absolute ethanol in dogs. *Jpn J Surg*, 1990;20(5):602-605
- 5 Kubo S, Matsui I, Otani S, Morisawa S, Kinoshita H, Sakai K. Effect of portal branch ligation on polyamine metabolism in rat liver. *Life Sci*, 1986;38(20): 1835-1840

# Interaction between cisplatin, 5-fluorouracil and vincristine on human hepatoma cell line (7721) \*

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**Subject headings** liver neoplasms; cisplatin; 5-fluorouracil; vincristine; cell line

## Abstract

**AIM** To evaluate the killing effects of CDDP, 5-Fu and VCR on human hepatoma cell line (7721).

**METHODS** The median-effect principle was used.

**RESULTS** Killing effects of the individual drug were enhanced as the median concentration increased. Antagonism was produced when two drugs were used at a higher concentration ( $CI > 1$ ), and synergism was achieved when  $CI < 1$ . Finally, the effect was influenced by both the ratios of drug concentration and the sequence of administration.

**CONCLUSION** The drug administration order and drug concentrations are significant factors that need to be considered in clinical practice.

## INTRODUCTION

The combined chemotherapy for malignant carcinoma is desired to produce efficacious synergism between each drug, alleviate side effects of drugs and delay drug resistance. Clinically, the interaction (namely synergism, summation and antagonism) of different anticancer drugs in combination is usually evaluated by Chou-Talalay's combination index (i.e., median-effect principle)<sup>[1-9]</sup>. In this paper the combination effect between Cisplatin (Cis), 5-Fluorouracil (5-Flu) and Vincristine (VCR) on human hepatoma cell line 7721, was analyzed *in vitro*.

## MATERIALS AND METHODS

### Cell line and culture conditions

A human hepatoma cell line (7721 cells) was obtained from the Department of Bioengineering, Chongqing University. The cell line was cultured in RPMI 1640 (GIBCO, Grand Island, NY) with 10% (v/v) fetal calf serum, and 100 U/mL penicillin-streptomycin antibiotics, and kept at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Agents

Cis was purchased from Yunnan Gejiu Biochemical Pharmarcetics, 5-Fu from Tianjin Renming Pharmaceutical plant and VCR from Shanghai 11th Pharmaceutical Factory. These drugs were freshly prepared, dissolved in 0.9% NaCl solution before use, at concentrations of 40, 20, 10, 5, 2.5 mg/L for Cis, 5-Fu 400, 200, 100, 50, 25 mg/L, and VCR 4, 2, 1, 0.5, 0.25mg/L, respectively.

### Dose-effect test

The cytotoxicity of the drugs on human hepatoma cell line 7721 was examined by MTT assay<sup>[10]</sup>. Cells ( $1 - 5 \times 10^5$ /mL) were suspended in 10% FCS RPMI1640 medium and 200µl/well suspension was plated to 96 well microplate. Negative control and treatment groups were set up for each experiment.

### Effect of anticancer drug used alone or in combination

When the drug was used alone, each drug at five different concentrations were added to 96 well microplate at 20 µl/well in three duplicates. When used in pairs, Cis and 5-Fu, Cis and VCR, 5-Fu and VCR at a ratio of 1×1 were added to microplate at a total of 20µl/well (each drug 10µl at two times

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\*Project supported by Chinese Ministry of Public Health.

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Received 1998-05-25

concentration that of the drug used alone). Cells were incubated at 37°C, 5% CO<sub>2</sub> for 3 days.

#### Concentration dependence relationship in drugs used in combination

Cis at five different concentrations 2.5, 5, 10, 20 and 40 mg/L were combined with 5-Fu 100, 200 mg/L or VCR 1, 2 mg/L, respectively; VCR 0.25, 0.5, 1, 2 and 4 interacted with 5-Fu at 100 and 200 mg/L. The process lasted 3 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

#### Time-order dependence relationship in drugs used in combination

The time order for Cis and 5-Fu, Cis and VCR administration was as follows: two drugs were given simultaneously and cells were cultured for 3 days; and one drug was given first, another was then administered, and cells incubated for 2 days.

#### Data analysis

OD values of control and treatment groups were detected by Bio-Rad E2550 EIA Reader. Cytotoxicity = 1 - (mean OD of treatment group/mean OD of control group).

According to the median equation  $fa/fu = (D/D_m)^m$ , the median effect curve,  $Y = bx + a$  ( $Y = \log fa/fu$ ,  $x = \log D$ ) was plotted.  $D$  is the dose,  $D_m$  is the median effect dose required for 50% cytotoxicity,  $fa$  represents the fraction affected by the dose  $D$ ,  $fu$  defined the fraction unaffected equals  $1-fa$ , and  $m = b$  is the slope. The median effect dose is calculated by  $\log D_m = -a/m$ ,  $a = Y - bX$ . The interaction of two drugs is determined quantitatively by combination index (CI):

$$CI = (D_1)^x / (D_x)_1 + (D_2)^x / (D_x)_2 + \alpha (D_1)^x (D_2)^x / (D_x)_1 (D_x)_2$$

$D_1$  and  $D_2$  are the doses of drug 1 and 2, which are required to produce  $x\%$  effect in combination.  $(D_x)_1$  and  $(D_x)_2$  are the dose of drug 1 and drug 2 required to produce  $x\%$  effect individually. If the drugs are mutually exclusive,  $\alpha = 0$  and non-exclusive,  $\alpha = 1$ .  $CI = 1$ , summation;  $CI < 1$ , synergism; and  $CI > 1$ , antagonism<sup>[11]</sup>.

## RESULTS

#### Dose-effect relationships of three drugs used alone and in combination

The cytotoxicity of three drugs progressively increased with increasing doses, either used alone or in pairs (Table 1). According to the median-effect equation, the median effect doses of these drugs used alone or in combination can be calculated. When used alone, Cis was 1.158 mg/L, 5-Fu 17.056 mg/L and VCR 5.22 mg/L, respectively. In contrast to the drug used alone, the median-effect doses were lower in combination. Cis plus VCR was

0.85 mg/L (containing Cis 0.82 mg/L and VCR 0.03 mg/L); Cis plus 5-Fu 7.43 mg/L (containing Cis 0.31 mg/L and 5-Fu 7.21 mg/L); and 5-Fu plus VCR was 8.45 mg/L (containing 5-Fu 8.44 mg/L and VCR 0.01 mg/L). The combined effect of two drugs at ratio 1:1 can be illustrated for synergism, summation or antagonism (Figure 1).

Table 1 Dose-effect relationships of three drugs used alone and in combination

Cis	5-Fu	VCR	Cis+5-Fu	VCR+5-Fu	Cis+VCR
(2.5)0.662	(25)0.600	(0.25)0.178	(2.5+25)0.709	(2.5+25)0.647	(2.5+25)0.527
(5.0)0.809	(50)0.784	(0.50)0.204	(5+50)0.859	(5+50)0.713	(5+50)0.781
(10.0)0.867	(100)0.834	(1.00)0.282	(10+100)0.877	(10+100)0.810	(10+100)0.844
(20.0)0.893	(200)0.853	(2.00)0.429	(20+200)0.902	(20+200)0.839	(20+200)0.873
(40.0)0.928	(400)0.871	(4.00)0.446	(40+400)0.951	(40+400)0.892	(40+400)0.898

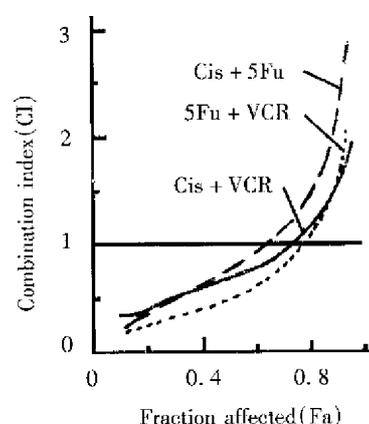


Figure 1 The relationship between CI and fraction affected of Cis+5-Fu, VCR+5-Fu and 5-Fu+VCR.

#### Concentration dependence relationship in combination

**Cis and 5-Fu** The interaction of 5-Fu at a dose of 200 mg/L and Cis at five concentrations exhibited antagonism, the same results can be seen when 5-Fu at 100 mg/L was combined with Cis at  $\geq 20$  mg/L, but synergism was shown when Cis at 20 mg/L.

**Cis and VCR** Most cases in which Cis at various concentrations and VCR at 1 mg/L (except for Cis at 2.5 mg/L), were used, showed antagonism. When Cis at various concentrations (except for 2.5 mg/L) was combined with VCR at 2 mg/L, synergistic effect appeared.

**VCR and 5-Fu** The combination of VCR at  $> 0.5$  mg/L and 5-Fu at 100 mg/L showed synergism. In contrast, when VCR at 0.5 mg/L or  $< 0.5$  mg/L and 5-Fu 100 mg/L, were used in combination antagonism occurred. Moreover, when 5-Fu at up to 200 mg/L was combined with Cis at various concentrations (except for Cis. the highest one) the interaction was also antagonism (Table 2).

**Table 2 Fa and CI of drug combinations at different ratios**

Drug 1 (mg/L)	Drug 2 (mg/L)	fa	CI	Drug 1 (mg/L)	Drug 2 (mg/L)	fa	CI
Cis 2.5	5-Fu 100	0.821	0.75	Cis 2.5	VCR 2	0.554	1.15
5	100	0.856	0.85	5	2	0.858	0.80
10	100	0.877	0.87	10	2	0.862	0.74
20	100	0.883	1.09	20	2	0.873	0.71
40	100	0.893	1.23	40	2	0.904	0.66
Cis 2.5	5-Fu 200	0.839	1.85	VCR 0.25	5-Fu 100	0.657	2.39
5	200	0.867	2.93	0.5	100	0.761	1.15
10	200	0.899	2.95	1	100	0.810	0.77
20	200	0.902	3.00	2	100	0.826	0.64
40	200	0.907	3.36	4	100	0.850	0.51
Cis 2.5	VCR 1	0.498	0.32	VCR 0.25	5-Fu 200	0.759	2.13
5	1	0.825	1.38	0.5	200	0.783	1.79
10	1	0.844	2.05	1	200	0.825	1.28
20	1	0.852	2.09	2	200	0.839	1.10
40	1	0.894	3.40	4	200	0.867	0.92

As can be seen in Table 2, the more prominent efficacy can be achieved when 5-Fu at lower concentration was used with other two drugs, while VCR could be used at higher concentrations.

#### Time-order dependence relationship in drugs used combination (Table 3)

**Cis and 5-Fu** When Cis and 5-Fu were used simultaneously, the effect was more efficacious than that of Cis used for 24h followed by 5-Fu or 5-Fu followed by Cis (*t* test,  $P < 0.05$ ). In contrast, there was no difference between Cis and 5-Fu no matter which was used first ( $P > 0.05$ ).

**Cis and VCR** When Cis was given 24h after VCR, the fraction affected was less prominent than that of two drugs treated simultaneously or Cis first ( $P < 0.05$ ). There was no apparent difference between two drugs used simultaneously and Cis used first ( $P > 0.05$ ).

**Table 3 Relationship between time-order and effect in drugs used in combination ( $\bar{x} \pm s$ )**

Drugs (mg/L)	Simultaneous exposure	Pre-Cis exposure	Post-Cis exposure
<b>Cis+5-Fu</b>			
2.5 25	0.709±0.015	0.683±0.016	0.598±0.010
5 50	0.859±0.011	0.807±0.027	0.773±0.015
10 100	0.877±0.013	0.824±0.012	0.793±0.026
20 200	0.902±0.014	0.831±0.017	0.805±0.016
40 400	0.951±0.012	0.853±0.015	0.822±0.010
<b>Cis+VCR</b>			
2.5 0.25	0.527±0.025	0.470±0.023	0.199±0.023
5 0.5	0.781±0.034	0.707±0.022	0.407±0.017
10 1	0.844±0.015	0.789±0.025	0.533±0.013
20 2	0.873±0.017	0.818±0.029	0.651±0.021
40 4	0.898±0.025	0.893±0.020	0.752±0.025

## DISCUSSION

Cis, a broad spectrum in anti-cancer drug, inhibits RNA and protein synthesis of tumor cells by cross-

linking with DNA. 5-Fu, an antimetabolic agent, halts DNA and RNA synthesis by inhibiting the activity of thymine nucleotide synthetase at various times in the cell cycle, especially in S phase. Thus, the common mechanism of the two drugs Cis and 5-Fu can stop RNA synthesis. VCR can inhibit cell microtubule integrity and therefore stops nucleospindle formation in cell division. *In vitro*, these three drugs exhibited pronounced cytotoxicity when used alone, in human hepatoma cell line 7721, but higher doses were needed. Compared with the drugs used alone, both the median effect concentration and the dose required were much lower when used in combination. The results indicate that lower dose in combination can achieve higher efficacy with less side-effects. When used in combination, the time order of administration affected drug cytotoxicity. If the cytotoxicity of the drug was pronounced when used alone, there was no difference when used in combination disregarding which was administered first. If one drug efficacy is more prominent than another, when used alone a higher combined effect can be achieved when the more efficacious drug was used first.

Anticancer drug sensitivity can analyze quantitatively the drug interaction, i.e. synergism, summation and antagonism, and hence guiding the clinical treatment.

**ACKNOWLEDGEMENT** We would like to thank Professor FAN Wei-Ke for preparing the manuscript.

## REFERENCES

- Chou TC, Talalay P. Quantitative analysis of dose-effects relationships: the combined effect of multiple drugs on enzyme inhibitors. *Adv Enzyme Regul.* 1984;27-55
- Chang TT, Gulati SC, Chou TC, Vega R, Gandcoa L, Ezzat Ibrahim SM *et al*. Synergistic effect of 4-hydroperoxy-cyclophosphamide and etoposide on a human promyelocytic leukemia cell line (HL-60) demonstrated by computer analysis. *Cancer Res.* 1985;45(6):2434-2439
- Chang TT, Gulati S, Chou TC, Colvin M, Clarkson B. Comparative cytotoxicity of various drug combination for human leukemic cells and normal hematopoietic precursors. *Cancer Res.* 1987;47(1):119-122
- Chang BK, Gutman R, Chou TC. Schedule-dependent interaction of  $\alpha$ -difluoromethylornithine and cis-diamine dichloroplatinum (II) against human and hamster pancreatic cancer cell lines. *Cancer Res.* 1987;4(9):2247-2250
- Kohn N, Ohnuma T, Kancko M, and Holland J.F. Interactions of doxorubicin and cis platin in squamous carcinoma cells in culture. *Br J Cancer.* 1988; (3):330-334
- Howell SB, Hom D, Sanga R, Vick JS, Abramson LS. Comparison of the synergistic potentiation of etoposide, doxorubicin, and vinblastine cytotoxicity by dipyrindamole. *Cancer Res.* 1989;49(12):3178-3183
- Hofs HP, Wagener DJ. Concentration and sequence dependent synergism of ethyldeshydroxyparasomycin in combination with antitumor agents. *Anti-cancer Drugs.* 1994;5(1):35-42
- Smith KS, Folz BA. Synergistic and additive combination of several antitumor drugs and other agents with the potent alkylating agents adozclesin. *Cancer Chemother Pharmacol.* 1995;35(6):471-485
- Lee JT, Park S, Lee JH. Efficacy of in vitro treatment of chronic myelogenous leukemia cell line K562 cells using 4-hydroperoxy-cyclophosphamide, alpha-interferon and gamma interferon. *J Korean Med Sci.* 1996;11(1):26-32
- Tang WX, Luo YP, Wang RX. Determination of anticancer drug sensitivity test by the MTT Colorimetric assay for human solid tumors. *J Chongqing Med Univ.* 1992;17(21):103-108
- Han R. The drug prophylaxis and chemotherapy for tumor. Beijing: United Publishing House of BMU and PUMC, 1991:315-320

# Transduction of Fas gene or *Bcl-2* antisense RNA sensitizes cultured drug resistant gastric cancer cells to chemotherapeutic drugs

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**Subject headings** stomach neoplasms; Fas gene; *Bcl-2* gene; antisense nucleic acid; drug resistance, multiple; gene transduction; apoptosis

## Abstract

**AIM** To compare the expression level of *Fas* gene and *Bcl-2* gene in gastric cancer cells SGC7901 and gastric cancer multidrug resistant cells (MDR) SGC7901/VCR, to transduce *Fas* cDNA and *Bcl-2* antisense nucleic acid into SGC7901/VCR cells respectively, and to observe the expression of two genes in transfectants and non-transfectants as well as their drug sensitivity.

**METHODS** Eukaryotic expression vector pBK-*Fasc* cDNA and pDOR-anti *Bcl-2* were constructed and transfected into SGC7901/VCR cells by lipofectamine, respectively. Northern blot and Western blot were used to detect the expression of mRNA and protein in SGC7901/VCR and SGC7901 cells and transfectants, and drug sensitivity of transfectants for VCR, CDDP and 5-FU was analyzed with MTT assay.

**RESULTS** After gene transfection, 80 for *Fas* and 120 for antisense *Bcl-2* drug-resistant clones were selected from  $2 \times 10^5$  cells, transfection rate being 0.04% and 0.06%. Two clones of SGC7901 *Fas*/VCR cells and SGC7901 anti *Bcl-2*/VCR cells were randomly selected for further incubation. Hybridization results showed that the expression level of *Fas* mRNA and protein in SGC7901/VCR cells was much lower, but that of *Bcl-2* mRNA and protein was higher than that in SGC7901 cells. The expression of *Fas* mRNA and protein in SGC7901 *Fas*/VCR cells was higher, and of *Bcl-2* mRNA and protein was lower in SGC7901 anti *Bcl-2*/VCR cells than that in non-transfectants. MTT assay showed

that transfectants were more sensitive to VCR, CDDP, 5-FU than non-transfectants.

**CONCLUSION** *Bcl-2* gene displayed high expression while *Fas* gene had low expression in drug resistant gastric cancer cells. Expression of *Bcl-2* protein was effectively blocked in SGC7901 anti *Bcl-2*/VCR cells by gene transfection. In contrast, the expression of *Fas* mRNA and protein in SGC7901 *Fas*/VCR cells increased. *Fas* gene and *Bcl-2* antisense nucleic acid transfection sensitized drug resistant gastric cancer cells to chemotherapeutic drugs. These results suggest cell apoptosis plays an important role in the mechanism of MDR, and enhancing apoptosis might reverse MDR.

## INTRODUCTION

Chemotherapy is one of the major methods in tumor treatment, but it often does not work due to multidrug resistance (MDR). Recent studies indicated that inhibition of cancer cell apoptosis and longer cell life may be one of MDR mechanisms<sup>[1]</sup>. So it is assumed that inducing apoptosis might reverse MDR. We transfected *-Fas-* gene and *Bcl-2* antisense nucleic acid into drug resistant gastric cancer cells, and observed the expression of target genes in transfectants and the sensitivity of transfectants to chemotherapeutic agents in order to find the ground for reversing gastric cancer MDR.

## MATERIALS AND METHODS

### Material

Drug resistant gastric cancer SGC7901/VCR cells and JM109 bacterial strain were kept in our department. Retrovirus vector pDOR-SV40 and expression vector pBK-CMV were generous gifts from Dr. Cui Da-Xiang, Department of Biochemistry, Fourth Military Medical University. The pBluescript *Bcl-2* cDNA plasmid was from our department. The pBluescript *Fas*-cDNA was presented by professor Itoh N, Japan. *Eco* RI, *Bam* HI, *Sal* I, *Xho* I, CIP, RNaseA, T4 DNA ligase, PMSF, Aprotinin, SDS, ABC kit, probe labeled kit and guanidinium isothiocyanate were

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Received 1998-06-06

purchased from SABC and Promega, and Lipofectamine from Gibco BRL. MTT, Protease k, DEPC, MOPS, G418, FCM and nitrocellulose filter were products of Sigma. [ $\alpha^{32}\text{P}$ ]dATP was derived from Yahui Ltd, Beijing, primer and oligonucleotides from Sangon, Shanghai, and rat anti-human *Bcl-2* monoclonal antibody and rabbit anti human *Fas* polyclonal antibody from Orient Ltd, Beijing.

## Methods

### Construction and identification of recombinant vectors

1.9kb *Bcl-2* cDNA was inserted into pBluescript KS at *Eco* RI site and 2.5kb *Fas* cDNA into pBluescript KS at *Xho* I site. According to the construction protocol, primary plasmids were digested by enzyme. The results of agarose gel electrophoresis showed that primary plasmids contained complete *Bcl-2* cDNA and *Fas*-cDNA. We used *Eco* RI and *Bam* HI to cut pBluescript *Bcl-2* cDNA, *Sal* I and *Eco* RI to cut pBluescript *Fas*-cDNA, and obtained 0.622 kb *Bcl-2* cDNA and 1.83 kb *Fas*-cDNA fragments by frozen-thaw methods. Under T4 DNA ligase, cDNA fragments were connected with the corresponding pDOR and pBK-CMV vectors digested by enzyme, the products were transformed into competent cells for routine culture. Ampresistant colonies and mini-prepared plasmids were selected randomly, and recombinant plasmids were identified through enzyme digestion and agarose gel electrophoresis. Three pairs of primers designed according to *Fas*-cDNA sequence were used for PCR amplification of pBK-*Fas*-cDNA, and sequence of PCR product was analyzed to confirm the correctness of open reading frame.

### Gene transfection and clone selection

Purified pDOR-anti-*Bcl-2* or pBK-*Fas* cDNA (0.5 $\mu\text{g}$ ) diluted in 100 $\mu\text{l}$  RPMI 1640 was mixed with 5 $\mu\text{l}$  lipofectamine, and placed at room temperature for 10min. The mixture was then transfected into  $2 \times 10^5$  SGC7901/VCR cells. The cells were selected by 500 mg/L G418 24 hours later. At the same time, pDOR or pBK-CMV vector which lacked target genes transfected cells and non-transfectants served as negative controls.

### Southern blot and Northern blot

Genomic DNA and total RNA were obtained from the well growing  $1 \times 10^7$  cells. Genomic DNA digested with *Eco* RI ran agarose gel electrophoresis. The denatured total RNA underwent formaldehyde denaturation and gel electrophoresis. DNA and RNA were transferred to nitrocellulose filter by vacuum aspiration. [ $\alpha^{32}\text{P}$ ]dATP labeled probe was made for prehybridization, hybridization and autoradiography.

### Western blot

Half cell lytic protein was used for SDS-PAGE, and comassie brilliant blue stained, the other half was electrotransferred 100v overnight, nitrocellulose filter denatured by SDS and visualized by ABC method.

### Drug sensitivity assay

Cells ( $10^3 - 10^4$ ) diluted with 200 $\mu\text{l}$  10% RPMI 1640 were seeded into 96-well plates, respectively, added cisplatin (1 $\mu\text{g}$ , 10 $\mu\text{g}$ , 100 $\mu\text{g}$ ), 5-FU (7  $\mu\text{g}$ , 70 $\mu\text{g}$ , 700  $\mu\text{g}$ ), and VCR (0.1 $\mu\text{g}$ , 1.0 $\mu\text{g}$ , 10 $\mu\text{g}$ ) according to the clinically established plasma peak concentration. Three days later, 20  $\mu\text{l}$  MTT solution (5g/L) was dropped into plates, the supernatant was discarded after 4 hours, and 150 $\mu\text{l}$  DMSO was added to melt crystal. OD value was read at 590nm wavelength.

## RESULTS

### Construction of eukaryotic expression vector

As shown in Figure 1, 1.9 kb *Bcl-2* cDNA and 2.5kb *Fas*-cDNA were separated from primary plasmids digested with *Eco* RI and *Xho* I, 0.622kb anti-sense *Bcl-2* fragment and 6.5kb vector from recombinant vector pDOR-anti-*Bcl-2* digested by *Eco* RI and *Bam* HI, 1.8 kb *Fas* cDNA and 4.5kb vector from recombinant vector pBK-*Fas*-digested by *Eco* RI and *Sal* I. Electrophoresis of PCR product (Figure 2) indicated that three pairs of primers amplified 500 bp, 850 bp, 700 bp fragments respectively, which were consistent with our expectation.

### Establishment of transfectants

When transfected cells were cultured selectively by G418 for 4-5 weeks, resistant clones formed gradually, the number of stable clones reached about 120, with a transfection rate of above 0.05%. In contrast, all non-transfectants died two weeks after G418 selection. Resistant clones were further incubated in the presence of low-dose G418 for 40-50 days. We got two resistant clones, SGC7901-*Fas*/VCR cells and SGC7901 anti-*Bcl-2*/VCR cells.

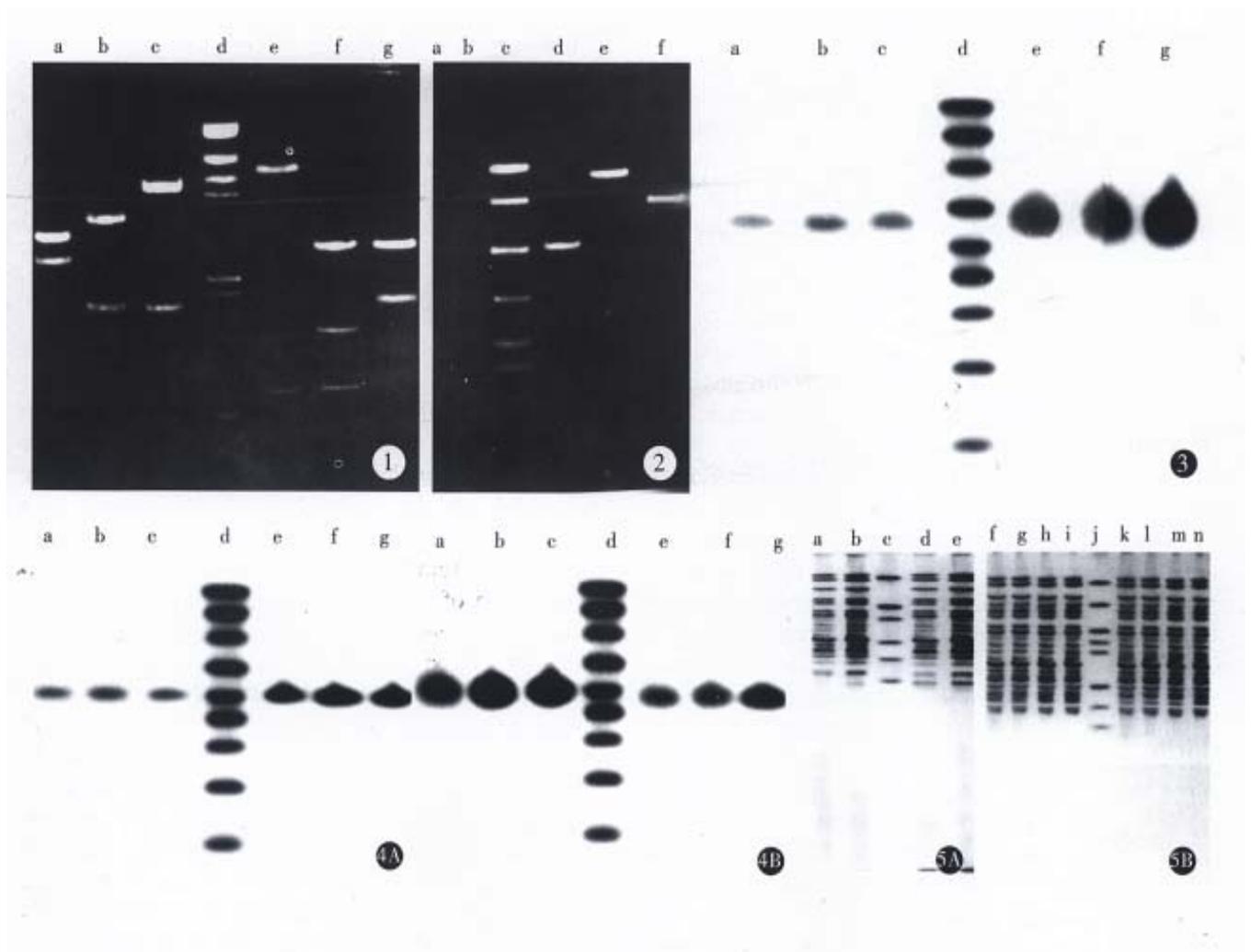
### Expression of target genes

Northern blot was performed using a single strain cDNA fragment of *Fas* or *Bcl-2* or antisense *Bcl-2* as probes, respectively. The results (Figures 3 and 4) showed that SGC7901 cells had weak signals of *Fas* and strong signal of *Bcl-2*, while SGC7901/VCR had weak signal of *Fas* and very strong signal of *Bcl-2*. The signal of *Fas* became stronger in SGC7901-*Fas*/VCR than that in SGC7901/VCR, which was also dependent on cell number. The signal of antisense *Bcl-2* was stronger in SGC7901 anti-*Bcl-2*/VCR than in SGC7901/VCR and non-transfectants, but the signal of sense *Bcl-2*

was a little weaker than that in non-transfectants. SDS-PAGE and Western blot (Figure 5) showed that a band of Mr 36000-40000 was found in transfectants, but not in non-transfectants, the size of which was consistent with Mr of *Fas*-protein. A band of Mr 25000, which equaled to that of *Bcl-2* was found in non-transfectants, but not in antisense *Bcl-2* transfected cells.

### Drug sensitivity assay

The survival rates of transfectants treated with cisplatin or 5-FU were obviously lower than non-transfectants, however, the survival rates of transfectants treated with VCR were only lowered lightly, compared with non-transfectants (Table 1).



**Figure 1** Identification of recombinant vectors digested with enzymes. a. pBluescript *Fas/Xho*I, b. pBluescript *Fas/Eco*RI+*Sal*I, c. pBK-*Fas/Eco*RI+*Sal*I, d.  $\gamma$ DNA Marker/*Hind*III, e. pDOR-*Bcl-2/Eco*RI+*Bam*HI, f. pBluescript *Bcl-2/Eco*RI+*Bam*HI, g. pBluescript *Bcl-2/Eco*RI  
**Figure 2** Electrophoresis of pBK-*Fas*- PCR product. a. PCR of competent cells as template, b. PCR of empty vector transformed bacterial, c. PCR marker, d. PCR1 of pBK-*Fas* (500bp), e. PCR2 of pBK-*Fas* (850bp), f. PCR3 of pBK-*Fas* (700bp)  
**Figure 3** Northern blot with *Fas*-cDNA probe. a. SGC7901/VCR/ $3 \times 10^6$  cells, b. empty vector transfectant/ $5 \times 10^6$  cells, c. SGC7901/ $3 \times 10^6$  cells, d. RNA Marker, e. SGC7901 -*Fas*/VCR/ $1 \times 10^6$  cells, f. SGC7901 -*Fas*/VCR/ $2 \times 10^6$  cells, g. SGC7901 *Fas*/VCR/ $3 \times 10^6$  cells.  
**Figure 4** Northern blot with *Bcl-2* probe. A. antisense probe, B. sense probe  
a. SGC7901/ $3 \times 10^6$  cells, b. empty vector transfectant/ $2 \times 10^6$  cells c. SGC7901/ $3 \times 10^6$  cells, d. RNA Marker, e. SGC7901 anti-*Bcl-2*/VCR/ $2 \times 10^6$  cells, f. SGC7901 anti-*Bcl-2*/VCR/ $3 \times 10^6$  cells, g. SGC7901 anti-*Bcl-2*/VCR/ $4 \times 10^6$  cells  
**Figure 5** SDS-PAGE and Western blot. A. *Fas* transfectants, B. anti-*Bcl-2* transfectants  
a. SGC7901/VCR/ $3 \times 10^6$  cells, b. SGC7901/ $3 \times 10^6$  cells, c. High molecular weight protein marker, d. SGC7901-*Fas*/VCR/ $1 \times 10^6$  cells, e. SGC7901-*Fas*/VCR/ $2 \times 10^6$  cells, f. SGC7901/ $1 \times 10^6$  cells, g. SGC7901/ $2 \times 10^6$  cells, h. SGC7901/VCR/ $1 \times 10^6$  cells, i. SGC7901/VCR/ $2 \times 10^6$  cells, j. Middle molecular weight protein marker, k. SGC7901 anti-*Bcl-2*/VCR/ $5 \times 10^5$  cells, l. SGC7901 anti-*Bcl-2*/VCR/ $1 \times 10^6$  cells, m. SGC7901 anti-*Bcl-2*/VCR/ $2 \times 10^6$  cells, n. SGC7901 anti-*Bcl-2*/VCR/ $3 \times 10^6$  cells

**Table 1 Survival rates of transfectants and non-transfectants treated with chemotherapeutic drugs (%)**

Cells	n	CDDP (mg/L)			5-FU(mg/L)			VCR(mg/L)		
		5	50	500	35	350	3500	0.5	5	50
SGC7901 Fas/VCR	10	45.1 <sup>a</sup>	40.5 <sup>a</sup>	32.0	43.5 <sup>a</sup>	38.6 <sup>a</sup>	30.7 <sup>a</sup>	55.0	46.7 <sup>a</sup>	40.0 <sup>a</sup>
SGC7901 anti-Bcl-2/VCR	10	50.2 <sup>a</sup>	45.1 <sup>a</sup>	40.5	50.1 <sup>a</sup>	44.6 <sup>a</sup>	39.5 <sup>a</sup>	60.3	55.1 <sup>a</sup>	49.2 <sup>a</sup>
SGC7901/VCR	10	70.2	61.3	52.0	71.1	62.2	55.3	72.5	67.4	60.1

In comparison with SGC7901/VCR cells group, <sup>a</sup>P<0.05.

## DISCUSSION

Apoptosis and mitosis both are important for keeping the balance of organism *in vivo*, which act in opposite way. Recent studies indicated that inhibition of cancer cell apoptosis and disturbance of apoptosis related genes can increase the anti-apoptosis protein or decrease pro-apoptosis protein, which is one of the mechanisms of tumorigenesis. Therefore, induction of apoptosis may treat tumors. Many chemotherapeutic agents are found to act through damaging DNA or regulating apoptosis related genes *p53*, *c-myc*, *Bcl-2*, *c-H-ras* to trigger apoptosis<sup>[2-4]</sup>. But MDR becomes an obstacle in tumor treatment. Recent researches have shown that MDR is the result of many mechanisms, in addition to the changes of Pgp, MRP, LRP, Topo II and GST/GSH, deregulation of apoptosis also produces cross drug resistance of cancer cells<sup>[1,5]</sup>. The ability of drug resistant cells repairing damaged DNA and anti-apoptosis has strengthened greatly<sup>[6]</sup>. Introduction of *Bcl* xL gene may induce resistance of drug sensitive cells, and overexpression of *Bcl-2* is linearly related to overexpression of Pgp, therefore, it is supposed that the expression level of apoptosis related genes determines the MDR phenomena of cancer cells<sup>[7]</sup>. Chinese hamster drug resistant ovarian fibroblastoma LR73/20E cells, which overexpress MDR and Pgp, are not sensitive at all to apoptosis inducing colchicine, but become sensitive to apoptosis after treated with drug resistant reversing agent verapamil<sup>[8]</sup>. Apoptosis can not be induced in drug resistant breast cancer MCF-7/ADR cells which overexpress *Bcl-2* mRNA and protein by sugar starvation<sup>[9]</sup>. Drug resistant leukemia HL-60/AR, HL-60/VCR, HL-60/TAX1000 cells, overexpressing MRP, Pgp, *Bcl-2*, and *Bcl*-xL, can inhibit the intercellular aggregation of chemotherapeutic agent paclitaxel and prohibit apoptosis. *Bcl-2* and *Bcl*-xL transfected drug sensitive cells can antagonize apoptosis, but can not affect the intercellular aggregation of chemotherapeutic agent paclitaxel<sup>[10]</sup>. P53 protein and *Fas* antigen promote cell apoptosis, but they display low expression in drug resistant cells<sup>[1,11]</sup>. These results show that high expression of *Bcl-2* and low expression of *Fas* are important for drug resistant cells to antagonize apoptosis and resist

chemotherapeutic agents.

Although antiapoptosis gene *Bcl-2* can not promote cell proliferation, it prolongs cell life. Its inhibition of apoptosis is caused by changing the permeability of Ca<sup>2+</sup> channel and the expression of ICE<sup>[12]</sup>, and is related with regulation of Bax, TNF and *Fas*<sup>[13]</sup>. Controlling the transcription of *Bcl-2* mRNA by antisense nucleic acid technology can effectively suppress the expression of *Bcl-2* and retard tumor cell growth<sup>[14]</sup>.

*Fas* gene product, a type-I membrane protein, belongs to the TNF receptor family. *Fas* molecule on the surface of cell transmits death message into cell through combining *Fas* ligand or anti *Fas* monoclonal antibody, resulting in the death of cells in several hours<sup>[15]</sup>. Compared with normal tissues, malignant tumor cells, especially metastatic tumor cells present extremely lower expression of *Fas*, while most benign tumors are similar to their original tissues in the expression of *Fas*. It is hypothesized that malignant tumors might suppress *Fas* expression or lose *Fas* molecule in order to avoid the surveillance of *Fas* ligand. This explains the lack of *Fas* expression in drug resistant cells<sup>[1]</sup>. In one word, transduction of proapoptosis *Fas* gene or antisense *Bcl-2* gene into drug resistant cells is obviously helpful in increasing the drug sensitivity. Chen XQ transiently transduced *Bcl-2* antisense RNA into human leukemia cells (CEM), and found that intrinsic *Bcl-2* protein was decreased, and transfected cells were more sensitive to etoposide and a lot of apoptotic bodies and small DNA fragments formed when cells died.

To study the significance of *Fas* and *Bcl-2* protein in drug resistant gastric cancer SGC7901/VCR cells, we constructed the eukaryotic expression vectors pBK *Fas* and pDOR-anti-*Bcl-2* which were respectively transfected into SGC7901/VCR cells. After G418 selection, resistant clones overexpressing *Fas* protein or lacking *Bcl-2* protein were obtained. Molecular hybridization showed drug resistant gastric cancer SGC7901/VCR cells expressed a tiny amount of *Fas* mRNA and protein, but highly expressed *Bcl-2*. Compared with control cells, *Fas* mRNA and protein expression of *Fas* transfected cells was obviously increased. In anti-*Bcl-2* transfected cells, *Bcl-2* mRNA expression was

inhibited slightly, but *Bcl-2* protein was blocked greatly. Drug sensitivity assay demonstrated that transfectants were more sensitive to VCR, CDDP and 5-FU than non-transfectants. It was concluded that suppression of apoptosis play an important role in inducing MDR of tumor cells, and promoting apoptosis could reverse MDR to some extent.

## REFERENCES

- 1 Friesen C, Fulda S, Debatin KM. Deficient activation of the CD95 (APO-1/*Fas*) system in drug-resistant cells. *Leukemia*, 1997 Nov;11(11):1833-1841
- 2 Arceci RJ. Tumor cell survival and resistance to therapy. *Curr Opin Hematol*, 1996 Jul;3(4):279-287
- 3 Ketley NJ, Allen PD, Kelsey SM, Newland AC. Modulation of idarubicin-induced apoptosis in human acute myeloid leukemia blasts by all-trans retinoic acid, 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub>, and granulocyte-macrophage colony-stimulating factor. *Blood*, 1997;90(11):4578-4587
- 4 Nooter K, Boersma AW, Oostrum RG, Burger H, Jochemsen AG, Stoter-G. Constitutive expression of the c-H-ras oncogene inhibits doxorubicin-induced apoptosis and promotes cell survival in a rhabdomyosarcoma cell line. *Br J Cancer*, 1995;71(3):556-561
- 5 Van-Waardenburg RC, Meijer C, Burger H, Nooter K, De-Vries EG, Mulder NH, De-Jong S. Effects of an inducible anti-sense c-myc gene transfer in a drug-resistant human small cell-lung-carcinoma cell line. *Int J Cancer*, 1997;73(4):544-550
- 6 Colvin OM. Drug resistance in the treatment of sarcomas. *Semin-Oncol*, 1997; 24(5):580-591
- 7 Minn AJ, Rudin CM, Boise LH, Thompson CB. Expression of *Bcl-xL* can confer a multidrug resistance phenotype. *Blood*, 1995;86(5):1903-1910
- 8 Robinson LJ, Roberts WK, Ling TT, Lamming-D, Sternberg SS, Roepe PD. Human MDR 1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. *Biochemistry*, 1997;36(37):11169-11178
- 9 Lee-YI, Galoforo SS, Berns CM, Tong WP, Kim HR, Corry PM. Glucose deprivation-induced cytotoxicity in drug resistant human breast carcinoma MCF-7/ADR cells: role of *c-myc* and *Bcl-2* in apoptotic cell death. *J Cell Sci*, 1997;110(Pt5):681-686
- 10 Huang Y, Ibrado AM, Reed JC, Bullock G, Ray S, Tang C, Bhalla K. Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. *Leukemia*, 1997;11(2):253-257
- 11 Reeve JG, Xiong J, Morgan J, Bleehen NM. Expression of apoptosis-regulatory genes in lung tumour cell lines: relationship to p53 expression and relevance to acquired drug resistance. *Br J Cancer*, 1996;73(10):1193-1200
- 12 Shimizu S, Eguchi Y, Kamiike W, Matsuda H, Tsujimoto Y. *Bcl-2* expression prevents activation of the ICE protease cascade. *Oncogene*, 1996;12(12):2251-2257
- 13 Allouche M, Bettaieb A, Vindis C, Rouse A, Grignon C, Laurent G. Influence of *Bcl-2* overexpression on the ceramide pathway in daunorubicin-induced apoptosis of leukemic cells. *Oncogene*, 1997;14(10):1837-1845
- 14 Webb A, Cunningham D, Cotter F, Clarke PA, di-Stefano F, Ross P *et al.* *Bcl-2* antisense therapy in patients with non-Hodgkin lymphoma. *Lancet*, 1997; 349(9059):1137-1141
- 15 Nagada S, Golstein P. The Fas death factor. *Science*, 1995;267(5160):1449-1455

# Modulation of hypothalamic arcuate nucleus on gastric motility in rats

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**Subject headings** gastric motility; arcuate nucleus; neural pathways; vagus nerve; sympathetic nerve; electrical stimulation

## Abstract

**AIM** To investigate whether the arcuate nucleus (ARC) could modulate gastric motility, and if so, what are the mechanisms or pathways.

**METHODS** Wistar rats, anaesthetized with urethan, parameters of stimulation and electrolytic lesion sites were determined according to the Paxinos and Watson "ATLAS of rat brain in stereotaxic coordinate". Intra-gastric pressure (IGP) and gastric motility were measured by Reybould's method.

**RESULTS** Electrical stimulation of ARC could obviously decrease the IGP by  $42.2\% \pm 5.4\%$ ,  $n = 15$ ,  $P < 0.01$ , and the phasic gastric contractions disappeared. The analysis showed that the locus coeruleus (LC) and dorsal raphe (DR) nuclei may be involved in central, but without the involvement of  $\beta$ -endorphinergic neurons rich in the ARC, while in periphery, the peripheral neural pathways are both vagus and sympathetic nerves. The fibers in vagus may be non-cholinergic. Humoral factors may also be involved. At the receptor level, Tonic action of adrenergic nerve in the stomach is mainly inhibitory;  $\beta$ -receptors, which may be present on the stomach wall and mediate inhibition; and  $\alpha$ -receptors, which come into play through vagus, mediate inhibition, but those present on the smooth muscle mediate sympathetic excitation. Microinjection of TRH into ARC could significantly increase the IGP by  $183.02\%$  ( $0.53 \text{ kPa} \pm 0.08 \text{ kPa}$  vs  $1.5 \text{ kPa} \pm 0.6 \text{ kPa}$ ,

$n = 10$ ,  $P < 0.001$ ), the rate and amplitude of phasic gastric contraction were also increased (3 cpm vs 6cpm - 8cpm). The peripheral pathway of such excitatory effects were transmitted with cholinergic vagus nerve mediated by M-receptor.

**CONCLUSION** ARC could modulate gastric motility biphasically, inhibitory and excitatory, depending on the nature of stimuli.

## INTRODUCTION

Arcuate nucleus (ARC) is the third largest nucleus of the hypothalamic nuclei with a volume of  $0.94 \text{ mm}^3$  in rats. It is located at the base of hypothalamus, and surrounds the ventral part of the third ventricle. At least 15 neurotransmitters and neuropeptides, e.g.  $\beta$ -endorphin, enkephalin, etc. have been found in ARC. The ARC is anatomically organized to communicate primarily with the pituitary gland, hypothalamus, limbic system, certain thalamic nuclei, the midbrain periaqueductal gray and autonomic nuclei of the brainstem. This general organization leads us to hypothesize that it may play a key role in integrating autonomic functions. But so far there has been no report showing that ARC could modulate gastric motility. This study was designed to clarify whether ARC modulates gastric motility, and if so, to analyse their mechanisms or pathways.

## MATERIALS AND METHODS

Wistar rats, both sexes, weighing 250 g - 300 g, fasted 24h before experiment, but free to have water, were anesthetized with urethan and paralysed with flaxedil.

### IGP and gastric motility

Intra-gastric pressure (IGP) and gastric motility were measured according to Reybould's method through pressure transducer (type YH-II, Institute of Space Medicine Engineering), and recorded by two channels physiological pen-recorder (type LSM-2B, Chengdu Instrument Factory). The rate of IGP changes are calculated according to the following equation:

$$\% \text{ of IGP change} = \frac{\text{IGP after stimulation} - \text{IGP before stimulation}}{\text{IGP before stimulation}} \times 100$$

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Received 1998-03-26

### Operations

Subdiaphragmatic vagotomy was performed according to Mordes (1978) method, and extirpation of celiac neural plexus by Moraes (1978) method. Experiments began 2-3 weeks after operation and sham operation used as control.

### Electrical stimulation and electrolytic lesion

Rats were fixed at stereotaxic instrument (type SN-2, Narishige, NIHON, KONDON). The reference coordinates were: for ARC, p 3.5 mm - 3.8 mm, H 10.0 mm - 10.5 mm, L or R 0.2 mm; and for LC, p 9.5 mm - 9.8 mm, H 7.0 mm - 7.1 mm, L or R 1.1 mm - 1.3 mm; for DR, p 7.8 mm, H 6.5 mm - 6.8 mm, L or R 0.0 mm; for LCV (lateral cerebroventricle), p 0.8 mm, H 4.0 mm - 4.5 mm, L or R 1.2 mm - 1.3 mm. Electrical stimulating pulse was produced by electrotonic stimulator (type SEN-3201, NIHON, KONDON), passing through isolator, then got constant current output.

Stimulating electrode: unipolar co-axial electrode was used, with a diameter of 0.4 mm, length of naked tip 0.1 mm. Stimulating parameters: monophasic square wave, 0.3 ms duration, 100Hz, 0.1mA-0.3mA for 2min. Time between two stimulations: not less than 15min. For electrolytic lesions, direct positive current 1 mA-1.5mA for 60 s - 90 s. Experiments were carried out for 8 min - 10 min after electrolytic lesion or LCV injection.

### Histological examination

At the end of the experiment, the brain was removed and the position of stimulating and/or lesion sites limits were verified histologically.

### Drugs

Atropine sulfate (No.10 Shanghai Pharmaceutical Factory), phentolamine (CIBA), propranolol (Second Shanghai Pharmaceutical Factory), TRH (Chinese Academy of Med Sci), naloxone (Shanghai Med University).

All data were given as  $\bar{x} \pm s_x$  and analysed statistically by paired Students' *t* test,  $P < 0.05$  was considered to be significant.

## RESULTS

### Electrical stimulation experiment

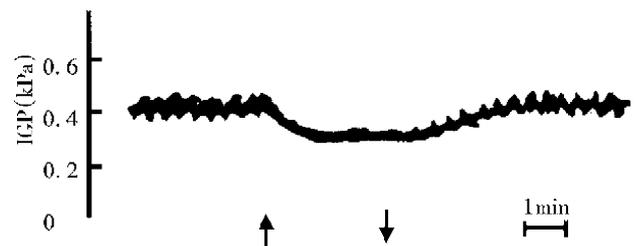
**The effects of ARC stimulation on IGP.** Before stimulation, IGP was quite stable, with 3 cpm gastric phasic contraction. During stimulation, IGP decreased significantly ( $42.2\% \pm 5.4\%$ ,  $n = 15$ ,  $P < 0.01$ ), the latent period was 6 s-8 s and restored  $4.8\text{min} \pm 0.7\text{min}$  after stop of stimulation. The phasic contractions also disappeared during stimulation (Figure 1).

In case the stimulating electrode was not

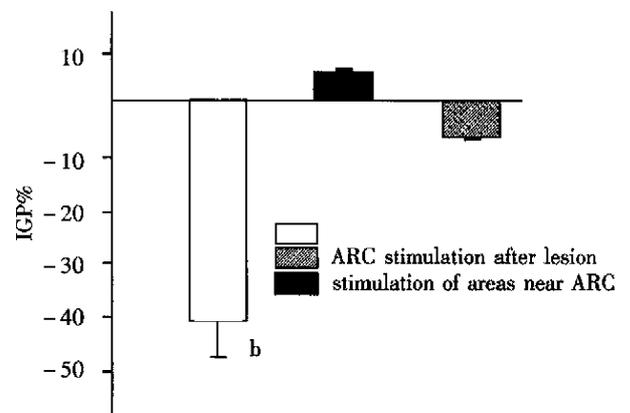
located exactly in the ARC, but near the ARC ( $n = 7$ ), or stimulation took place after electrolytic action ( $n = 5$ ), the decreasing effect of stimulation were all non-significant (Figure 2).

The time course of the variation of blood pressure during stimulation was not correlated with that of IGP.

The above results indicated that the depression effect of IGP during stimulation of ARC unique special, neither due to the diffusion of stimulating current nor to the changes of cardiovascular activities.



**Figure 1** Effect of stimulation of ARC on intragastric pressure (IGP).  $\uparrow/\downarrow$  onset and stop of stimulation.



**Figure 2** Effect of ARC stimulation on IGP,  $^b P < 0.01$ , vs before stimulation.

### Mechanisms analysis of the decreasing effect of ARC stimulation

Centrally, lesion of LC or DR led to IGP decrease by  $15.7\% \pm 3.6\%$  and  $19.6\% \pm 2.5\%$  ( $P < 0.01$ ,  $P < 0.05$ ) compared with control group. After intra-cerebroventricular injection of naloxone, the decreasing effect was not changed.

These results showed that both LC and DR, and  $\beta$ -endorphinergic neurons rich in ARC, were involved in the central mechanism (Table 1).

**Table 1 Effect of various treatments on the reduction of IGP inducet by ARC stimulation**

Treatment	n	IGP(%)
ARC stimulation	15	-42.2±5.4
ICV of saline	8	-43.5±8.1
ICV of naloxone	10	-40.6±6.4
Sham lesion of LC	8	-39.1±6.3
Lesion of LC	8	-15.7±3.6 <sup>b</sup>
Sham lesion of DR	8	-36.3±5.4
lesion of DR	8	-19.6±2.5 <sup>a</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, vs control group.

Peripherally, after vagotomy, the decreasing rate of IGP was  $24.3\% \pm 3.2\%$  ( $P<0.05$ ) compared with the corresponding group, but the decreasing effect was not abolished by i. m. atropine. Extirpation of celiac neural plexus or phentolamine i. m could obviously reduce the decreasing effect, while propranolol i. m) did not. After vagotomy plus sympathectomy, the decreasing effect still existed. Such results indicated that the peripheral pathway of decreasing effect of ARC on IGP were through both sympathetic and vagus nerve. The fibers in vagus mediating the reduction of IGP may be non-cholinergic. Humoral factors may also be involved in the peripheral mechanisms (Table 2).

**Table 2 The peripheral pathways of reduction IGP induced by ARC stimulation**

Treatment	n	IGP(%)
Stimulation of ARC	15	-42.2±5.4
Normal saline	10	-39.1±5.0
Sham operation	6	-40.2±8.0
Cervical vagotomy	13	-25.7±3.4 <sup>a</sup>
Subdiaphragmatic vagotomy	8	-24.3±2.2 <sup>a</sup>
Atropine	9	-30.6±5.0
Extirpation of celiac nerve plexus	11	-19.1±3.8 <sup>a</sup>
Phentolamine	7	-22.8±5.2 <sup>a</sup>
Propranolol	8	-44.4±6.5
Cervical vagotomy+extirpation of celiac nerve plexus	8	-12.9±3.9 <sup>b</sup>
Subdiaphragmatic vagotomy+phentolamine	5	-15.3±3.8 <sup>a</sup>

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, vs stimulation of ARC.

At receptor level,  $\alpha$ -receptor antagonist phentolamine did not significantly increase the IGP of intact rats, but obviously decreased the IGP of rats with vagotomy.  $\beta$ -receptor antagonist propranolol increased the IGP of both intact and vagotomized rats (Tables 3-4).

Such results showed that tonic action of adrenergic nerve in stomach was mainly inhibitory.  $\beta$ -receptor may be present on stomach muscle and mediated inhibition.  $\beta$ -receptor, which came into play through vagus, mediated inhibition while those

presenting on the stomach wall mediated sympathetic excitation.

**Table 3 Effects of phentolamine and propranolol on IGP**

Drug	n	IGP (kPa)		
		BT	AT	%
Normal saline	9	0.735±0.088	0.735±0.078	+0.6±1.6
Phentolamine	8	0.725±0.078	0.735±0.078	+2.3±2.7
Propranolol	8	0.892±0.069	0.960±0.059	+7.9±2.3 <sup>a</sup>

BT: Berore treatment; AT: after treatment, <sup>a</sup>P < 0.05 vs normal saline group.

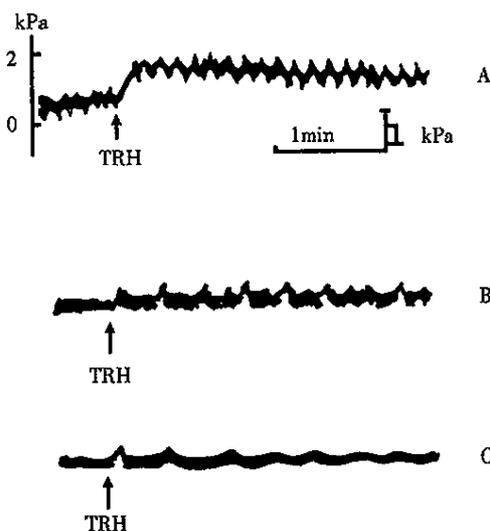
**Table 4 Effect of phentolamine and propranolol on IGP of rats with vagotomy**

Treatment	n	IGP (kPa)		
		BT	AT	%
Sham operation+ phentolamine	7	0.617±0.098	0.588±0.088	-1.1±2.2
Vagotomy+ phentolamine	7	0.8333±0.157	0.707±0.137	-15.6±5.1 <sup>a</sup>
Sham operation+ propranolol	4	0.568±0.069	0.637±0.069	+17.6±2.5
Vagotomy+ propranolol	5	0.784±0.176	0.970±0.216	+28.3±7.9

<sup>a</sup>P<0.05 vs sham operation group.

### Chemical stimulation experiment

Microinjection of thyrotropin releasing hormone (TRH)  $10\mu\text{g}/1\mu\text{l}$  per rat into ARC within 1 min could increase IGP ( $0.53\text{ kPa} \pm 0.08\text{ kPa}$  vs  $1.5\text{ kPa} \pm 0.06\text{ kPa}$ ) by 183.02% ( $n = 10$ ,  $P < 0.001$ ), latent period 8s - 10s, lasting 40min - 50min. The amplitude and frequency of phasic contractions also increased (3 cpm - 4 cpm vs 6cpm) (Figure 3).

**Figure 3 Effect of Microinjection on gastric motility.**

A. Microinjection of TRH.

B. Microinjection of TRH after vagotomy.

C. Microinjection of TRH after atropine(im).

Mechanism analysis of this increasing effects: a. Microinjection of same volume saline ( $n = 7$ ), b. iv TRH ( $n = 7$ ), c. im phentolamine ( $n = 9$ ).

## DISCUSSION

ARC is a very complex nucleus, both of its morphology and physiology.

Morphologically, ARC communicates extensively with its neighboring nuclei of thalamus; hypothalamus; midbrain; brainstem; pituitary gland; and limbic system; and also with extensive intrinsic organization the arcuate cells are small and fusiform, or in more lateral position larger and polygonal. The ARC contains many transmitters and peptides and shows phenomena of colocalization of transmitters.

Physiologically the ARC involved in integrating emotional, endocrine, sensory and pain processing and vegetative homeostatic autonomic functions (chronwall, DEPTLDES, 6: Suppl 2, 1-11, 1985). In this study, we showed that the ARC could modulate gastric motility biphasically, both inhibitory and excitatory, The underlying mechanisms responsible for such differences e.g whether due to the different nature of stimulus, and/or the excitation of different ARC cells, and/or liberation of certain transmitters. etc, await further investigations.

It was reported that the effects of adrenergic drugs on the gastro intestinal smooth muscle depend on the nature and location of the receptors.  $\beta$ -receptor, located in the smooth muscle, after i.m.  $\beta$ -receptor antagonist-propranolol, the IGP showed obviously increased, indicated that the  $\beta$ -receptor mediated inhibition.  $\alpha$ -receptor, is located both in the nerve plexus of gastric wall as well as in the smooth muscles, the former one by means of pre-synaptic modulation inhibite the release of ACh from the vagus nerve endings, hance inhibit the gastric motility, The  $\alpha$ -receptor depend upon the presence of intact vagus. This demonstrated that  $\alpha$ -receptor located in nerve plexus can mediate inhibition. In intact rat, after i.m.  $\alpha$ -receptor antagonist phentolamine, the IGP only showed some increase, but without statistically significant, this result can be

explained as follows: in intact animal with intact vagus nerve, all  $\alpha$ -receptors, both in the nerve plexus and smooth muscle were blocked, The two opposite actions, excitation and inhibition cancelled out each other (Table 3); while in the vagotomized rats, those  $\alpha$ -receptor located in nerve plexus loss their acting target, the inhibitory action could not expressed, so, under the action of phentolamine, the blocking action of those  $\alpha$ -receptors, located in the smooth muscles result in the decrease of IGP (Table 4). This indicated that in intact animal, those  $\alpha$ -receptors located in the smooth muscles, mediate excitation. In short, there are three types of receptors:  $\alpha$ -excitatory,  $\alpha$ -inhibitory, and  $\beta$ -inhibitory. These conclusions came from *in vitro* experiments and used receptor ligands, e. g. adrenaline and noradrenaline, such drugs may alter the physiological body function, thus the conclusion obtained may not be fitted to the normal intact organism.

In view of this, in the present study, we used *in vivo* experiment and specific antagonists in an attempt to analyse the adrenergic receptors on the gastric wall in intact organism under physiological condition.

## REFERENCES

- 1 Ma R, Xu GY. Anatomy and physiology of the hypothalamic arcuate nucleus. *Acta Univer Med Anhui (in Chinese)*, 1992;27(2):162-164
- 2 Ma R, Zhang JX, Xu GY, Qian XI. Effect of electrical stimulation of hypothalamic arcuate nucleus on intragastric pressure in rats. *IBID*, 1989; 24(1):6-8
- 3 Ma R, Xu GY. Regulation of arcuate nucleus on intragastric pressure in rats. *Chin J Appl Physiol (in Chinese)*, 1991;7(3):272-273
- 4 Ma R, Xu GY. Involvement of Locus Coeruleus and Dorsal Raphe nucleus in the reduction of intragastric pressure induced by ARC stimulation. *Acta Physiol Sinica (in Chinese with English abstract)*, 1991;43(5):489-493
- 5 Ma R, Ling WH, Zhang JX, Xu GY. No effect upon cerebroventricular injection of naloxone on reduction of intragastric pressure induced by stimulation of arcuate nucleus in rats. *Acta Univer Med Anhui (in Chinese)*, 1990;25(2): 92-93
- 6 Ma R, Xu GY. Effects of stimulation of arcuate nucleus on intragastric pressure and peripheral pathway analysis in rats. *Acta Physiol Sinica (in Chinese with English abstract)*, 1991;43(4):376-383
- 7 Ma R, Xu GY. An investigation on adrenergic receptors in rat stomach. *IBID*, 1990;42(2):397-400
- 8 Cao Q, Xu GY, Qian XI, Zhang JX. Gastric motive regulation to microinjection of TRH into ARC in rats. *Acta Univer Med Anhui (in Chinese with English abstract)*, 1991;26(3):161-164

# Changes in erythrocyte membrane ATPases and plasma lipid peroxides in upper abdominal surgery under intravenous procaine-balanced anesthesia

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**Subject headings** erythrocyte membrane/enzymology; anesthesia; procaine; Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase; Na<sup>+</sup> K<sup>+</sup>-ATPase; lipid peroxides

## Abstract

**AIM** To observe the changes in erythrocyte membrane ATPases and plasma lipid peroxides (LPO) patients with in abdominal surgery under intravenous procaine<sup>a2</sup> balanced anesthesia.

**METHODS** By determining the ATPase activities of erythrocyte membrane, effects of upper abdominal surgery under intravenous procaine-balanced anesthesia on the function of erythrocytes were observed in 15 patients undergoing cholecystectomy and gastrectomy (5 males and 10 females, aged 45.9±10.20 years and weighed 60.60kg±11.93kg). All patients were free from severe renal, hepatic, pulmonary, cardiac, metabolic and endocrinological diseases and acute infection for at least 2 weeks before surgery. Patients receiving any drug known to affect carbohydrate metabolism prior to anesthesia were excluded from the study.

**RESULTS** Erythrocyte membrane Na<sup>+</sup>, K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase, Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase activities were not significantly changed 60min-90min after incision as compared with 30min before anesthesia, but were decreased markedly 10min and 24 hours after completion of operation ( $P<0.01$ ). Plasma lipid peroxides (LPO) were

increased significantly 24 hours after surgery ( $P<0.01$ ) following an initially marked but transient reduction. Plasma LPO changes were not correlated with erythrocyte membrane ATPase activities,  $r = -0.0396$ ,  $-0.0097$  and  $0.4383$ , respectively ( $P>0.05$ ).

**CONCLUSION** Abdominal surgical trauma under intravenous procaine-balanced anesthesia may be associated with the decreased ATPase activities of erythrocyte membrane and increased LPO in plasma.

## INTRODUCTION

Red blood cells are responsible for the transportation of oxygen and carbon dioxide into and out of tissues and organs of the body. Its shape, deformability, exchanges of intra and extra-cellular electrolyte homeostasis and membrane integrity, and the normal erythrocyte functions all depend upon normal activities of the three kinds of ATPases on erythrocyte membranes. It has been demonstrated that anesthesia and surgical trauma can lead to a significant decrease in the activities of these ATPases<sup>[1-3]</sup>. The mechanism of decreased activities of ATPases, however, is still unclear. In the study, we observed perioperative alterations in the activities of erythrocyte membrane Na<sup>+</sup>, K<sup>+</sup>-ATPase, Mg<sup>2+</sup> ATPase and Ca<sup>2+</sup>, Mg<sup>2+</sup> ATPase and plasma lipid peroxides (LPO), attempting to probe into the mechanism mentioned above.

## MATERIALS AND METHODS

Fifteen patients underwent elective upper abdominal surgery (5 males and 10 females, aged 45.90±10.20 years and weighed 60.60kg±11.93kg) including cholecystectomy (14) and gastrectomy (1). All patients had been free from significant renal, hepatic, pulmonary, cardiac, metabolic and endocrinological diseases and acute infection for at least two weeks before surgery. Patients receiving any drugs known to affect carbohydrate metabolism prior to anesthesia were excluded from this study.

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\*Project supported by the National Natural Science Foundation of China, No.38970700.

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Received 1998-04-01

After fasting for 12-14 hours and premedicating with phenobarbitone 0.1 g and atropine 0.5 mg intramuscularly 30min before anesthesia, general anesthesia was induced with intravenous diazepam 0.2 mg·kg<sup>-1</sup>, and thiopentone 4 mg·kg<sup>-1</sup> - 6 mg·kg<sup>-1</sup> and fentanyl 0.1 mg, 100 mg succinylcholine (SCC) were given to facilitate intubation. Anesthesia was maintained with an intravenous combined solution of 1% procaine and 0.06% SCC at a rate of 0.1ml·kg<sup>-1</sup>·min<sup>-1</sup>. Intravenous fentanyl 10 μg·kg<sup>-1</sup> and droperidal 0.15mg·kg<sup>-1</sup> were administered prior to incision. Additional thiopentone was given if needed. Lactated Ringer's solution was used during the intra and post operative periods. Ventilation was controlled to a PET CO<sub>2</sub> of 4.67kPa-5.33kPa at the same time, electrocardiogram (ECG), heart rate and blood pressure were continuously monitored with Datex Cardiacap.

Venous blood were collected in heparin (20 U/ml) 30min before induction of anesthesia (T0), 60min-90min after incision (T1), 10min (T2) and 24 hours (T3) after completion of operation. Plasma samples were collected for the detection of LPO (TBA method). According to Beutler's method<sup>[4]</sup>, the packed red blood cells were filtered through a special cotton column to remove white blood cells and platelets, and subsequently washed and centrifuged three times with 50mmol/L tris-EDTA-NaCl buffer (EDTA -1mmol/L and NaCl 150mmol/L, pH 7.4), and finally the packed red blood cells were made into 50%-60% red blood cell suspension (au procedures were performed below 4°C) and preserved separately in liquid nitrogen for assay.

Samples taken out of liquid nitrogen were put into ice-water to melt for assay. Red blood (0.5ml) cell suspension was drawn, diluted precisely with more than 20-fold red cell membrane washing solution (10mmol/L tris-EDTA buffer, EDTA

1mmol/L, pH 7.4, 0°C-4°C) and centrifuged (10000r/min for 20min, at 0°C-4°C) for 4 times. Opalescent red cell membranes were obtained and made into 2ml red blood cell membrane suspension with a 50mmol/L tris-sucrose-buffer (sucrose 75mmol/L, pH 7.4, 0°C-4°C). According to the modified Lowry's method<sup>[5]</sup>, the contents of membrane protein were determined and adjusted to 1.5g/L approximately. The activities of red blood cell membrane Na<sup>+</sup>, K<sup>+</sup> ATPase, Mg<sup>2+</sup> ATPase and Ca<sup>2+</sup>, Mg<sup>2+</sup>-ATPase were determined by Charalambous' method<sup>[6]</sup> and expressed by nmolPi·mgPro<sup>-1</sup>·min<sup>-1</sup>.

## RESULTS

### *Changes in activities of red blood cell membrane ATPase*

There were no significant changes of the activities of three kinds of ATPases on red blood cell membranes since the initial anesthesia to 60min-90min after incision. Up to 10min after the completion of surgery, however, Na<sup>+</sup>, K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase and Ca<sup>2+</sup>, Mg<sup>2+</sup> ATPase activities decreased significantly to 2.962 ± 1.245, 4.125 ± 1.006 and 16.642 ± 11.346 from 3.745 ± 1.233, 5.050 ± 1.115 and 32.705 ± 9.585 nmolPi·mgPro<sup>-1</sup>·min<sup>-1</sup> (*P*<0.001, respectively) and were still at a lower level 24 hours after completion of surgery

### *Changes in plasma LPO*

Plasma LPO increased significantly following an initially marked but transient reduction. 60min-90min after incision, plasma LPO decreased to 8.831 ± 1.180 from 9.815nmolMDA/ml ± 1.100 nmolMDA/ml prior to anesthesia (*P* < 0.05), and then gradually increased and reached to the peak (11.782 nmolMDA/ ml ± 1.218 nmolMDA/ ml) 24 hours after surgery.

**Table 1 Results of red blood cell membrane ATPases and plasma LPO**

	T0	T1	T2	T3
Na <sup>+</sup> , K <sup>+</sup> -ATPase (nmolPi·mgPro <sup>-1</sup> ·min <sup>-1</sup> )	3.745±1.233	3.546±1.466	2.962±1.165 <sup>b,c</sup>	2.900±1.055 <sup>a,b</sup>
Mg <sup>2+</sup> -ATPase (nmolPi·mgPro <sup>-1</sup> ·min <sup>-1</sup> )	5.050±1.150	4.905±1.192	4.125±1.008 <sup>b,c</sup>	3.978±1.183 <sup>b,c</sup>
Ca <sup>2+</sup> , Mg <sup>2+</sup> -ATPase (nmolPi·mgPro <sup>-1</sup> ·min <sup>-1</sup> )	32.71±5.85	32.93±7.61	16.64±11.35 <sup>b,c</sup>	10.22±6.46 <sup>b,c,d</sup>
LPO (nmolMDA·ml <sup>-1</sup> )	9.815±1.100	8.831±1.180 <sup>a</sup>	9.135±1.174	11.78±1.22 <sup>b,c,e</sup>

Compared with T<sub>0</sub>, <sup>a</sup>*P*<0.05; <sup>b</sup>*P*<0.01; compared with T<sub>1</sub>, <sup>c</sup>*P*<0.01; compared with T<sub>2</sub>, <sup>d</sup>*P*<0.05; <sup>e</sup>*P*<0.01.

## DISCUSSION

The result showed that the activity of red blood cell membrane  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase decreased significantly 10min and 24 hours after completion of the operation. It was in accordance with other reports. Changes in activities of  $\text{Mg}^{2+}$ -ATPase and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -ATPase were similar to  $\text{Na}^+$ ,  $\text{K}^+$  ATPase. The mechanisms why the activities of red blood cell membrane ATPases are affected postoperatively, however, are still unclear. There is no doubt that the inhibition of ATPases, to some extent, reflects the fact that the noxious factors were produced in the blood during and after anesthesia and/or surgical trauma, which may directly and/or indirectly destruct or inhibit red cell membrane functions or ATPase activities. It was demonstrated that many lipid-soluble anesthetics and tranquilizers reversibly bind themselves to red blood cell membranes, and this is accompanied by alterations in membrane lipid-protein interaction and conformation, and alterations of coagulo-soluble state between the cytoplasmic side of the red blood cell membrane and cytoplasm, leading to decrease of membrane fluidity and activities of various membrane bound enzymes. Hudgins and Bond<sup>[7]</sup> reported that highly or lipid-soluble low anesthetics could reduce the activities of various ion pumps by competitive or non-competitive mechanism, such as dibucaine and procaine. However, their inhibitive mechanisms differ from each other according to the various ion pumps involved. For instance, the highly lipid soluble agents (e.g. dibucaine) inhibit various enzymes by nonspecific binding to red blood cell membranes. On the other hand, low lipid-soluble agents, such as procaine, might interact more specifically with monovalent cation binding sites or with one of the two conformations. The size of inhibited enzyme activities is also greatly correlated with the effect and toxicity of the anesthetics employed. Red blood cell membranes might be directly or indirectly destructed by disturbances in hormone homeostasis, and the release of various body fluid factors such as complements, oxygen radicals and LPO, and the increased activities of phospholipidase  $\text{A}_2$ <sup>[8]</sup> after anesthesia and/or surgical trauma. The injured membranes might be associated with disturbances in renewal of normal red cell membrane lipid and alterations in membrane lipid-protein interactions and conformations, together with changes in red blood cell morphology and a decrease in deformability, which finally leads to a decrease in membrane fluidity and enzymatic activities.

It was demonstrated that significantly inhibited activities of red cell membrane ATPases and

significant increases in plasma LPO after surgery were found in surgical patients under intravenous balanced anesthesia<sup>[2]</sup>. Palmer<sup>[9]</sup> suggested that decreased activity of  $\text{Na}^+$ ,  $\text{K}^+$ ATPase might be correlated with direct destructive effects of some fluid factors such as complements, oxygen radicals and/or LPO on enzymatic molecular conformations and functions. Our data, however, showed that decreased activities of  $\text{Na}^+$ ,  $\text{K}^+$  ATPase,  $\text{Mg}^{2+}$  ATPase and  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  ATPase were not related to perioperative alterations in plasma LPO ( $r = -0.0396$ ,  $-0.0097$  and  $-0.4383$ ,  $P > 0.05$ , respectively). Plasma LPO increased significantly following an initially marked but transient reduction. It was likely that significantly decreased LPO during intraoperative period was related to blood dilutions caused by intraoperative massive infusion, and/or to inhibited LPO oxidation induced by protective effects of anesthetics such as procaine hydrochloride on red blood cell membranes. The significantly increased LPO after surgery demonstrated that noxious fluid factors such as LPO, complements and oxygen radicals were produced during and after anesthesia and/or surgical trauma due to the absorption of necrotizing tissues and broken cells from the sites of surgical trauma, inflammatory and non-inflammatory responses after surgical trauma, potential injuries resulting from highly fractional oxygen, and direct or indirect effects of some anesthetics such as thiopentone. These factors may directly or indirectly result in the activation of the complement system in the body and the loss of neutrophil granulocyte functions, and then induce a series of oxidative reactions "respiratory bursts" by activated complements, lysosome enzymes, hydrogen peroxidase and myeloperoxidase<sup>[10]</sup>. As a result, plasma LPO increased significantly after surgery. The increased LPO may further lead to decreased activities in red blood cell membrane ATPases and other enzymes.

The intra- and post-operative inhalation of high fractional oxygen (more than 80%) may be directly associated with potential injuries to red blood cell membranes ATPases due to increases in superoxide anion ( $\text{O}_2^-$ ) and other toxic oxygen intermediates<sup>[11-13]</sup>.

The energy of ATPases directly depends upon intracellular ATP, which is only synthesized in the glycolytic pathway, and this energy accounts for about 30% - 50% of the total energy consumption of red blood cells. Consequently, decreased synthesis of intracellular ATP may be directly accompanied by decreased activities in red blood cell membrane ATPases and a reduction in red blood cell

deformability<sup>[14-16]</sup>.

The decreased activities of membrane ATPases may result in disturbances in intra and extra-cellular electrolyte homeostasis and in increased permeability of red blood cell membranes by various ions and small molecular substances, and then induce massive potassium exflux and sodium influx<sup>[1,16]</sup>, thereby leading to a reduction of red cell membrane fluidity and deformability and an increase in membrane fragility. Such alterations may directly affect gas exchanges between the blood and tissues, and microcirculatory functions, resulting in failures in microcirculation and in multiple organ system.

## REFERENCE

- 1 Hirayama T, Herlitz H, Jonsson O, Roberts D. Deformability and electrolyte changes of erythrocytes in connection with open heart surgery. *Scand J Thor Cardiovasc Surg*, 1986;20(3):253-259
- 2 Jia B, Zhang ST, Li GF. Lipid peroxidation injury to red blood cells during extracorporeal circulation: mechanism and protection. *Chung Hua I Hsueh Tsa Chih*, 1994;74(11):689-691,711
- 3 Ildan F, Oner A, Polat S, Isbir T, Gocer AT, Kaya M, Karadayi A. Correlation of alterations Na<sup>+</sup>, K<sup>+</sup>/Mg<sup>2+</sup>-ATPase activity, lipid peroxidation and ultrastructural findings following experimental spinal cord injury with and without intravenous methylprednisolone treatment. *Neurosurg Rev*, 1995;18(1):35-44
- 4 Beutle E, Blume KG, Kaplan JC, Lohr GW, Ramot B, Valentine WN. ICHS: Recommended methods for red cell enzyme analysis. *Br J Haematol*, 1997;35(3): 331-340
- 5 Markwell MAK, Hass SM, Bieber LL, Tolbert NE. A method of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem*, 1978;87(1):206-210
- 6 Charalambous BM, Mir MA. An improved procedure for the preparation and measurement of Na<sup>+</sup>, K<sup>+</sup>ATPase in human erythrocytes. *Biochim Biophys Acta*, 1982;691(1):71-82
- 7 Hudgins PM, Bond GH. Inhibition of dog kidney Na<sup>+</sup>, K<sup>+</sup>-ATPase activity by procaine, tetracaine and dibucaine. *Biochem Pharmacol*, 1984; 33 (11): 1789-1996
- 8 Yamaguchi H, Allers M, Roberts D. The effect of urea on red cell deformability during cardiopulmonary bypass. *Scand J Thor Cardiovasc Surg*, 1984;18 (2):119-122
- 9 Palmer GC, Palmer SJ, Christie-Pope BC, Callahan AS, Taylor MD, Eddy LJ. Classification of ischemic-induced damage to Na<sup>+</sup>, K<sup>+</sup>-ATPase in gerbil for brain. *Neuropharmacology*, 1982;24(6):509-516
- 10 El-Maallam H, Fletcher J. Effects of surgery on neutrophil granulocyte function. *Infect Immun*, 1981;32(1):38-41
- 11 Spragg RG, Hinshaw DB, Hyslop PA, Schraufstatter IU, Cochrane CG. Alteration in adenosine triphosphate and energy change in cultured endothelial and P388D1 cells after oxidant injury. *J Clin Invest*, 1985;76(4):1471-1476
- 12 Rohn TT, Hinds TR, Vincenzi FF. Inhibition of the Ca pump of intact red blood cell by tbutyl hydroperoxide: importance of glutathione peroxidase. *Biochim Biophys Acta*, 1993;1153(1):67-68
- 13 Hebbel RD, Shalev O, Foker W, Pank BH. Inhibition of erythrocyte Ca<sup>2+</sup>-ATPase by activated oxygen through thiol and lipid-dependent mechanisms. *Biochem Biophys Acta*, 1986;862(1):8-16
- 14 Marikovsky Y. The cytoskeleton in ATP-deleted erythrocytes: the effect of shape transformation. *Mech Ageing Dev*, 1996;86(2):137-144
- 15 Bossi D, Giardina B. Red cell physiology. *Mol Aspects Med*, 1996; 17 (2): 117-128
- 16 Weed RL. Metabolic dependence of red cell deformability. *J Clin Invest*, 1969;48 (4):795-804

# Sequencing of PCR amplified HBV DNA pre-c and c regions in the 2.2.15 cells and antiviral action by targeted antisense oligonucleotide directed against sequence \*

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**Subject headings** hepatitis B virus; gene, viral; DNA, viral; antisense oligonucleotide; gene expression; polymerase chain reaction

## Abstract

**AIM** To study the specific inhibition of HBV gene expression by liver-targeting antisense oligonucleotide (ASON) directed against pre-c and c regions in a sequence specific manner.

**METHODS** According to the result of direct sequencing of PCR amplified products, a 16-mer phosphorothioate analogue of the antisense oligonucleotide (PS-ASON) directed against the HBV U-5-like region was synthesized and then linked with one liver-targeting ligand, the galactosylated poly-L-lysine. Their effect on the expression of HBV gene was observed using the 2.2.15 cells.

**RESULTS** HBV DNA in the 2.2.15 cells was from HBV with surface antigen subtype ayw 1 by sequencing so that antisense oligonucleotides could bind specifically to the target sequence through base pairing. Under the same experimental conditions, the inhibitory rates of PS-ASON to HBsAg and HBeAg were 70% and 58% at a concentration of 10  $\mu\text{mol/L}$ , while by ligand-PS-ASON they were 96% and 82%, the amount of HBV DNA in cultured supernatant and cells was reduced significantly. An unrelated sequence oligonucleotide showed no effectiveness.

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\*Project supported by the National Natural Science Foundation of China, No.39370648.

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Received 1998-05-11

All the oligonucleotides had no cytotoxicity.

**CONCLUSION** Antisense oligonucleotides complexed by the liver-targeting ligand can be targeted to cells via asialoglycoprotein receptors, resulting in specific inhibition of HBV gene expression and replication.

## INTRODUCTION

Many studies have shown that antisense oligonucleotides (ASONS) can efficiently inhibit HBV DNA replication and expression *in vitro* and may become a new generation of anti-HBV drugs<sup>[1]</sup>. However, as a potential therapeutic agent, synthetic ASONS must fulfil three main requirements (i.e., the three S rule): ① solubility, they must be water soluble and yet cross the lipophilic cell membrane; ② stability, they must resist enzymatic degradation to reach the target at an effective concentration; and ③ selectivity, they must bind specifically to the target sequence through base pairing.

Basing on the three S rule, we first synthesized a 16mer phosphorothioate analogue of ASON (PS-ASON) directed against the HBV U5-unlike region according to the sequencing result, and then linked PS-ASON with one liver-targeting ligand, the galactosylated poly-L-lysine (Gal-PLL). The effect of PS-ASON and ligand PS-ASON on the expression of HBV gene was observed and compared by using the 2.2.15 cells. The results of this experiment and reported below.

## MATERIALS AND METHODS

### PCR and direct sequencing of amplified products

HBV DNA in the 2.2.15 cells was tested by PCR. Primers were designed according to the pre c and c regions of HBV genome. The sequences of primers are P<sub>1</sub> (5'-TTCCCGATACAGAGCTGAGGCC) and P<sub>2</sub> (5'-AAGGTCTTTGTAGGAGGC).

PCR amplified products were purified with Magic™ PCR Preps DNA pure system (Promega). Direct sequencing of pure products was carried out according to the manual of Pharmacia T<sub>7</sub> kit.

### Cell culture

The 2.2.15 cell was the hepatoblastoma cell line HepG2 transfected with cloned HBV DNA. Various parameters of the replicative cycle can be quantitated in the transfected HepG<sub>2</sub> cell e.g., the secretion of HBsAg or HBeAg and the amount of episomal HBV DNA. Cells were grown in RPMI1640 (Sigma) medium supplemented with 15% fetal bovine serum, 2mmol/L L-glutamine, 10<sup>5</sup>U/L penicilin, 10<sup>5</sup>U/L streptomycin and the neomycin analogue G418 (380mg/L, Sigma). Cell cultures were maintained at 37°C in 5% CO<sub>2</sub> atmosphere.

### Preparation of targetable antisense DNA

Conjugate Gal-PLL was prepared according to the reductive amination<sup>[2]</sup>. Laotose and poly-L-lysine were reacted by using borohydride sodium (the molar ratio of lactose/ poly-L-lysine/ borohydride sodium were 50:1:300). Sugar and amino group was determined at a molar ratio of 10:1 in the reaction products (Gal-PLL) according to the Lee YC's method<sup>[3]</sup>.

A 16-mer oligodeoxynucleotide, complementary to U<sub>5</sub>-like region, corresponding to nucleotides 1980-1905 of the sequenced viral genome, was synthesized on automated nucleotide synthesizer (Applied Biosystems) using phosphorothioate linkages. As a control, a random 16-mer sequence was prepared in an identical fashion. Antisense DNA was titrated with conjugte to form soluble complex using an agarose gel retardation system as described previously<sup>[4]</sup>.

### Antisense DNA and viral gene expression

To determine the effect of antisense DNA on viral gene expression, after the 2.2.15 cells were seeded for 60h, culture fluid was taken out. Then the cells were incubated for 72h in medium containing antisense DNA alone, complexed antisense DNA, complexed random DNA and medium alone. All media containing DNA, the DNA concentration was 10μM. The medium was changed to RPMI1640 without oligodeoxynucleotides and the cells culture was continued for another 72h. Supernatant (200μl) was collected and assayed for HBsAg and HBeAg by ELISA (ABC) method as described by the manufacturer. HBV DNA was tested by dot hybridization.

## RESULTS

### PCR and sequencing

Total DNA was isolated from the 2.2.15 cells. PCR amplification of pre-c and c regions was done with P<sub>2</sub> and P<sub>2</sub> primers. The length of amplified sequence was limited at 260 base pairs by the primers. Agarose gel electrophoresis showed that amplified products located between nucleotides 221 and 298,

which corresponded well with our needs (Figure 1).

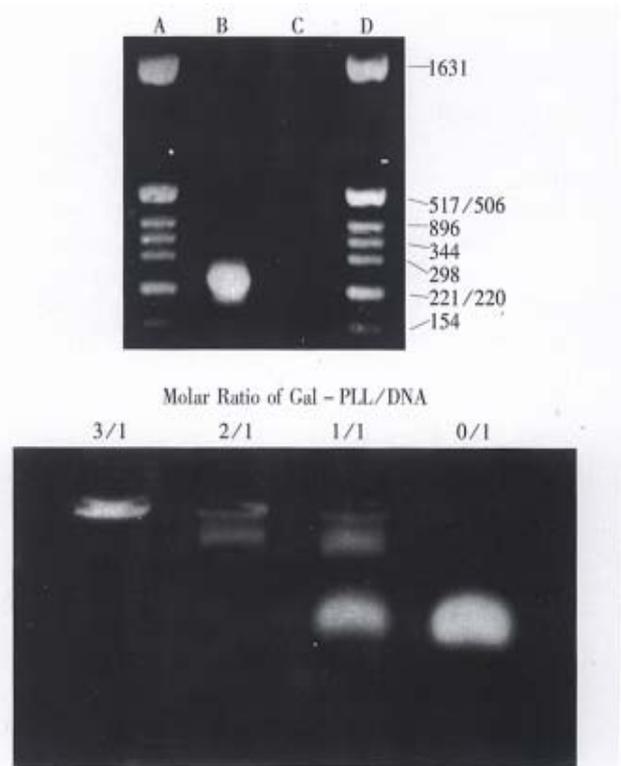
The purified PCR products were directly sequenced with T<sub>7</sub> DNA sequencing system. One hundred and thirty-two base pairs could be read on the film of autoradiograph as follows:

```
CCAGCACCATGCAACTTTTTACCTCTGCC
TAATCATCTCTTGTTCATGTCCTACTGTTC
AAGCCTCCAAGCTGTGCCTGGGTGGCTTT
GGGGCATGGACATCGACCCTTATAAAGAAT
TTGGAGCTACTG
```

The sequence was the same as what HBV (ayw1 subtype) had, including pre-c sequence (1816-1902), U<sub>5</sub>-like region (1857-1918) and part of poly-A addition single sequence (1919-1962).

### Molar ratio of Gal-PLL/DNA

Purified Gal-PLL was incubated with DNA by means of increasing concentrations. The extent of Gal-PLL: DNA complex formation was measured by agarose gel electrophoresis. Judged on the basis of charge neutralization, as seen by the reduction of electrophoretic mobility of the DNA, interaction between the Gal-PLL and DNA started at a molar ratio of 1:1 (Figure 2). The DNA migration is completely retarded at molar ratios of 2:1 and greater.



**Figure 1** Amplification of HBV DNA in the 2.2.15 cells with PCR. The PCR products were resolved on 1.5% agarose gel. A and D: Marker (PBR322/Hinf I); B: HBV DNA in cells; C: HBV DNA (adr subtype)

**Figure 2** Agarose gel electrophoresis analysis of complex formation. DNA: Gal-PLL complex was made with increasing molar ratios of Gal-PLL to DNA; 0/1 represents DNA only. DNA was visualized with ethidium bromide.

### Effect of antisense DNA HBV gene expression

To determine whether the targeted antisense DNA was functional, effects on HBV gene expression were evaluated. Table 1 shows that the influence of oligodeoxynucleotides on the secretion of HBsAg and HBeAg was determined 8.5 days after seeding. HBsAg and HBeAg secretions from cells were inhibited by 70% and 50% respectively at antisense DNA alone concentration of 10 $\mu$ M. The inhibition rates gradually increased to 96% and 82% when the complexed antisense DNA (Gal-PLL:ASON) was used. HBsAg and HBeAg secretions were not markedly inhibited by the complexed random DNA (19% and 10%). The amount of HBV DNA in the culture supernatant and cells was reduced significantly with complexed antisense DNA compared with the complexed random DNA and antisense DNA alone (data not shown).

**Table 1 Effect of antisense DNA on HBsAg and HBeAg synthesis in the 2.2.15 cells (P/N value  $\bar{x}\pm s$ )**

Treatment	HBsAg	Inhibitory rate (%)	HBeAg	Inhibitory rate (%)
Untreated control	9.40 $\pm$ 0.16		15.10 $\pm$ 0.15	
Antisense DNA alone	4.30 $\pm$ 0.25	70	7.60 $\pm$ 1.10	58
Complexed antisense DNA	2.40 $\pm$ 0.26	96	4.50 $\pm$ 0.42	82
Complexed random DNA	8.10 $\pm$ 0.13	19	13.80 $\pm$ 0.76	10

### DISCUSSION

Specificity of antisense oligonucleotides depends on the selectivity of Watson-Crick or other types of base pairing. The affinity associated with a mismatched base pair varies with the function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. Gibbs free energy of binding induced by a single mismatch decreased from 0.2 to 4.9kcal/mol at 100mM NaCl. Thus, a single base mismatch may result in affinity change by approximately 500 fold<sup>[5]</sup>. We identified that HBV DNA in the 2.2.15 cells were from HBV with surface antigen subtype syw-2 by sequencing so that antisense DNA could bind specifically to the target sequence through base pairing without any mismatch.

Oligonucleotides may be degraded by exonucleases and endonucleases, which exist extensively in serum, cells and fluid of body. Work from many laboratories has demonstrated that a wide range of modification may be used to enhance the stability of oligonucleotides. Phosphorothioate oligonucleotides have been shown to be extremely stable in media, cells and cell extracts, serum, various tissues, urine and stable to most nucleases. In this experiment, we chose phosphorothioate analog to study.

Phosphorothioates are negatively charged, but because of the sulfur atoms they may be slightly more lipophilic than phosphodiesteres and tend to bind nonspecifically to serum proteins, those may effect on the action of oligonucleotides.

Many techniques have been developed to introduce foreign DNA into cells *in vitro*. For example, methods such as electroporation, microinjection, liposomes. Some of these methods have been used successfully *in vivo*. The approach on specificity of DNA delivery has been to take advantage of cell surface receptors as natural internalization sites for targeting substances to specific cells. There have been particularly interests in liver cells because of the presence on these cells of unique receptors that are able to recognize galactose-terminal (asialo) glycoproteins. Wu GY *et al*<sup>[6]</sup> reports that asialoglycoprotein (asialoorosomucoid, ASOR)-poly(L-lysine) conjugates can be used to target genes in a soluble form resulting in specific delivery to cells possessing surface asialoglycoprotein receptors.

In this study an artificial ligand, Gal-PLL conjugate, was used for targeting antisense DNA to the 2.2.15 cells. With increasing proportions of conjugate in the sample, more DNA was retained by the Gal-PLL conjugate in the wells. A 2:1 molar ratio of the conjugate to DNA optimized the complex formation, and this molar ratio was the same as what Wu GY *et al* reported using ASOR<sup>[7]</sup>.

In the same experimental conditions, the inhibitory effects of HBsAg and HBeAg by PS-ASON were 70% and 58%, while by ligand PS-ASON were 96% and 82%. HBsAg and HBeAg secretion were not markedly inhibited by the random DNA. The results indicate that antisense oligonucleotides complexed by a soluble DNA-carrier system can be targeted to cells via asialoglycoprotein receptors in specific inhibition of HBV gene expression and replication.

Gal-PLL, as a hepatotropic carrier of DNA, has some advantages: ① It is obtain by a simple synthetic method; ② It might not cause allergy *in vivo*. So it is very important to study synthetic low-molecular weight carriers.

### REFERENCES

- 1 Korbe B, Wells F, Jones K, Engle R, Buckler-white A, Gerin I. Inhibition of hepatitis B virus replication *in vitro* by antisense oligonucleotides. *Antiviral Res*, 1994;20(Suppl 1):78
- 2 Schwartz BA, Gray GR. Proteins containing reductively aminated disaccharides synthesis and characterization. *Arch Biochem Biophys*, 1997;181(2):542-549
- 3 Lee YC. Synthesis some cluster glycosides suitable for attachment to protein or solid matrices. *Carbohydr Res*, 1978;67(2):509-514
- 4 Critiano RJ, Smith LC, Woo SL. Hepatic gene therapy: Adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. *Proc Natl Acad Sci USA*, 1993;90(12):2122-2126
- 5 Crooke ST. Therapeutic applications of oligonucleotides. *Annu Rev Pharmacol Toxicol*, 1992;32(3):329-376
- 6 Wu GY and Wu CH. Evidence for targeted gene delivery to HepG<sub>2</sub> hepatoma cells *in vitro*. *Biochem*, 1988;27(6):887-892
- 7 Wu GY, Wu CH. Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligonucleotides. *J Biol Chem*, 1992; 267 (18): 12436-12439

# Protection of gastric mucosa from ethanol induced injury by recombinant epidermal growth factor in rats \*

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**Subject headings** stomach ulcer; gastric mucosa; epidermal growth factor-urogastrone;

## Abstract

**AIM** To determine whether recombinant human epidermal growth factor (rhEGF) can protect gastric mucosa against ethanol induced injury in rats.

**METHOD** Fifty-four SD rats weighing 200g - 500g each were divided into six groups after fasting for 24 hours. Three groups received different doses of oral rhEGF (30, 60 and 120  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ ), one group was given cimetidine, one subcutaneous rhEGF (rhEGF IV) and one received saline as control.

**RESULTS** Acute gastric dilatation developed in the control and cimetidine groups and bloody gastric juice was found in the control group. The ulcer index was 58 in control group, 53 in rhEGF I, 46 in rhEGF II ( $P<0.01$ ), 11 in rhEGF III ( $P<0.01$ ), 19 in rhEGF IV ( $P<0.01$ ), and 39 in cimetidine group ( $P<0.05$ ).

**CONCLUSION** rhEGF protected gastric mucosa against ethanol induced damage. The effect was dose-dependent with blood levels of epidermal growth factor (EGF) at a dosage range of 60  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  - 20  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ . It was more effective by injection than via oral route at the same dosage.

## INTRODUCTION

Epidermal growth factor (EGF) is a single-chain polypeptide that is secreted by submandibular and Brunner's glands and is a powerful mitogen and an inhibitor of gastric acid secretion. Recent studies demonstrated that EGF is also capable of protecting the gastric mucosa from the damage caused by various irritants and promoting healing of chronic gastric and duodenal ulcers<sup>[1,2]</sup>. This study was designed to determine whether recombinant human EGF (rhEGF) could protect gastric mucosa against ethanol induced injury in rats. Our aim is to find a new method of gastric mucosa protection which may serve as a treatment for peptic ulcer.

## MATERIAL AND METHODS

SD rats weighing 200 to 250g were used in the study of gastric protection and gastric ulcer.

**Experiments with acute gastric mucosal lesion.** Acute gastricleision was induced by absolute ethanol in experiments with three sets of rats (control, rhEGF and cimetidine). The control group was given 0.9% saline for 3 days and then 100% ethanol (9 rats). Cimetidine group was treated with cimetidine and then with 100% ethanol (9 rats). The rhEGF group was divided into 4 subgroups, each received oral rhEGF 30, 60 or 120  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  except one subgroup which was given 60  $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$  rhEGF subcutaneously. Three days later 1mL of absolute ethanol was administered to all rats. One hour after ethanol administration, the rats were killed by cervical fracture. The stomach was dissected out and opened along the greater curvature, and the area of ulceration was determined. The amount of damage was expressed as ulcer index. All planimetric determinations were performed blindly by the same observer.

**The measurement of serum EGF and gastrin level.** Rat blood of 2 ml - 4 ml was collected in tube without anticoagulant. 3 hours later the serum was collected and EGF and gastrin measured. EGF kit was obtained from Amersham, U.K. and Depu Co, China.

## RESULTS

### Gastric ulcer index

Two rats in cimetidine group died in less than 1 hour. After administration of ethanol, acute gastric dilatation developed in the control and cimetidine groups and bloody gastric juice was found in the control group. The ulcer index was 58 in the control group, 53 in rhEGF I, 46 in rhEGF II ( $P<0.01$ ), 11

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\*Project supported by the National Science Committee of China, No. 96-C02-01-01.

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Received 1998-04-01

in rhEGF III ( $P < 0.01$ ), 39 in rhEGF IV ( $P < 0.01$ ), and 39 in cimetidine group ( $P < 0.05$ , Table 1).

**The levels of serum EGF.** The levels of serum EGF in rhEGF III, IV and cimetidine groups were higher than that of control group ( $P < 0.05$ ), especially in rhEGF III group ( $P < 0.001$ ). After giving the same dose of rhEGF, the serum EGF level in the subcutaneous group was significantly higher than that of the oral groups (Table 2).

**The values of serum gastrin.** The values of serum gastrin in rhEGF IV group was increased significantly than the control group ( $P < 0.05$ , Table 3).

**Table 1 Index of gastric ulcer in all groups (M)**

Groups	n	Range	Index
Control (p.o)	9	34-79	58
rhEGF I (30 $\mu$ g, p.o)	9	6-69	53
rhEGF II (60 $\mu$ g, p.o)	9	25-59	46 <sup>b</sup>
rhEGF III (120 $\mu$ g, p.o)	9	0-26	11 <sup>b</sup>
rhEGF IV (60 $\mu$ g, s.c.)	9	0-29	19 <sup>b</sup>
Cimetidine (p.o)	7	32-52	39 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , vs control group; <sup>b</sup> $P < 0.01$ , vs control group.

**Table 2 The levels of serum EGF in all groups (ng/L)**

Groups	n	Frang	$\bar{x} \pm s$
Control	9	0.51-0.72	0.63 $\pm$ 0.09
rhEGF I	9	6-69	53
rhEGF II	9	25-59	46 <sup>a</sup>
rhEGF III	9	0-26	11
rhEGF IV	9	0-29	19
Cimetidine	7	31-52	39 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , vs control group; <sup>b</sup> $P < 0.01$ , vs control group.

**Table 3 The values of serum gastrin in all groups (ng/L)**

Groups	n	Range	$\bar{x} \pm s$
Control	6	40-112	75 $\pm$ 28
rhEGF I	7	50-160	83 $\pm$ 39
rhEGF II	7	46-139	93 $\pm$ 43
rhEGF III	7	49-170	80 $\pm$ 42
rhEGF IV	6	81-283	149 $\pm$ 83 <sup>a</sup>
Cimetidine	6	18-101	49 $\pm$ 28

<sup>a</sup> $P < 0.05$ , vs control group.

## DISCUSSION

EGF is a 53-aminoacid peptide isolated for the first time from male mouse salivary glands by Cohen *et al* in 1962. Later it was also found in submandibular and duodenal Brunner's glands. Recent studies demonstrated that EGF is a powerful mitogen which is capable of promoting DNA, RNA and protein synthesis and inhibiting gastric acid secretion. The effect of EGF on gastric secretion and cell proliferation suggests that EGF could be responsible for maintaining the structural integrity of the gastrointestinal mucosa and preventing mucosal injury by noxious agents<sup>[3]</sup>.

This study observed gastric cytoprotection of rhEGF at various dosage, orally or subcutaneously. The degree of injury in rat stomach was significantly less severe in rhEGF group than in control group. The protective effect of rhEGF is dose-dependent at the range of 60  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  d<sup>-1</sup> - 120  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  d<sup>-1</sup>. The result suggested that home-made rhEGF can

protect gastric mucosa against ethanol injury. Besides, the concentration of rat serum EGF was related to the gastric cytoprotection effect. EGF is an effective protective factor of gastrointestinal epithelia.

Cytoprotective action was first claimed by Jacobson *et al* in the late 70s. From then on some brain intestinal peptides, such as gastrin, somatostatin, etc. were found to have a similar role in preventing injury from noxious agents. EGF belongs to another kind of substance eliciting the same protection of gastric mucosa. Previous studies had showed that its cytoprotective action was not mainly accomplished through inhibition of gastric secretion<sup>[4]</sup>. Experimental study in animals showed that EGF could ameliorate acute gastric injury caused by aspirin or stress by small non-antisecretory dosage to stomach<sup>[5]</sup>. Extirpation of submandibular glands will lower the EGF levels in the gastrointestinal tract markedly by more than 80%, with significant reduction of DNA and RNA contents of the gastric mucosa thus rendering gastric mucosa more susceptible to ulcerogenic agents. Exogenous EGF given parenterally or orally at doses that would stimulate the growth of gastroduodenal mucosa, enhanced ulcer healing in rats with intact salivary glands and could completely reverse the delay in ulcer healing in sialoadenectomized animals<sup>[6,7]</sup>. So the gastric protective action of EGF was chiefly related with increase of cellular DNA, RNA and protein synthesis.

This study also showed that a similar dosage of rhEGF when given orally or subcutaneously had different cytoprotective effect. The protection of gastric mucosa from ethanol induced injury in subcutaneous group was significantly greater than oral group, and serum EGF and gastrin levels were also higher in the former group. Our results suggested that cytoprotection of EGF might also be related to gastrin level besides serum EGF level.

In conclusion, rhEGF plays a significant role in the protection of gastric mucosa from ethanol induced injury. Its effect is dose-dependent at a dose range of 60  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  d<sup>-1</sup> - 120  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  d<sup>-1</sup>. rhEGF might be a new endogenous drug for the treatment of peptic ulcer.

## REFERENCES

- Konturek PC, Konturek ST, Brzozowski T, Ernst H. Epidermal growth factor and transforming growth factor- $\alpha$ : role in protection and healing of gastric mucosal lesions. *Eur J Gastroenterol Hepatol*, 1995;7(10):933-973
- Tepperman BL, Soper BD. Effect of sialoadenectomy on gastric mucosal integrity and growth in the rat. *Dig Dis Sci*, 1990;35(8):943-949
- Konturek JW, Brzozowski T, Konturek ST. Epidermal growth factor in protection, repair and healing of gastroduodenal mucosa. *J Clin Gastroenterol*, 1991;13(Suppl):S88-97
- Konturek ST. Role of growth factors in gastroduodenal protection and healing of peptic ulcers. *Gastroenterol Clin North Am*, 1990;19(1):41-65
- Konturek SJ, Denbinski A, Warzecha Z, Brzozowski T, Gregory H. Role of epidermal growth factor in healing of chronic gastroduodenal ulcers in rats. *Gastroenterology*, 1988;94(4):1300-1307
- Sarosiek J, Bilski J, Murty VL. Role of salivary epidermal growth factor in the maintenance of physicochemical characteristics of oral and gastric mucosal mucus coat. *Biochem Biophys Res Commun*, 1988;152:1421
- Finke U, Rutten M, Murphy RA. Effects of epidermal growth factor on acid secretion from guinea pig gastric mucosa: *in vitro* analysis. *Gastroenterology*, 1985;88:1175

# Clinical and experimental study of effect of *Raondix Salviae Militiorrhiza* and other blood-activating and stasis-eliminating Chinese herbs on hemodynamics of portal hypertension

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**Subject headings** hypertension, portal; liver cirrhosis; hemodynamics; drugs, Chinese herbal; blood activating and stasis eliminating

## Abstract

**AIM** To study the effects of *Radix Salviae Militiorrhiza* (RSM), other blood-activating and stasis-eliminating Chinese herbs on hemodynamics of portal hypertension.

**METHODS** Portal pressure of cirrhotic dogs after chronic common bile duct ligation was measured directly; portal blood flow in patients with liver cirrhosis were detected by ultrasound Doppler.

**RESULTS** After administration of RSM and *Radix Angelicae Sinensis* (RAS) by intravenous infusion in cirrhosis dogs, the portal venous pressure (Ppv), wedge hepatic venous pressure (WHVP), hepatic venous pressure gradient (HVPg), were significantly decreased ( $P < 0.05-0.01$ ), but the mean arterial pressure (MAP), and the heart rate (HR) remained unchanged. When nifedipine was used, Ppv, WHVP, MAP and HR were significantly decreased ( $P < 0.05$ ), and the MVPg unchanged ( $P < 0.05$ ). After administration of RSM, RSM+nifedipine and RSM+Hirudin+Nifedipin for 10-12 weeks, the diameter of portal vein (Dpv), spleen vein (Dsv), the portal venous flow (Qpv) and splenic venous flow (Qsv) in patients with hepatic cirrhosis were significantly lowered ( $P < 0.05-0.01$ ), and the effect of RAS was weaker.

**CONCLUSIONS** The efficacy of decreasing Ppv by Chinese herbs—RSM, RAS, etc. as compared

with nifedipine, demonstrated that the Chinese herbs were slower in action than that of nifedipine, but more long-lasting and without side effects. Hence, long-term administration of Chinese herbs, would be more beneficial.

## INTRODUCTION

There has been no long-lasting and side effects free drugs to lower the portal hypertension in patients with liver cirrhosis so far. The blood vessel constrictive drugs can reduce the volume of blood flow and lower the portal pressure by contracting visceral blood vessels but can not improve the prognosis of the patients obviously because of their bad dynamic effects, whereas drugs dilating blood vessels such as calcium antagonist—nifedipine can lead to hypotension at large doses although it is effective for portal hemodynamics<sup>[1]</sup>. Yigan infusion which contains blood-activating and stasis-eliminating herbs such as large doses of RSM and RAM has some effects of shrinking the liver and spleen; and some of blood-activating and stasis-eliminating herbs can prevent and cure hepatic fibrosis<sup>[2-5]</sup>. Therefore, the effects of RAM and RSM in portal and systemic dynamics were investigated in the present study.

## MATERIALS AND METHODS

### Animal model

Sixteen healthy mixed bred dogs, female and male, weighing 12.5kg - 20kg, were divided into two groups randomly: portal hypertension model group ( $n = 12$ ) made by chronic bile duct ligation method described by Boschj *et al* and control group ( $n = 4$ ).

### Drugs and administration route

RSM injection (40%), nifedipine injection (50%) (the Ninth Shanghai Pharmaceutical Factory (batch no 940712123, original drug 1.5g/mL). The dosage for dogs is 20 times higher than for adult people. The dosage by intravenous drip (1/3 of oral doses) of RSM 6g/kg body weight, RAS 3mg/kg body weight, nifedipine 0.3mg/kg body weight in 10% glucose were infused through thigh vein in 10

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Received 1998-05-06

minutes, according to our earlier studies. The time was calculated after the infusion. The dogs were paralyzed with pentobarbital intravenous anesthesia at fast for 12h in 8-11 weeks after bile duct ligation, and tubes were inserted through vein for infusion, through femoral artery for measuring the mean arterial pressure and heart rate, through femoral vein to inferior vena cava for icvp, through mesentery vein to portal vein for Ppv and from right external jugular vein to hepatic vein for FHVP and HVPG. The tubes were washed by heparin to prevent from grume. The indexes were recorded by physiological recorder (RM-6200) synchronously before and 10, 30 and 60 minutes after administration of drugs according to Latin rank principle, 3 times per day, each drug being used for 90min-120min. The results were analyzed by *F* test and *q* test.

### Clinical study

**Patients** Fifty-nine patients with hepatic cirrhosis (hepatitis B, 58 cases; hepatitis C, 1; female 21, male 38, mean age 45.5 years mean course 106 years) were included in. All patients were consonant with the following conditions: patients with hard hepato-splenomegaly and esophagogastric varices; patients with portal diameter >1.4cm with slight or without ascites, no upper digestive tract bleeding and hepatic encephalopathy. The patients who took vaso-active drugs such as propranolol, nifedipine and diuretics recently were asked to stop taking these medicines for a week before entering this study.

### Grouping and course of treatment

All patients were divided into 4 groups. Group 1, RSM, 60g/day, 21 patients; group 2, RAS 30g, 15 patients; group 3, RSM 60g/day + nifedipine 30 mg/day, 12 patients; and group 4, RSM 60 g/day + nifedipine 30 mg/day + bloodsucker 3g/day, 11 patients. A whole day dose of RSM and RAS were added with 200ml water and immersed for 20 minutes and then added 600ml water, cooked with low intensity of fire for three times into 100ml and taken orally bid. Leech was baked and ground to fine powder and put in to capsules and taken orally, bid. Nifedipine 30 mg, tid, was administered for 10 to 12 weeks.

### Experimental apparatus and methods

The patients were examined by color Doppler ultrasonography (detector head 3.5MHz) at dorsal position quietly at fast. The Dpv, Vpv, Dsv and Vsv were measured before and 2.6 and 10 weeks after treatment. Qpv and Qsv were calculated by the formula:  $Q = \pi R^2 \cdot V \cdot 60$ . Q: quantity of blood flow, R: half vein diameter (D/2), V: mean velocity of blood stream.

## RESULTS

### Animal model

Two of 12 dogs died at week 2 and 3 respectively due to infection and 3 dogs died between week 7 to 8 due to hepatic failure. Seven of 12 dogs developed into liver cirrhosis with ascites and abdominal varices, with body weight loss by 1/4 after bile duct ligation at week 5. All dogs had liver cirrhosis with a large amount of ascites, vein of greater omentum congested and abnormal liver function (Bil  $83.5 \mu\text{mol/L} \pm 4.95 \mu\text{mol/L}$ , ALT  $211.67 \text{ U} \pm 44.8 \text{ U}$ ), there was a significant difference compared with normal animal,  $P < 0.01$ . The special characters of anatomy and histology were coincided to these of biliary cirrhosis. The mean portal pressure ( $2.56 \text{ kPa} \pm 0.30 \text{ kPa}$ ) with cirrhosis were increased significantly compared with normal dogs ( $1.18 \pm 0.02$ ,  $P < 0.001$ ).

### Effects of RSM and RAS on hemodynamics in health dogs

The portal and systemic circulation index in healthy dogs were not affected by intravenous administration of RSM and RAS. The MAP of dogs were lowered 5 - 10 minutes after RAS administration, but returned normal 60 minutes later. The portal pressure and MAP were decreased significantly 60 minutes after administration of nifedipine compared with those before the drug administration (Ppv  $1.50 \text{ kPa} \pm 0.45 \text{ kPa}$  vs  $1.27 \text{ kPa} \pm 0.61 \text{ kPa}$ ,  $P < 0.05$ ; MAP  $16.5 \text{ kPa} \pm 0.71 \text{ kPa}$  vs  $0.21 \text{ kPa} \pm 0.19 \text{ kPa}$ ,  $P < 0.05$ ).

### Effects of RSM and RAS on hemodynamics in dogs with liver cirrhosis

The Ppv, WHVP and HVPG were decreased significantly in dogs with liver cirrhosis 60 minutes after administration of RSM and RAS ( $P < 0.05-0.01$ ). The other indexes were not changed significantly,  $P < 0.05$ . The Ppv, MAP and HR decreased significantly 60 minutes after administration of nifedipine ( $P < 0.01$ ), but HVPG was not changed significantly ( $P > 0.05$ ). This result showed that RSM and RAS have selective effects on portal system in dogs with cirrhosis but without effects on blood pressure and HR, while nifedipine had effects on blood pressure, Ppv and HR (Table 1).

### Comparison in effects of RSM, RAS and nifedipine on Ppv and HVPG

The Ppv in dogs with liver cirrhosis were decreased significantly 10 minutes after administration of RSM and nifedipine as compared with that before ( $2.62 \text{ kPa} \pm 0.27 \text{ kPa}$  vs  $2.45 \text{ kPa} \pm 0.28 \text{ kPa}$ ,  $P < 0.05$ ,  $2.42 \text{ kPa} \pm 0.05 \text{ kPa}$  vs  $2.05 \text{ kPa} \pm 0.24 \text{ kPa}$ ,  $P < 0.05$ ). Although RSM could lower Ppv but not statistically ( $2.56 \text{ kPa} \pm 0.30 \text{ kPa}$  vs  $2.43 \text{ kPa} \pm 0.39 \text{ kPa}$ ,  $P > 0.05$ ).

**Table 1** Effect of RSM, RAS and nifedipinc on hemodynamics in dogs with liver cirrhosis(KPa)

	RSM treatment group		RAS treatment group	
	Before	After 60 min	Before	After 60 min
Ppv	2.56±0.30	1.82±0.33 <sup>b</sup>	2.42±0.05	1.38±0.32 <sup>b</sup>
FHVP	1.39±0.47	1.10±0.28	0.98±0.15	0.94±0.06
WHVP	2.17±0.36	1.70±0.30 <sup>b</sup>	2.33±0.09	1.90±0.33
HVPG	0.93±0.33	0.60±0.43 <sup>b</sup>	1.35±0.16	0.97±0.30
ICVP	1.02±0.34	0.99±0.29	1.05±0.35	1.02±0.32
MAP	13.75±2.40	13.30±2.34	14.07±3.18	13.13±2.48
HR(beat/min)	134.00±1.72	147.80±22.36	138.75±28.39	137.00±28.02

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 *vs* compared with petreatment.

**Table 2** Comparesion among the effect of RSM, RAS on Dpv HVPG

Treatment	RSM vs RSA		RSM vs nifedipine		RSM vs nifedipine	
	Ppv	HVPG	Ppv	HVPG	Ppv	HVPG
30 min	1.29	1.96	3.06	2.30	4.13 <sup>a</sup>	4.26 <sup>a</sup>
60 min	2.51		3.57 <sup>a</sup>		5.95 <sup>a</sup>	

<sup>a</sup>*P*<0.05, *P*<0.01 *vs* compared in any two group.

**Table 3** The effect of RSM, RAS and other drugs on portal hemodyamics in patients with liver cirrhosis

Group	Dpv (mm)		Dsv (m)		Qpv (ml/min)		Qsv (ml/min)	
	Before	1 0 weeks	Before	10 weeks	Before	10 weeks	Before	10 weeks
1	14.84±1.03	13.06±1.58 <sup>c</sup>	12.20±1.92	10.03±1.93 <sup>c</sup>	2346.95±1592.29	1686.93±1056.05 <sup>b</sup>	2065.50±1209.17	1421.38±924.99 <sup>b</sup>
2	14.80±0.92	14.37±0.09 <sup>b</sup>	11.10±1.67	10.38±0.186 <sup>b</sup>	1673.42±203.19	1564.49±267.08	1717.60±919.34	1099.04±744.82 <sup>a</sup>
3	15.03±1.29	13.62±0.09 <sup>c</sup>	11.78±3.16	10.97±0.28 <sup>b</sup>	2117.47±676.31	1753.40±593.46 <sup>a</sup>	1825.89±1151.13	1312.28±780.56 <sup>b</sup>
4	14.75±1.39	13.05±0.65 <sup>b</sup>	11.58±2.31	9.87±2.05 <sup>a</sup>	1794.74±641.06	1226.08±321.16 <sup>a</sup>	1781.92±1197.19	1087.74±70.45 <sup>a</sup>

Group 1: RSM; group 2: RAS; group 3: RSM nifedipine; group 4:RSM+bloodsucker+ nifedipine, compared in any two group <sup>a</sup>*P*<0.05 <sup>b</sup>*P*<0.01 <sup>c</sup>*P*<0.01.

**Table 4** Comparesion of the used drugs on Dpv

Administration	Group 1-2	Group 1-3	Group 1-4	Group 2-3	Group 2-4	Group 3-4
2 weeks	-0.254	-4.220c	-2.637a	-3.633c	-2.265a	0.881
6 weeks	3.042b	-0.153	-2.635	-2.917b	-2.941b	-0.415
10 weeks	4.563c	1.217	-0.098	-3.055b	-3.628c	-0.982

Group 1: RSM; group 2: RAS; group 3: RSM+nifedipine; group 4:RSM+leach+ nifedipine, compared in any two group.

<sup>a</sup>*P*<0.05 <sup>b</sup>*P*<0.01 <sup>c</sup>*P*<0.01.

Table 2 shows that RAS has a more powerful effect in Ppv and HVPG 30 minutes after treatment than nifedipine ( $P < 0.05$ ) whereas there was no significant difference in effects for Ppv and HVPG among RSM, RAS and nifedipine ( $P > 0.05$ ). It is worthy noticing that RSM and RAS were more effective for Ppv 60 minutes after administration than nifedipine ( $P < 0.05$ ), but there was no significant difference between RSM and RAS. The result demonstrated that the action of nifedipine on portal and blood pressure is quick and short whereas RSM and RAS last long in action but without effects on MAP and HR.

### Clinical study

#### Effects of RSM and RAS on portal hemodynamics in patients with cirrhosis

The Qpv, Dsv, Qpv and Qsv in patients with cirrhosis were lowered significantly after using RSM ( $P < 0.01-0.001$ ), while Dpv, Dsv and Qsv were decreased markedly after using RAS ( $P = 0.05-0.01$ ), but no significant effects on Qpv ( $P > 0.05$ ). The Dpv, Dsv, Dpv and Qsv were all decreased significantly in the groups of RSM + nifedipine and RSM + bloodsucker + nifedipine 10 weeks after treatment ( $P < 0.05-0.01$ ). The velocity of blood flow of portal and spleen vein were not changed significantly ( $P > 0.05$ ) (Table 3).

#### Comparison of effects of RSM, RAS on Dpv

Dpv decreased significantly in 2 weeks after treatment in the group RSM + nifedipine and group RSM + leech + nifedipine. The rank order in effect from strong to weak is RSM + leech + nifedipine > RSM and RSM + nifedipine > RAS ( $P < 0.05-0.01$ ). (Table 4)

### Side effects

There was no side effects in the group taking the medicinal herbs. A few patients had headache, dizziness and so on in the group taking herbs and nifedipine in combination, but all recovered after termination of medicine.

### DISCUSSION

Calcium antagonist—nifedipine is one of the most common drugs used to lower portal hypertension, but it can also lower the blood pressure at large oral doses and some patients had bad tolerance to it. More and more attention has been paid to the better effect of RSM and RAS in portal hemodynamics in patients with hepatic cirrhosis. The present study showed that RSM and RAS used intravenously could lower Ppv (kPa,  $2.56 \pm 0.30$ ,  $1.82 \pm 0.33$ ,  $2.43 \pm 0.05$ ,  $1.38 \pm 0.32$ ,  $P < 0.01$ ), WHVP (kPa,  $2.17 \pm 0.36$ ,  $1.70 \pm 0.30$ ;  $2.33 \pm 0.09$ ,  $1.90 \pm 0.33$ ), and HVPG (kPa,  $0.93 \pm 0.33$ ,  $0.60 \pm 0.43$ ;  $1.35 \pm 0.16$ ,  $0.97 \pm 0.30$ ) in dogs with experimental liver cirrhosis, but without any influence on MAP and HR in normal dogs. Although nifedipine could

reduce Ppv, WHVP and HR in dogs with liver cirrhosis, RSM and RAS had more powerful effects in lowering portal hypertension and without effect on systemic pressure as compared with nifedipine. The combination of RSM, RAS, RSM + nifedipine, RSM + nifedipine + leech could reduce Dpv, Dsv, Qpv and Qsv in patients with liver cirrhosis. RSM and RAS could also improve the patients' symptoms and liver function. Rapid and prolonged effects could be obtained, when combined therapy of the herbal medicine and western drugs was used. This should be further studied. The effects of nifedipine in lowering portal hypertension is rapid, which appeared 10 minutes after intravenous and 2 weeks oral administration but with the disadvantage of reducing the blood pressure and HR. The effect of intravenous RAS in reducing Ppv in dogs with liver cirrhosis became stronger than nifedipine with the prolonged time of drug administration ( $P < 0.05$ ), but without changes of blood pressure and HR. Although the effect of RSM in reducing portal hypertension appeared in 30 minutes intravenously and 6 weeks orally it last longer and became stronger, and peaking at 60 minutes intravenously ( $2.56 \text{ kPa} \pm 0.30 \text{ kPa}$ ,  $1.82 \text{ kPa} \pm 0.33 \text{ kPa}$ ,  $P < 0.01$ ) and 10 weeks orally ( $14.84 \text{ kPa} \pm 1.03 \text{ kPa}$ ,  $13.06 \text{ kPa} \pm 1.58 \text{ kPa}$ ,  $P < 0.001$ ). The result showed a long course of treatment or intravenous administration is necessary in RSM. The effect of combination of RSM, leech and nifedipine in treatment of portal hypertension appeared rapidly and more powerful, which is a drug regimen of choice for patients with high Ppv.

The mechanism of RSM and RAS in lowering portal hypertension has not been well understood yet. RSM can prevent from liver fibrosis if it is used for a long time. It was reported that RSM can inhibit fibroblast cells. Large doses of RSM can activate collagenase and help blockage the extracellular matrix<sup>[4]</sup>. The value P-III-P and lamin were decreased in patients with liver disease after oral treatment of RSM. The present study demonstrated that long-term oral treatment of RSM for 10-12 weeks can reduce the portal vein and spleen diameters and blood flow, but the velocity of blood flow did not change. The effect become more and more powerful with time. The present study suggested that combination of RSM, RAS, leech and nifedipine is effective in lowering hypertension and without side effects in treatment of liver cirrhosis.

### REFERENCES

- Hu Y, Wang XL, Li DG, Lu HM, Gu HD, Shi PW *et al* Experimental study on the effects of  $\text{Ca}^{2+}$  antagonists on rat model of cirrhosis with portal hypertension. *Chin J Dig* 1995;15(2):89-91
- Song JB, Li SB. Experimental study of injection angelica and natrii angelicae on rat portal pressure. *Chin J Hepatol*, 1996;4(3):158-160
- Huang ZP, Go B, Liang KH. Effects of radix angelicae sinensis on systemic and portal hemodynamics in cirrhotics with portal hypertension. *Chin J Med*, 1996;35(1):15-18
- Li XT, Bai WY, Wang HM, Yao XX. Study of effects of Radix salviae miltiorrhiza on portal hypertension in experimental cirrhotic dogs. *New J Dig Dis*, 1997;5(7):421-422
- Yao XX, Li XT, Li YW, Zhang XY. Clinical and experimental study of radix salviae miltiorrhiza and other Chinese herbs of blood-activating and stasis-eliminating effects on hemodynamics of portal hypertension. *Chin J Dig*, 1998;18(1):24-27 (in Chinese)

# Effect of combined therapy of Yinchenhao Chengqi decoction and endoscopic sphincterotomy for endotoxemia in acute cholangitis \*

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**Subject headings** cholangitis; endotoxemia; sphincterotomy; endoscopy; Yin Chen Hao Cheng Qi decoction

## Abstract

**AIM** To evaluate the therapeutic mechanism of Yinchenhao Chengqi ( YCHCQ ) decoction (containing mainly *Herba Artemisia capillaris*) combined with endoscopic sphincterotomy (EST) for endotoxemia (ETM) in acute cholangitis.

**METHODS** Twenty-one cases of acute cholangitis with ETM were divided randomly into two groups: group A, 10 patients treated with YCHCQ decoction combined with EST, group B, 11 patients treated with EST. The incidence rate of ETM, plasmic ET, serum superoxide dismutase (SOD) activity, malonyldialdehyde (MDA), complement C3 and C-reactive protein (CRP) were studied respectively.

**RESULTS** The ET level of group A (35.92ng/L±8.30 ng/L) was significantly reduced after 7 days of treatment ( $P<0.05$ ) in contrast to that of group B (47.8ng/L±11.62ng/L), so did the level of MDA and CRP. But the SOD activity and C<sub>3</sub> level in group A increased significantly ( $P<0.05$ ).

**CONCLUSION** YCHCQ decoction combined with EST had a beneficial effect for ETM in acute cholangitis.

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\*Project supported by the National Natural Science Foundation of China, No.38970871.

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Received 1998-05-21

## INTRODUCTION

Endotoxemia (ETM) is one of the most important physiopathologic causes of acute cholangitis and it is the trigger of cytokines and inflammatory factors. In recent studies it has been found that Yinchenhao Chengqi (YCHCQ) decoction has a beneficial effect on ETM in acute cholangitis. With the development of endoscopic surgery, endoscopic sphincterotomy (EST) has become an effective replacement for some operations in the treatment of acute cholangitis<sup>[1]</sup>. The effect of YCHCQ decoction combined with EST on ET, oxygen free radical and complement C<sub>3</sub> was observed in order to find out its therapeutic mechanism.

## MATERIALS AND METHODS

### Clinical materials

Twenty-one cases of acute cholangitis with endotoxemia were divided randomly into two groups: group A, 10 patients (6 males and 4 females) treated with YCHCQ decoction and EST; and group B, 11 patients (6 males, 5 females) treated with EST (Tables 1,2).

**Table 1** Disease composition of acute cholangitis

Group	No. of patients	Common bile duct stones (%)	Benign stenosis of Oddi's sphincter (%)	Biliary ascariasis (%)
Group B	11	6(55)	4(36)	1(9)
Group A	10	6(60)	3(30)	1(10)

**Table 2** Comparison of general condition

Group	No. of patients	WBC in peripheral blood (10 <sup>9</sup> /L)	Percent of neutrophil cell	Temperature (°C)	Age (years)
Group B	11	15.47±6.83	0.85±0.08	38.92±1.32	52.43±13.61
Group A	10	15.36±7.26	0.83±0.09	38.87±1.24	56.21±14.17
<i>P</i>		>0.05	>0.05	>0.05	>0.05

### Methods

**EST treatment** EST was performed on the patients with benign stenosis of Oddi's sphincter. Besides EST, stone extraction using Dormia basket, retrieval balloon or mechanical lithotripter was also followed in the patients with common bile duct stones, and ascaris lumbricoides extraction using forceps was conducted in those with biliary ascariasis.

**Combined treatment of EST and YCHCQ decoction** The patients in group A took one YCHCQ decoction

a day, 100mL in the morning and 100mL at night. YCHCQ dection was composed of *Herba Artemisiae capillaris* 30g, *Fructus Gardeniae* 15g, *Cortex Magnoliae Officinalis* 15g, *Fructus Aurantii* 15g, *Radix et rhizoma Rhei* 15g (added later) and *Natrii Sulfas* 10g (taking with hot decoction).

**Assay of ET, superoxide dismutase (SOD), malonyldialdehyde (MDA), complement C<sub>3</sub> and C-reactive protein (CRP) in peripheral blood** Plasmic ET was measured using limulus ozo-group development process, serum SOD was determined using xanthine oxidase process, and MDA by the thiobarbituric acid clorimetric method. CRP and C<sub>3</sub> were detected with fully-automatic instruments for biochemical analysis.

**Definition of ETM** ET in peripheral blood of 50 normal volunteers was measured. The result was 32.53 ng/L ± 10.32 ng/L. Plasmic ET of 95% normal volunteers ranged from 0 ng/L to 53.84 ng/L. If it is above 54 ng/L, it is defined as ETM.

**Statistical analysis**

The results were expressed as  $\bar{x} \pm s_{\bar{x}}$ . Data were analyzed using Student's *t* test. The incidence rate of ETM was analyzed using  $\chi^2$  test. P value less than 0.05 was considered significant.

**RESULTS**

The average days of hospitalization in group A were 7 days, 9 recovered and 1 improved, and no complication occurred, while, the average days of hospitalization of group B were 12 days, 9 recovered and 3 improved, and complications occurred in 2 cases, including one case of acute peritonitis, and one case of common bile duct stones at the ampulla of Vater who underwent an operation to remove the stones.

**Effect of different treatment on ETM**

In Table 3, plasmic ET level of the two groups was markedly higher than that of the normal volunteers, and ETM occurred. On the 3rd day after treatment, the incidence rate of ETM in group A was lowered significantly, as compared with that before treatment. The incidence rate of group B also decreased, but there was no significant difference as compared with before until the 5th day after treatment. The plasmic ET level of the two groups declined obviously on the 3rd day, but without significant difference between the two groups. On the 5th and 7th day, ET level was lower in group A than in group B.

**Effect of different treatment on the activity of serum SOD**

Before treatment, the activity of serum SOD was lower in both groups than normal (104.2 kNU/L ± 18.8 kNU/L). There was nosignificant difference

between the two groups, although on the 3rd and 7th day after treatment, the activity of serum SOD in the two groups obviously increased, that of group A being much higher than that of group B (*P* < 0.05). On the 7th day, serum SOD values of group A had already turned normal (Table 4).

**Table 3 Changes of the incidence rate of ETM**

Group	No.of patients	Before treatment (%)	After treatment(%)		
			d3	d5	d7
Group B	11	11/11(100)	9/11(82)	1/11(64) <sup>a</sup>	3/11(27) <sup>a</sup>
Group A	10	10/10(100)	6/10(60) <sup>a</sup>	2/10(20) <sup>ac</sup>	

<sup>a</sup>*P* < 0.05, comparison of intra-group; <sup>c</sup>*P* < 0.05, comparison among groups.  $\chi^2$  test.

**Table 4 Changes of plasmic ET, serum SOD and MDA ( $\bar{x} \pm s$ )**

Group	No.of patients	Before treatment	After treatment		
			d3	d5	d7
Plasmic ET (ng/L)					
Group B	11	97.12±15.20	72.84±10.36 <sup>a</sup>	62.61±10.08 <sup>a</sup>	47.80±11.62 <sup>a</sup>
Group A	10	98.67±15.54	67.07±13.50 <sup>a</sup>	48.18±11.46 <sup>ac</sup>	35.92±8.30 <sup>ac</sup>
Serum SOD (kNU/L)					
Group B	11	62.52±8.01	72.38±10.52 <sup>a</sup>	86.89±13.56 <sup>a</sup>	
Group A	10	63.68±9.45	96.47±15.35 <sup>ac</sup>	105.18±13.21 <sup>ac</sup>	
Serum MDA (μmol/L)					
Group B	11	38.50±2.29	24.40±2.43 <sup>a</sup>	12.47±1.70 <sup>a</sup>	
Group A	10	37.32±3.80	6.40±1.28 <sup>ac</sup>	4.33±1.03 <sup>ac</sup>	

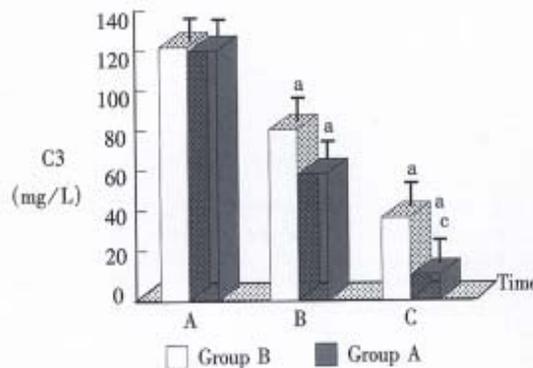
<sup>a</sup>*P* < 0.05, comparison intra-group; <sup>c</sup>*P* < 0.05, comparison among groups. Student's *t* test.

**Effect of different treatment on the level of serum MDA**

It is shown in Table 4 that the level of serum MDA lowered in various degrees in both groups after treatment. MDA level in group A became nearly normal on the 7th day.

**Effect of different treatment on the level of serum CRP**

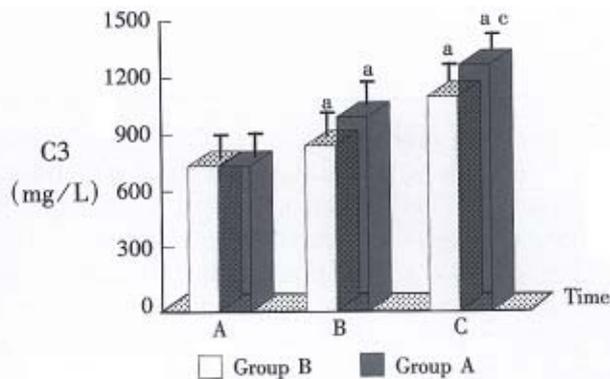
There was no difference in the CRP level between the two groups, which was both obviously higher than the normal level before treatment. The CRP level obviously decreased after treatment on the 3rd and 7th day. However, the CRP value in group A declined much more significantly than that in group B (*P* < 0.05) (Figure 1).



**Figure 1** Changes of serum CRP. <sup>a</sup>*P* < 0.05, comparison of intra-group; <sup>c</sup>*P* < 0.05, comparison among groups. Student's *t* test. A: Before treatment; B: The 3rd day after treatment; C: The 7th day after treatment.

### Effect of different treatment on serum C<sub>3</sub>

As shown in Figure 2, there was no significant difference in serum C<sub>3</sub> of the two groups which was both lower than the normal before treatment. However, patients treated with combined YCHCQ decoction and EST had significantly higher serum SOD than group B.



**Figure 2** Changes of serum C<sub>3</sub>. <sup>a</sup> $P < 0.05$ , comparison of intra-group; <sup>c</sup> $P < 0.05$ , comparison among groups. Student's *t* test. A: Before treatment; B: The 3rd day after treatment; C: The 7th day after treatment.

## DISCUSSION

### Therapeutic mechanism of YCHCQ decoction on ETM in acute cholangitis

As an antagonistic agent, YCHCQ decoction can reduce ET production and absorption. *Redix et Rhizoma Rhei* and *Natrii Sulfas* could get rid of abdominal mass, bacteria and ET with an effect of “Tongligongxia” in TCM to reduce the ET. *Redix et Rhizoma Rhei*, *Fructus Gardeniae* and *Cortex Magnoliae Officinalis* have a more powerful bacteriostatic effect to reduce the production of ET and the incidence of ETM derived from the gut<sup>[2]</sup>. *Redix et Rhizoma Rhei*, *Herba Artemisiae Scopariae* and *Fructus Gardeniae* are also cholagogues, antispasmodics and anti-inflammatory agents. They can lower the incidence rate of ETM which resulted from bile duct obstruction because the inflammatory bile in the bile duct flows into the intestinal tract. Some studies have confirmed that *Redix et Rhizoma Rhei* has a more powerful antagonistic effect on ET. The destroyed reticular structure of ET by *Redix et Rhizoma Rhei* was observed under electron microscope.

YCHCQ decoction could reduce the production of oxygen free radical. ET could activate the respiratory burst of leukocyte. A large amount of oxygen free radicals strongly damage the histocyte, particularly the gut barrier<sup>[3]</sup>. Peroxide, such as MDA etc, is produced because of lipid peroxidation by oxygen free radical which could attack multiple unsaturated fatty acid on the biological membrane<sup>[4]</sup>. YCHCQ decoction may reduce the production of peroxide MDA, increase the activity of SOD, decrease the permeability of capillary, and

promote microcirculation. Therefore, it can reduce the production of oxygen free radical, keep the balance of oxidation and antioxidation, lessen the damage to the gut barrier, reduce the production and absorption of ET, inhibit the cascade effect of ET and oxygen free radical, stop pernicious circulation, keep the stabilization of the internal environment, raise the ability of antioxidation and alleviate the damage of peroxidation to organisms.

YCHCQ decoction could enhance immunologic function, and promote the recovery of the function of the complement system, the macrophage system and the inactivation of ET.

### Effect of combined YCHCQ decoction and EST on ETM in acute cholangitis

This study confirms that YCHCQ decoction combined with EST had a better therapeutic effect on ETM in acute cholangitis. Its advantages are that: EST could incise part of the papilla so as to clear common bile duct stones with mechanical litherpsy instrument, and to eliminate the factors of mechanical obstruction and bile duct stenosis. On the basis of this action, the therapeutic effect of YCHCQ decoction could be fully exerted. Thus, the most important factors of acute cholangitis were eliminated because common bile duct stones were successfully removed with the combined treatment. In addition, endoscopic retrograde biliary drainage or endoscopic nasobiliary drainage were performed after EST, in order to reduce the pressure of the bile duct, drain the inflammatory bile, remove the “Damp-Heat” and the mass on the abdomen, normalize secretion and discharge the bile, reduce jaundice, and preserve the function of the liver and the kidney.

EST is an established non-<sup>2</sup>surgical method of management for patients with acute cholangitis from biliary obstruction or various causes. The results show that the patients undergoing EST do not require surgical operation and rarely suffer from complications. They need only a short time of hospitalization, experience little pain, and obtain a rapid recovery. Also, this treatment dose not have the limitations caused by repeated surgical operations or the conditions of patients<sup>[5]</sup>. YCHCQ decoction could reduce or eliminate the occurrence of ETM in acute cholangitis. With its therapeutic effect of “Tongligongxia” and clearing away “damp-heat” and “Fuzhengquxie” in TCM, YCHCQ decoction can reduce the production of oxygen free radical and lipid peroxidation, thus inhibiting the production and absorption of ET.

## REFERENCES

- Boender J, Nix GA, Ridder MA, Dees J, Schutte HE, Buuren HR *et al.* Endoscopic sphincterotomy and biliary drainage in patients with cholangitis due to common bile duct stones. *Am J Gastroenterol*, 1995;90(2):233-238
- Chen HL, Zhou JY, Guan FL, Guo PL. Clinical study of therapeutic effect of Compound Dachengqi Tang on ETM in obstructive jaundice. *J Integr Med*, 1991;11(12):724-726
- Chen HL, Wu XZ. Cytokines and Multiple system organ failure. *Foreign Med Sci (Sec Physiol Pathol Clin)*, 1995;15(4):261-264
- Borregaard N, Miller LJ, Springer TA. Chemoattractant-regulated mobilization of a novel intracellular compartment in human neutrophils. *Science*, 1987;237(9):1204-1206
- Prat F, Tennenbaum R, Ponsot P, Altman C, Pelletier G, Fritsch J *et al.* Endoscopic sphincterotomy in patients with liver cirrhosis. *Gastrointest Endosc*, 1996;43(2):127-131

**Review**

# Inflammatory bowel disease: definition, epidemiology, etiologic aspects, and immunogenetic studies

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**Subject headings** inflammatory bowel disease/epidemiology; inflammatory bowel disease/etiology; inflammatory bowel disease/genetics; inflammatory bowel disease/immunology

## DISEASE DEFINITION

Several causes exist for inflammation of the gut such as infection, toxin, autoimmune reaction, radiation and ischemia. When none of these causes are identified, a group of diseases with unknown etiology remains which are called chronic inflammatory bowel diseases (IBD). The chronic inflammatory bowel diseases include two distinct entities, ulcerative colitis (UC) and Crohn's disease (CD), although there is a small group of patients with an intermediate form.

UC was first described in 1859 by Walks<sup>[1]</sup>. It is an inflammatory process confined to the colon in all instances, which presents itself clinically with bloody diarrhea, mucus in the stools, abdominal pain, and weight loss. The colonic inflammation is usually superficial, continues diffusely and often begins in the rectum. Microscopic changes show edema and congestion of mucosa, infiltration of lymphocytes, plasma cells and polymorphonuclear granulocytes, crypt abscesses, crypt architecture distortion, and ulceration. When the inflammation is confined to the rectum, the condition is called proctitis. When the inflammation does not extend beyond the descending colon, it is called left-sided colitis. If the inflammation extends proximal from the rectum to at least the hepatic flexure (sometimes defined as beyond the splenic flexure), it is termed pancolitis or total colitis. It is called reverse ileitis or backwash ileitis, when inflammation of the entire colon extends to the terminal ileum as well.

Crohn, Ginsberg and Oppenheimer described ileitis as a pathological and clinical entity different

from intestinal tuberculosis in 1932<sup>[2]</sup>. It was named Crohn's disease after the name of the first author. However, in 1913, Dalziel gave a remarkable accurate description of the disease<sup>[3]</sup>. CD can affect any part of the digestive tract from the mouth to the anus. The terminal ileum is the commonest site for the disease. Lockhart-Mummery and Morson first described the colon localization of CD in 1960<sup>[4]</sup>. CD is clinically presented with abdominal pain, diarrhea and weight loss. Occasionally with an abdominal mass, intestinal obstruction, or fistula. The inflammation is focal, segmental and transmural, often complicated by fissures, fistulas, abscesses, and intestinal obstruction. The macroscopic changes of CD reveal aphthous ulcers and the microscopic features are lymphoid aggregates, chronic inflammatory cell infiltration and epithelioid granulomas.

Terminal ileitis refers to CD limited to the last part of the ileum. It is called regional or segmental enteritis, when the disease involves several segments of the small intestine. Granulomatous colitis refers to the colonic involvement of the disease.

About 10% of the patients with colonic inflammation can not be classified as either CD or UC. These patients are categorized as "undeterminate colitis"<sup>[5,6]</sup>. Follow-up of these patients has shown that the majority of these patients developed UC<sup>[6]</sup>.

## EPIDEMIOLOGY

The incidence of IBD varies greatly in different geographic areas of the world<sup>[7-31]</sup>. A high incidence is seen in North-West Europe and North America. It is uncommon in Asia and Africa. The incidence of IBD in western countries is about 2 to 15 per 100000/year for UC and 0.9 to 11.6 per 100000/year for CD. The peak age for UC is 30 years and for CD is 20 years. Women generally have a 20%-30% higher risk than men in developing CD. The reports on sex differences in UC are variable, but there seems to be a tendency to a male preponderance.

Data from Copenhagen from 1962-1987, show that the incidence of UC remained stable at a mean of 8.1/100000, whereas the incidence of CD increased by 6 times, from less than 1/100000 to

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Received 1998-08-27

4.1/100000<sup>[13,14]</sup>. A recent epidemiological study of the European collaborative study group on IBD gave 10.4/100000 (95% CI 7.6-13.1) as the incidence for UC and 5.6/100000 (95% CI 2.8-8.3) for CD from the South to the North of Europe<sup>[29]</sup>. In this study, higher overall incidence rates were found in Northern Europe than in Southern Europe, but the magnitude of the difference was less than expected on the basis of previous studies<sup>[7-24]</sup>. One explanation is that the results may reflect a recent increase in incidence of IBD in Southern Europe and a stable incidence in the North.

The prevalence of IBD varies widely from 30-200/100000 for UC and 1.2 - 106/100000 for CD<sup>[30]</sup>. Determining accurate prevalence rates, referring to the number of cases at one moment divided by the total population of the area, creates certain problems. Incidence figures, especially of prospective studies, are therefore more likely to be correct than prevalence figures<sup>[31]</sup>.

In areas with a high incidence of UC, the incidence of CD also appears to be high. The ratio of UC to CD is approximately 2:1. No data are available about the exact incidence of IBD in developing countries. IBD was thought to be a rare disease in Asian countries in the past. However, the number of UC and CD cases has surprisingly increased over the past 10 years<sup>[32]</sup>. There is a need for epidemiological studies in these areas as to judge the strength of genetic and environmental factors in the pathogenesis of IBD.

## ETIOLOGY

The etiology of IBD is not yet known but is likely to be multifactorial. The pathogenesis of the disease is largely determined by environmental and immunological factors on a genetically predisposed host.

### *Environmental factors*

Since UC and CD were first described, much effort has been made to search for infectious agents. Although several bacteria can induce identical symptoms and pathologic changes reminiscent to those found in UC and CD, bacterial infection has different features with a self-limited course, effectiveness of antibiotic treatment and returning to normal histology after treatment<sup>[33]</sup>. *Campylobacter jejuni*, *Shigella*, *Salmonella* and pathogenic coli are the most common causes for self-limited colitis<sup>[33,34]</sup>. Parasites (Amoebae, schistosomiasis) and viruses (e. g. herpes, cytomegalovirus, rotavirus, Norwalk agent and influenza) are also explored as possible causative agents in IBD or as inducing relapses of IBD, but no definite evidence has been provided<sup>[35]</sup>. Most recently, measles virus has been proposed as a causative agent for CD<sup>[36-40]</sup>. The connection

remains disputed<sup>[41-44]</sup>. Mycobacterium paratuberculosis has been isolated from the bowel of some patients with CD<sup>[45-47]</sup> and identified in more than 50% of biopsy specimens, in less than 20% in the healthy control group and in UC<sup>[48,49]</sup>, but recent studies do not support the hypothesis that Mycobacterium paratuberculosis is involved in the etiology of CD<sup>[50,51]</sup>. Most antimycobacterial treatment in CD patients has been unsuccessful<sup>[52-55]</sup>.

Other factors, including smoking, use of oral contraceptives, socio-economic status, nutrition and dietary habit, blood transfusion and perinatal infections, have been suggested as risk factors in IBD<sup>[31]</sup>. However, all factors should be reconfirmed except smoking. Smoking has been considered as the strongest exogenous risk factor for IBD. In this context UC and CD seem to be opposites. Non-smokers are associated with UC and ex-smokers are at even higher risk of developing of UC than never-smokers<sup>[56-61]</sup>. The protective effect of smoking in UC seems to be related to nicotine, but clinical trials have shown variable results<sup>[62-64]</sup>. In contrast to UC, smoking is associated with the development of CD by thrombogenic and vasculitic effects<sup>[58-61,65]</sup>, and apparently influences the clinical course and quality of life of the patient<sup>[66,67]</sup>.

### *Immunological factors*

Immunology is one of the most actively studied aspects in IBD. Disturbance of immune reaction often has been observed in the patients, such as increased numbers of immune cells in the lamina propria, the demonstration of humoral and cellular immune activation, association with other immune-related diseases, and effective treatment with steroids and immunosuppressive agents. Thus, abnormalities of the intestinal mucosal immune function have been postulated to explain the clinical and histopathological nature of IBD.

The immunological hypothesis proposes that IBD represents an abnormal immune response to a normal stimulus in a genetically susceptible host<sup>[68]</sup>. This hypothesis was strongly supported by recent experiments on knockout mice in which deletion of immune-related genes has resulted in chronic colitis<sup>[69-71]</sup>. Alternatively the infections hypothesis proposes that some unidentified pathogens cause IBD and that the immune system is just responding appropriately to these pathogenic stimuli. Even though no such pathogens have been found, this hypothesis is still possible since chronic gastritis has recently been confirmed to be caused by *Helicobacter pylori* infection<sup>[72,73]</sup>. Moreover, intestinal inflammation develops in mice as a consequence of an abnormal immune response in the presence of a single pathogen, such as *Helicobacter*

*hepaticus* or *Helicobacter bilis*<sup>[74,75]</sup>. However, in these two hypotheses the immune system will respond to ubiquitous antigens. In CD, the response may be elicited by luminal constituents, whereas in UC, an autoimmune response may be the causative factor<sup>[76]</sup>.

The antigenic challenge to the gastrointestinal immune system is enormous, including pathogenic bacteria, normal resident intestinal flora, bacterial products, toxin, viruses, ingested chemicals, even food and drinks. Thus, the immune system of the gut has evolved at least two different directions. On one hand, it has to provide the host with protective mechanisms against invasion of pathogens across the surface of the mucosa, and on the other hand it has to take up large nutrient substances and tolerate the normal intestinal flora, all of which may potentially be immunogenic. For this purpose, the gut mucosal immune system has developed specialized structures, such as Peyer's patches and isolated lymphoid follicles, which is termed the gut-associated lymphoid tissue (GALT), lamina propria, intra-epithelial lymphocytes and mesenteric lymph nodes. Intestinal antigens pass through membrane cells (M-cells) and are presented by antigen-presenting cells (APC) in conjunction with the MHC class I or class II antigens in Peyer's patches. The interaction between the MHC-peptide complex on the APC and T cell receptor (TCR) determines the nature of the T cell responses. Because of an enormous antigen challenge in the intestine, it is apparent that the mucosal immune system is in a constant state of response, mainly down regulating mechanisms of inflammation.

Recently, a defective epithelial barrier in the intestinal mucosa has been postulated as one of the etiologic factors in IBD<sup>[76]</sup>. The evidence of enhanced mucosal permeability in CD<sup>[77-79]</sup> and colonic mucin and sulfur compound alteration in UC<sup>[80-82]</sup> support this hypothesis. However, other<sup>[83-86]</sup> can not confirm the increased intestinal permeability in CD. A defective epithelial barrier may allow large uptake of antigens and pro-inflammatory molecules, such as luminal bacteria and bacterial products, n-formyl-methionyl-leucyl-phenylalanine (FMLP), and peptidoglycan-polysaccharide polymers, and lipopolysaccharide (LPS), and give more opportunities for antigens to initiate immunological and inflammatory responses.

One of the important immunoregulatory abnormalities in IBD is related to the T cell response<sup>[76,87,88]</sup>. The intestinal lamina propria contains approximately two thirds of CD4+T cells and one-third of CD8+T cells in a proportion similar to the peripheral blood lymphocytes<sup>[68]</sup>. Activation of T cells by different stimuli shows a rapid increase of the expression of several surface antigens. CD3 is present on all T cells and is used

as a marker for the T cell lineage. CD4 is present on those  $\alpha/\beta$  T cells that interact with the human leukocyte antigen (HLA) class II molecules and CD8 is present on  $\alpha/\beta$  T cells that interact with HLA class I molecules<sup>[89,90]</sup>. Most of the lamina propria T cells have the CD45RO + CD45RA-phenotype characteristics of memory T cells. Functionally, lamina propria lymphocytes are characterized by their reduced proliferative and cytokine responses through the TCR, but increased reactivity through the alternative CD2 activation pathway. When T cells are activated, they express interleukin-2 (IL-2) receptor alpha chains (IL-2R $\alpha$  or CD25), HLA-DR, CD98 (an activation molecule recognized by the monoclonal antibody 4F2) and the transferrin receptor. They also produce many cytokines, and thus take part in the regulation of B and T cell responses and other immune activities<sup>[68,87,91-93]</sup>.

Histopathology of IBD has shown an increased number of lymphocytes in the lamina propria. Immunohistochemical studies on frozen sections of intestinal tissue or cytofluorometric analysis of isolated intestinal lamina propria mononuclear cells from IBD patients shows no significant changes in the CD4 + and CD8 + T cell subpopulations<sup>[94]</sup>. There are, however, no obvious defects in these helper and suppresser T cell functions in isolated lamina propria lymphocytes from CD patients on pokeweed mitogen stimulation<sup>[95]</sup>. However, memory T cells expressing CD45RO + are increased in the intestinal mucosa of IBD patients<sup>[68,91,96]</sup>. The T-cell population from the peripheral blood or lamina propria of intestinal mucosa in CD patients showed early activation markers: IL-2R, CD98 and the transferrin receptor T9<sup>[97]</sup>, and HLA-DR antigens. Some studies have shown that HLA-DR is not increased in lamina propria T cells<sup>[68]</sup>. One study from Korea describes an increase of IL-2R, the transferrin receptor, CD3 and HLA-DR in lamina propria lymphocytes in UC, whereas CD3, TCR $\alpha/\beta$ , and TCR $\alpha/\beta$  was decreased in CD<sup>[98]</sup>. In another study, higher numbers of T cells expressing IL-2R are found in CD patients, whereas macrophages express IL-2R predominantly in UC patients<sup>[99]</sup>. These phenomena may be related to different activation patients observed in UC and CD. High concentrations of circulating soluble IL-2R in serum or high expression of IL-2R in mucosa is strongly indicative for an active stage of CD and UC<sup>[99-102]</sup>.

CD4 positive T cells can be divided into two subpopulations, Th1 cells and Th2 cells according to their secreted cytokines. Cytokines are small soluble peptides with molecular weight ranging from 5 to 50kDa, which regulate the function of target cells through binding to specific cell surface receptors. A large number of different cytokines have been

identified and each cytokine has special functions and cell origin. Cytokines act in conjunction and form complex interactions in a cytokine network. It is interesting that CD has selectively activated Th1 cells which produce pro-inflammatory mediators, such as IL-2 and IFN- $\gamma$ , and UC has selectively activated Th2 lymphocytes which produce anti-inflammatory cytokines, such as IL-4 and IL-10<sup>[76,103]</sup>. These observations also suggest that CD and UC have different patterns of abnormal immunity. Recently a Th3 subset of T cells has been reported to exist in GALT which plays a key role in the production of transforming growth factor beta (TGF $\beta$ ). This cytokine is an active suppression component of oral tolerance, a state of unresponsiveness to immunity, and has downregulatory effects on Th1 cells<sup>[104,105]</sup>. Activation of human mucosal T cells has been shown to cause tissue injury in organ cultures<sup>[106]</sup>, indicative of the importance of T cell activation in the mucosal lesions for IBD.

Another important immunoregulator abnormality in IBD is related to the imbalance of cytokine regulation<sup>[76]</sup>. Although cytokines consist of a wide range of structurally distinct peptides, the majority are categorized into three major groups by their overall dominant effects<sup>[107]</sup>:

1) Pro-inflammatory cytokines, so called Th1 type: TNF $\alpha$ , IL-1, IL-2, IL-6, IL-12 and interferon  $\gamma$  (IFN- $\gamma$ ), lymphotoxin alpha (LT $\alpha$ ) or tumor necrosis factor beta (TNF $\beta$ ) are responsible for cell mediated immune responses. IL-1, IL-6, IL-8, and TNF $\alpha$  are predominantly monocyte or macrophage derived and have preferential pro-inflammatory activities<sup>[108]</sup>;

2) Whereas the Th2 type subset secretes immunomodulatory cytokines IL-1ra, IL-4, IL-5 and IL-10, and IL-13 predominantly synthesized by T cells and responsible for humoral or B cell immunity<sup>[108]</sup>;

3) Growth factor and regulatory cytokines, including the colony-stimulating factors, transforming growth factor beta (TGF $\beta$ ), epidermal growth factor (EGF) family, insulin like growth factors, fibroblast growth factors, have growth regulation and proliferative effects on different cell types.

An imbalance between positive and negative factors in favor of a proinflammatory response can initiate an inflammatory cascade.

One of the most interesting immunoregulatory balances in the IL-1/IL-1ra ratio, the former is a pro-inflammatory cytokine, whereas the latter is an anti-inflammatory cytokine which inhibits the actions of IL-1 by binding to IL-1 receptors without agonistic effects. A decreased IL-1ra/IL-1 ratio has been observed in the mucosa of IBD patients when compared with the ratio in the mucosa from healthy

individuals and from other inflammatory disorders<sup>[109]</sup>. The same study group had reported blockade of endogenous IL-1ra to exacerbate and to prolong inflammation in rabbit immune colitis<sup>[110]</sup> and deletion of the IL-1ra gene to increase susceptibility to experimental colitis in mice<sup>[111]</sup>. Holt and colleagues think that a decreased ratio of IL-1ra/IL-1 mRNA is the result of inflammation rather than a unique IBD-related abnormality<sup>[112]</sup>. However, Kojouharoff *et al* show that treatment with anti-IL-1 reagents, anti-TNF monoclonal antibodies, and dexamethasone leads to aggravation of acute colitis in mice induced by 5% sodium dextran sulphate, but with IL-1 activity-inhibiting reagents for chronic colitis failed to show any significant effect, whereas the other two significantly reduced the colitis<sup>[113]</sup>. This study suggests that TNF but not IL-1 plays a major role in perpetuation of chronic inflammation. Recently, several cytokines, such as IL-4, IL-10, and IL-13 were reported to be capable of inhibiting the pro-inflammatory cytokines<sup>[114-116]</sup>. Kucharzik *et al* did show that combinations of IL-10 plus IL-4 and IL-10 plus IL-13, respectively, inhibited IL-1 $\beta$ , and TNF $\alpha$  response of peripheral monocytes stimulated by pokeweed mitogen much more than IL-4, IL-10, or IL-13 alone<sup>[117]</sup>.

The classical anti-colon antibodies are found more common in UC patients than in CD patients, but are of minor diagnostic value<sup>[118,119]</sup>.

Anti-neutrophil cytoplasmic antibodies (ANCA) have been identified<sup>[120]</sup>. Perinuclear staining (pANCA) occurs in up to 70% of patients with UC, but in less than 20% of CD patients and in an even lower percentage of healthy controls.

Yang *et al* showed that pANCA-positive patients have a significantly increased frequency of HLA-DR2 as compared with pANCA negative patients, whereas pANCA-negative patients have a high frequency of HLA-DR4. These authors therefore proposed at least two genetically subclinical markers an HLA-DR2 associated pANCA positive group and an HLA-DR4 associated pANCA negative group<sup>[121]</sup>. These results could not be confirmed by other studies<sup>[122,123]</sup> (Bouma, personal communication). Recently, Abad *et al* reported the IBD associated ANCA is different from vasculitis-associated ANCA<sup>[124]</sup>.

Tropomyosin is an actin-binding cytoskeletal protein localized in the apical cytoplasm and brush border of colonic enterocytes and may possess epitopes cross-reacting with bacterial products<sup>[125]</sup>. Anti-tropomyosin antibody, a 40kD protein present in epithelial cells of the colon, skin and biliary tract was found in the circulation of more than 55% of UC patients and the cross-reaction may be able to induce complement activation mediated by IgG<sup>[125-127]</sup>. These data suggest that UC is an

autoimmune disorder, but CD is not. However, the notion that UC and CD are autoimmune disease has been supported by very little documentation<sup>[76]</sup>.

The final common pathway of immune activation in IBD is the local influx of lymphocytes, macrophages, and polymorphonuclear neutrophils (PMN) which induce tissue damage, and subsequently results in clinical manifestations of IBD. These cells produce many immune and inflammatory mediators that amplify the inflammatory reaction. Among these are cytokines, complement components, eicosanoids, platelet-activating factor (PAF), leukotrienes, adhesion molecules, neuropeptides, reactive oxygen metabolites (ROMs), and nitric oxide (NO).

ROMs are one of the key mediators in the inflammatory final common pathway<sup>[128]</sup>. Therefore, removal of large amounts of free oxygen radicals can relieve inflammation<sup>[129-131]</sup> and probably one of the major working mechanisms of 5-aminosalicylate and sulphasalazine used in the treatment of IBD<sup>[132]</sup>.

The unrestrained activation of the immune response and an imbalance in the immune regulation are characteristics of IBD. This process appear to be a secondary response following the initial unknown stimulation or primary trigger. From the recent development of experimental colitis in animal models with application of targeted deletion of genes (knockout mice) involved in immune processes and transgenic approaches<sup>[133-138]</sup> we have learned that:

- 1) The intestinal epithelial and mucosal immune system are necessary for maintaining normal intestinal homeostasis, and damage of the intestinal epithelial barrier and imbalance of mucosal immune regulation will contribute to the development of IBD;

- 2) In some of these models without normal intestinal flora or even without some challenge by an exogenous external agent, inflammation does not occur. This means that activation of the immune response in the intestinal mucosa needs persistent antigen stimulation. The antigens can be pathogenic bacteria, bacterial products, the normal intestinal flora, and even dietary antigens;

- 3) Genetic susceptibility of the host is also necessary to develop IBD as an important interaction between environmental factors and genetic predisposition.

As for the immune mechanism of IBD, Sartor proposes that the pathogenesis of IBD progresses through a series of steps: initiating events, perpetuating events, immunoregulatory abnormalities, tissue damage and clinical symptoms<sup>[76]</sup>. The initiating factors may be infections, toxins, and NSAIDs. These factors can break the intestinal epithelial barrier. Then the

inflammatory process may be continued by exposure to large amounts of resident luminal antigens and thereby induce a mucosal immune response and immunoregulatory abnormalities in genetically susceptible host, and finally tissue damage and clinical symptoms of IBD may occur. Although no specific initial pathogen has been found, and large and often conflicting results exist, it is now clear that IBD is a disease of or at least involving the mucosal immune system.

### *Genetic factors*

There is overwhelming evidence that genetic susceptibility plays a role in the development of IBD<sup>[139-143]</sup>. This is supported by several studies.

Firstly, there is an increased risk for the relatives of IBD patients to develop the disease. The frequency of a positive family history in first degree relatives of IBD is increasing in comparison with the normal population. Familial aggregation is more frequent in CD than in UC, suggesting that CD has a stronger genetic predisposition than UC. However, the data on familial aggregation do not fit a simple Mendelian pattern of inheritance<sup>[144,145]</sup>.

There is a higher concordance rate (which means that both twins suffer from the disease) in monozygotic twins than in dizygotic twins in both CD and UC, but this rate is higher in CD than in UC<sup>[146,147]</sup>. These twin studies also suggest the importance of genetic factors in the predisposition to IBD and can explain the phenomenon of familial aggregation. Since monozygotic twins share 100% of their genes, the observation that not all monozygotic twins are concordance for the disease, suggests that the penetrance of the IBD genotype is reduced and environmental factors also play a role in the development of IBD. Interestingly, one recent report first documented a case of UC and CD occurring in a monozygotic twin pair<sup>[148]</sup>. This case confirmed the important genetic contribution to the development of IBD, but also highlighted the role of environmental factors in dictating the IBD phenotype.

Using complex segregation analysis of a large population of IBD patients, two studies have concluded that up to 30% of IBD cases may result from a major gene defect. In CD, a major recessive gene has been suggested and in UC the results are consistent with the involvement of a major dominant gene<sup>[144,149]</sup>.

There is a limited number of reports on the risk of developing IBD in spouses of patients with IBD. These studies suggest that the incidence of IBD in spouses is not increased over the risk of disease in the general population and is dramatically less than in siblings<sup>[150-152]</sup>. The high rates of IBD in the first-degree relative versus the lack of high rates in spouses<sup>[153]</sup> also support the importance of the

genetic susceptibility to IBD.

Secondly, a large difference in the incidence and prevalence of IBD is found at different geographic locations and in various ethnic groups. Western countries, especially in North and West Europe and North America, have a higher incidence and prevalence of both UC and CD than the rest of the world<sup>[31,143]</sup>. As for certain ethnic groups, a high incidence of IBD was found in Caucasians and a low incidence in black and Asian people<sup>[31,154]</sup>.

The most interesting observation is that the Jewish population has a 2-9 times higher risk of developing IBD than other ethnic groups living in the same geographic area and during different time periods<sup>[155-158]</sup>. Furthermore, Ashkenazi Jews of European origin and American origin have a greater risk of coming down with IBD than the Jews of North African and Asian origin<sup>[158-160]</sup>. The prevalence of IBD is higher in Ashkenazi Jews of middle European origin than in those of Polish or Russian origin<sup>[161,162]</sup>. These ethnic aggregation phenomena can be explained by both genetic and environmental factors, but with such a consistently increased incidence and prevalence of IBD in Jewish people, it is hard to exclude genetic factors.

Thirdly, IBD is often associated with specific genetically determined syndromes, such as Turner's syndrome, Hermansky-Pudlak syndrome, and glycogen-storage disease type Ib. UC or CD are also often associated with several other immune-related disorders with a clear genetic component but otherwise unknown etiology, such as ankylosing spondylitis, primary sclerosing cholangitis, psoriasis, multiple sclerosis, coeliac disease, and autoimmune thyroid disease<sup>[143]</sup>. Ankylosing spondylitis, primary sclerosing cholangitis, multiple sclerosis and coeliac disease are linked to the genes of the MHC<sup>[163]</sup>.

Finally, recent whole genome screening studies using microsatellite markers which survey the entire genome for IBD-related loci in families with multiple IBD patients, have stressed the importance of genetic factors in the development of IBD. These are ongoing studies that have demonstrated a susceptibility locus for CD on chromosome 16<sup>[164,165]</sup>. The locus has been called IBD1. Later, Ohmen *et al* from the US confirmed this finding and described linkage with this locus only in non-Jewish CD sibpairs and not in Ashkenazi Jewish CD sibpairs<sup>[165]</sup>. Satsangi *et al* have detected the susceptibility loci for both CD and UC on chromosomes 3, 7 and 12<sup>[166]</sup>. Identification of susceptibility loci for IBD by these powerful technic is just beginning and progress will lead to a substantial understanding of the genetic contribution to the pathogenesis of IBD.

The experiments in transgenic mice and knockout mice to induce the phenotypically similar

intestinal and colonic inflammation also prove the contribution of genetic susceptibility and heterogeneity of IBD. Since the pathogenesis of IBD involves immune responses, it is not surprising that a large number of candidate gene studies are focusing on the associations between IBD and immunerelated genes, including MHC genes, cytokine genes, T cell receptor genes, complement factor genes, and genes encoding adhesion molecules, e. g. intercellular adhesion molecule-1 (ICAM-1).

## IMMUNOGENETIC FACTORS

To identify the genes that predispose to IBD, two methods are now widely used. These include population-associated studies and family-linkage studies. The population-associated studies are based on epidemiologic investigations and designed to compare the frequencies of genetic markers or polymorphic candidate genes between unrelated affected cases and unaffected controls. An association between a disease and a distinct genetic marker may be suggestive for a causal relationship of an associated gene (i. e., susceptibility gene), or may result for linkage disequilibrium, which may indicate the nearby location of the actual disorder gene. Linkage analysis tests the chromosomal location of disease susceptibility genes by identifying polymorphic markers of known location transmitted in families with multiple affected members. Linkage studies examine whether a certain allele of a genetic marker locus is transmitted within a family with the disease of interest. Existence of linkage between a given marker and the disease indicates that this marker is located in close physical proximity to that gene. Both the methods have been used to detect genetic markers in IBD.

### HLA genes

The major Histocompatibility Complex (MHC) in humans called the Human Leukocyte Antigen (HLA) complex, is located on the short arm of chromosome 6, which occupies a large segment of DNA extending about 3500 kilobases or 4 centimorgan. More than 100 different MHC genes have been identified in the HLA complex. The major HLA class I genes encode HLA-A, HLA-B and HLA-C molecules, and HLA class II genes encode HLA-DP, HLA-DQ and HLA-DR molecules. Class I molecules present peptides derived from proteins that are endogeneously synthesized in the cytosol to CD8+ T cells. Class II molecules present peptides derived from exogenous or membrane-bound proteins to CD4+ T cells. Each HLA gene encodes a cell surface molecule and most HLA genes are highly polymorphic. Since HLA molecules play an important role in antigen recognition and in the immune response, the genes are intensively studied in immunerelated diseases,

including IBD.

HL-A class I studies in Japan showed an increased prevalence of HLA-B5 and its subtype B52 in UC and this allele is in linkage disequilibrium with the class II HLA-DR2<sup>[167-171]</sup>. Studies from other countries have revealed the inconclusive results that HLA-A11 has increased the frequency in UC<sup>[172,173]</sup>. Two reports from China show that HLA-A31 was significantly more often detected in UC than in normal controls in the Chinese population<sup>[174,175]</sup>.

The studies of HLA-class II genes show a positive correlation of UC with HLA-DR2 in Japanese<sup>[169,171,176-178]</sup>. By the DNA-typing methods, HLA-DRB1-1502 has been found as the allele responsible for this association. HLA-DRB1-1502 is the most frequent subset of HLA-DR2 in Japanese. Several studies in European and North American populations confirm these results and revealed that HLA-DRB1-1501 is the most frequent subset of HLA-DR2 in Caucasians<sup>[179-183]</sup>. However, other studies did not show this association or even a negative association had been found<sup>[184-188]</sup>. Recently in some studies a positive association between HLA-DRB1-0103 and extensive UC has been observed<sup>[186,189]</sup>. It is interesting to know that the frequency of HLA-DR4 is significantly decreased in UC patients, suggesting that HLA-DR4 may have a protective effect against UC<sup>[170,179,186,190]</sup>.

In CD, results of studies are not uniform. Several studies have shown an increased frequency of HLA-B44<sup>[191-195]</sup>, HLA-B18<sup>[196]</sup>, whereas other studies do not find any association with HLA class I alleles<sup>[197-200]</sup>.

As for HLA class II genes, HLA-DR1<sup>[201]</sup>, -DR4<sup>[176,202-205]</sup>, -DR7<sup>[206]</sup>, -DRB3-0301<sup>[187]</sup> are found more often in CD, whereas HLA-DR3<sup>[201,206]</sup>, -DQA1 \* 0102<sup>[204]</sup>, and DPB1-0401<sup>[207]</sup> are found less frequent in CD. Of these genes, only HLA-DR4 was frequently related with CD in studies in the Japanese population. Ethnic variation as well as clinical heterogeneity in UC and CD may explain these confusing results.

HLA linkage studies have been conducted to evaluate co-segregation of HLA haplotypes in IBD families with multiple affected members. Increased haploypoe sharing among affected siblings has been found in some studies<sup>[186,208-210]</sup> but not in toher<sup>[145,199,211,212]</sup>.

### Cytokine genes

**TNF $\alpha$  genes and LT $\alpha$**  Tumor necrosis factor alpha (TNF $\alpha$ ) and tumor necrosis factor beta (TNF $\alpha$  or lymphotoxin alpha, LT $\alpha$ ) are potent cytokines with numerous biological functions such as the heemorrhagic necrosis of tumors, cytotoxicity and immunoregulation. The genes encoding TNF $\alpha$  and

LT $\alpha$  are tandemly arranged in the class III or central region of the MHC at the short arm of chromosome 6<sup>[213]</sup>. This region, encoding at least 30 different proteins that regulate macrophage and T cell function, B cell proliferation, and antibody production, plays a unique role in the regulation of the immune system<sup>[214]</sup>.

Several polymorphisms in TNF region have been described. Partanen and Koskimies reported a rare EcoRI restriction fragment length polymorphism (RFLP) in the LT $\alpha$  gene in 1988<sup>[215]</sup>. Later, Messer *et al* showed a NcoI RFLP in the first intron of the LT $\alpha$  gene in 1991<sup>[216]</sup> and thereafter Ferencik *et al* described an AspHI RFLP in the first intron of the LT $\alpha$  gene<sup>[217]</sup>. Of five polymorphisms in the TNF $\alpha$  gene, four were found to be G to A transition polymorphisms at positions 376<sup>[218]</sup>, -308<sup>[219]</sup>, -238<sup>[220]</sup>, and 163<sup>[218]</sup> in the promoter region of the TNF $\alpha$  gene, and one was a C insertion polymorphism in a C-stretch at position +7 in the first exon of the TNF $\alpha$  gene<sup>[221]</sup>. As for microsatellites in the TNF- region, five polymorphic loci have been found<sup>[222]</sup>. Two are located 3.5kb upstream of the LT $\alpha$  gene, one is present in the first intron of the LT $\alpha$  gene, and the positions of the other two are downstream of the TNF $\alpha$  gene. Recently, a compoundtetra, dinucleotide microsatellite polymorphism was found in TNF/LT locus<sup>[223]</sup>.

A limited number of studies on associations between the TNF $\alpha$  308 gene polymorphism and UC and CD have been carried out. Mansfield *et al* found no evidence for the involvement of this polymorphism in either UC or CD<sup>[224]</sup>. Allele 2 of the TNF $\alpha$  -308 polymorphism was slightly decreased in CD<sup>[225]</sup>. A specific allelic combination of five microsatellite alleles, linked with the HLA-DR1-DQ5 haplotype, was increased in CD patients<sup>[226]</sup>. Studies from our group have shown that in the Dutch population there are only five haplotypic combinations at four polymorphic sites in the TNF $\alpha$  and LT $\alpha$  genes<sup>[227]</sup>.

**IL-1 and IL-1ra genes** In man, the genes encoding interleukin-1 $\alpha$  (IL-1 $\alpha$ ) and IL-1 $\beta$ , and their antagonist, interleukin-1 receptor antagonist (IL-1ra) are located in each others vicinity on the long arm of chromosome 2 (2q13 - 14)<sup>[228]</sup>. Polymorphisms of IL-1 $\alpha$ <sup>[229-231]</sup>, IL-1 $\beta$ <sup>[232,233]</sup>, and IL-1ra<sup>[234]</sup> genes have been described in recent years. The IL-1ra gene polymorphism in intron 2 is characterized by an 86bp tandem repeat (VNTR), with five different alleles encountered in the Caucasian population. Mansfield *et al* from England studied the association between IBD and IL-1 $\alpha$ , IL-1 $\beta$  and IL-1ra genes. Allele 2 of the IL-1ra gene was significantly over-represented in UC patients,

especially in patients with total colitis, a similar situation was not seen in CD<sup>[224]</sup>. Our group did confirm that IL-1ra allele 2 is related with severity in UC<sup>[235]</sup>, and found non-carriers of IL-1 $\beta$  allele 2 to be more often present in carriers of IL-1ra allele 2 in UC and CD than in healthy controls<sup>[236]</sup>. This suggests that the imbalance of the IL-1/IL-1ra-ratio observed in IBD<sup>[109,237]</sup> has a biological basis. Brett *et al* found an increased frequency of IL-1ra allele 2 in UC, with the majority of the association arising from the pouchitis group, suggesting that the presence of allele 2 in patients with UC affects the disease outcome<sup>[238]</sup>. However, three other reports show no association between the allele 2 and UC<sup>[225,239,240]</sup>.

**Other candidate genes** From the United States, Yang *et al* have reported studies on single base polymorphisms in exons 4 and 6 of the ICAM-1 gene in IBD patients. The results show that these two polymorphisms are not associated with CD or UC. Only after stratifying patients by pANCA status, differences are demonstrated between subgroups of UC and CD<sup>[241]</sup>. The significance of this unconfirmed observation is uncertain.

Elmgreen *et al* have reported a study on a polymorphism in the complement C3 gene in IBD. A single nucleotide substitution in the C3 gene did result in a fast moving product on electrophoresis (C3F), that is more often present in CD patients, but not in UC patients<sup>[242]</sup>.

As for T cell receptor (TCR) gene, two studies show no associations of IBD with TCR $\alpha$  and  $\beta$ -chain polymorphisms<sup>[243,244]</sup>. One study found that the TCR constant beta gene was associated with UC<sup>[190]</sup> and two other studies found that TCR-V- $\beta$  8 was associated with CD<sup>[145,146]</sup>.

With regard to transporter associated with antigen processing (TAP) genes, which are located between HLA-DP and HLA-DQ, Heresbach *et al* found no difference between overall UC or CD and unrelated healthy controls<sup>[123,247]</sup>.

Above all, the results from these candidate gene studies are often conflicting and require further confirmation.

## SUMMARY

The etiology of IBD remains unclear. Environmental factors, a genetic predisposition and a disturbed immunological response all contribute to the development of the disease.

Even though pathogens have not been identified as a cause of IBD, persistent infections are highly suspected, including Mycobacterium paratuberculosis and measles virus in CD, and an unknown bacterial infection and/or bacterial products in UC. Smoking has been suggested to be a strong risk factor in CD, and in determining the

clinical course of UC and CD.

Disturbances of the mucosal immune system and an imbalance of immune regulation presented by cellular and/or humoral abnormalities as well as functions of pro-inflammatory cytokines and anti-inflammatory cytokines, and other mediators were observed in IBD patients and are considered to be fundamental to the pathogenesis of IBD. Because of the multiplicity and complexity of the interactions of all these elements, it is difficult to distinguish between primary and secondary, and pathogenic and non-pathogenic phenomena. With the development of transgenic and knockout rodent models, it is possible to define the critical immune elements necessary to develop IBD. Recent studies demonstrated that mucosal homeostasis is important and a deflection mucosal barrier may allow unrestrained uptake of luminal antigens and pro-inflammatory molecules and thus initiate immune responses. Some evidence for an imbalance of pro- and anti-inflammatory cytokines and of Th1/Th2 lymphocytes have been found in chronic intestinal inflammation. pANCA are present in more than 70% of UC patients, but their role as a subclinical marker of UC needs to be confirmed.

As to genetic factors, epidemiological studies have demonstrated the influence of genetic factors on the development of IBD by phenomena such as ethnic variations, familial aggregation, monozygotic twins versus dizygotic twins, and first degree relatives versus spouses. A genetic influence weighs more for CD than for UC. Much interest has been focused on association studies of HLA-genes, cytokine genes and other immunemediating genes. These studies do not explain the overall disease susceptibility to either UC or CD, but may in future studies shed more light on the relevance of these markers for subgroups of patients. The definition of clinical subgroups in IBD, relevant to these studies forms a challenge to the clinicians. Recent studies using systematic whole genome screening in families with multiple affected members found the first susceptibility locus for CD on chromosome 16, and susceptibility loci for both CD and UC on chromosomes 3, 7 and 12 have been reported. Further studies are needed to clarify the complex genetic mechanisms in IBD.

## REFERENCES

- 1 Wilks S. The morbid appearance of the intestine of miss Banks. *Med Times Gazette*, 1985;2:264-269
- 2 Crohn BB, Ginsberg L, Oppenheimer GD. Regional enteritis. A pathological and clinical entity. *JAMA*, 1932;99:1323-1329
- 3 Dalziel TK. Chronic interstitial enteritis. *Br Med J*, 1913; 2: 1068-1070
- 4 Lockhart-Mummery HE, Morson BC. Crohn's disease (regional enteritis) of the large intestine and its distinction from ulcerative colitis. *Gut*, 1960;1:87-105
- 5 Price AB. Overlap in the spectrum of non-specific inflammatory bowel disease: 'colitis indeterminate'. *J Clin Pathol*, 1978;31:567-577
- 6 Wells AD, McMillan I, Price AB, Ritchie JK, Nicholls RJ. Natural history of indeterminate colitis. *Br J Surg*, 1991;78:179-181

- 7 Moum B, Vatn MH, Ekblom A, Aadland E, Fausa O, Lygren I *et al*. Incidence of ulcerative colitis and indeterminate colitis in four counties of southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol*, 1996; 31:362-366
- 8 Moum B, Vatn MH, Ekblom A, Aadland E, Fausa O, Lygren I *et al*. Incidence of Crohn's disease in four counties in southeastern Norway, 1990-93. A prospective population-based study. The Inflammatory Bowel South-Eastern Norway (IBSEN) Study Group of Gastroenterologists. *Scand J Gastroenterol*, 1996;31:355-361
- 9 Kildebo S, Breckan R, Nordgaard K, Burhol PG, Jorde R. The incidence of Crohn's disease in northern Norway from 1983 to 1986. *Scand J Gastroenterol*, 1989;24:1265-1270
- 10 Kildebo S, Nordgaard K, Aronsen O, Breckan R, Burhol PG, Jorde R. The incidence of ulcerative colitis in Northern Norway from 1983 to 1986. The northern Norwegian study group. *Scand J Gastroenterol*, 1990; 25:890-896
- 11 Haug K, Schrupp E, Halvorsen JF, Fluge J, Hamre E, Hamre T. Epidemiology of Crohn's disease in western Norway. Study group of inflammatory bowel disease in western Norway. *Scand J Gastroenterol*, 1989;24:1271-1275
- 12 Ekblom A, Helmick C, Zack M, Adami HO. The epidemiology of inflammatory bowel disease: a large, population-based study in Sweden. *Gastroenterology*, 1991;100:350-358
- 13 Langholz E, Munkholm P, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of ulcerative colitis in Copenhagen county from 1962-1987. *Scand J Gastroenterol*, 1991;26:1247-1256
- 14 Munkholm P, Langholz E, Nielsen OH, Kreiner S, Binder V. Incidence and prevalence of Crohn's disease in the County of Copenhagen, 1962-87: A six fold increase in incidence. *Scand J Gastroenterol*, 1992;27:609-614
- 15 Martinez Salmeron JF, Rodrigo M, de Teresa J, Nogueras F, Garcia-Montero M, de Sola C *et al*. Epidemiology of inflammatory bowel disease in the province of Granada, Spain: a retrospective study from 1979 to 1988. *Gut*, 1993;34:1207-1209
- 16 Russel MGVM, Dorant E, Volvics A, Brummer RJM, Pop P, Muris JWM *et al*. High incidence of inflammatory bowel disease in the Netherlands: results of a prospective study. *Dis Colon Rectum*, 1998;41:33-40
- 17 Shivananda S, Pena AS, Mayberry JF, Ruitenberg EJ, Hoedemaeker PJ. Epidemiology of proctocolitis in the region of Leiden, The Netherlands. A population study from 1979 to 1983. *Scand J Gastroenterol*, 1987;22:993-1002
- 18 Shivananda S, Pena AS, Nap M, Weterman IT, Mayberry JF, Ruitenberg EJ *et al*. Epidemiology of Crohn's disease in region Leiden, the Netherlands. A population study from 1979 to 1983. *Gastroenterology*, 1987;93:955-974
- 19 Trallori G, Palli D, Saieva C, Bardazzi G, Bonanomi AG, d'Albasio G *et al*. A population-based study of inflammatory bowel disease in Florence over 15 years (1978-92). *Scand J Gastroenterol*, 1996; 31: 892-899
- 20 Tragnone A, Corrao G, Miglio F, Caprilli R, Lanfranchi GA. Incidence of inflammatory bowel disease in Italy: a nationwide population-based study. Gruppo Italiano per lo Studio del Colone del Retto (GISC). *Int J Epidemiol*, 1996;25:1044-1052
- 21 Vucelic B, Korac B, Sentic M, Milicic D, Hadzic N, Juresa V *et al*. Epidemiology of Crohn's disease in Zagreb, Yugoslavia: a ten years prospective study. *Int J Epidemiol*, 1991;20:216-220
- 22 Vucelic B, Korac B, Sentic M, Milicic D, Hadzic N, Juresa V *et al*. Ulcerative colitis in Zagreb, Yugoslavia: Incidence and prevalence 1980-1989. *Int J Epidemiol*, 1991;20:1043-1047
- 23 Manousos ON, Koutroubakis I, Potamianos S, Roussomoustakaki M, Gourtsoyiannis N, Vlachonikolis IG. A prospective epidemiologic study of Crohn's disease in Heraklion, Crete. Incidence over a 5-year period. *Scand J Gastroenterol*, 1996;31:599-603
- 24 Kyle J. Crohn's disease in the northeastern and northern Isles of Scotland: an epidemiological review. *Gastroenterology*, 1992; 103:392-399
- 25 Stonnington CM, Phillips SF, Melton LI, Zinsmeister AR. Chronic ulcerative colitis: Incidence and prevalence in a community. *Gut*, 1987;28:402-409
- 26 Stowe SP, Redmond SR, Stormont JM, Shan AN, Chessin LN, Segal HL *et al*. An epidemiologic study of inflammatory bowel disease in Rochester, New York. Hospital incidence. *Gastroenterology*, 1990;98:104-110
- 27 Kawai K, Higashi A, Watanabe Y, Tada M. Epidemiological study of inflammatory bowel disease in Japan. International Falk Workshop of Inflammatory Bowel Diseases in Asia. Hong Kong; 1996:27-29
- 28 Wright JP, Froggatt J, O'Kewefe EA, Ackerman S, Watermeyer S, Louw J *et al*. The epidemiology of inflammatory bowel disease in Cape Town 1980-1984. *S Afr Med J*, 1986;70:10-15
- 29 Shivananda S, Lennard-Jones J, Logan R, Fear N, Price A, Carpenter L *et al*. Incidence of inflammatory bowel disease: is there a difference between north and south. Results of the European collaborative study on inflammatory bowel disease. *Gut*, 1996;39:690-697
- 30 Calkins BM, Mendeloff AI. The epidemiology of idiopathic inflammatory bowel disease. In: Kirsner JB, Shorter RG, eds. *Inflammatory bowel disease*. Baltimore: Williams & Wilkins, 1995:31-68
- 31 Russel MGVM, Stockbrugger RW. Epidemiology of inflammatory bowel disease: an update. *Scand J Gastroenterol*, 1996;31:417-427
- 32 Shivananda S, Sutherland L. Epidemiology of bowel inflammation. International Falk Workshop on inflammatory bowel disease in Asia. Hong Kong; 1996:3-38
- 33 Miskovitz P, Rochwarger A. The evaluation and treatment of the patient with diarrhea. In: Miskovitz P, Rochwarger A, eds. *Infectious causes of diarrhea*. Boston: Andover Medical Publishers, 1993:45-96
- 34 Rubin PH, Present DH. Differential diagnosis of chronic ulcerative colitis and Crohn's disease of the colon: one, two or many diseases. In: Kirsner JB, Shorter RG, eds. *Inflammatory bowel disease*. 4 ed. Baltimore: Williams & Wilkins, 1995:355-379
- 35 Geboes K, Ectors N. The infectious track in inflammatory bowel disease: a controversial area. Research and Clinical Forums. *Wells Medical Ltd*, 1995;17:41-48
- 36 Wakefield AJ, Pittilo RM, Sim R, Cosby SL, Stephenson JR, Dhillon AP *et al*. Evidence of persistent measles virus infection in Crohn's disease. *J Med Virol*, 1993;39:345-353
- 37 Wakefield AJ, Ekblom A, Dhillon AP, Pittilo RM, Pounder RE. Crohn's disease: pathogenesis and persistent measles virus infection. *Gastroenterology*, 1995;108:911-916
- 38 Lewin J, Dhillon AP, Sim R, Mazure G, Pounder RE, Wakefield AJ. Persistent measles virus infection of the intestine: confirmation by immunogold electron microscopy. *Gut*, 1995;36:564-569
- 39 Ekblom A, Daszak P, Kraaz W, Wakefield AJ. Crohn's disease after in-utero measles virus exposure. *Lancet*, 1996;348:515-517
- 40 Thompson NP, Montgomery SM, Pounder RE, Wakefield AJ. Is measles vaccination a risk factor for inflammatory bowel disease. *Lancet*, 1995;345:1071-1074
- 41 Iizuka M, Nakagomi O, Chiba M, Ueda S, Masamune O. Absence of measles virus in Crohn's disease. *Lancet*, 1995;345:199
- 42 Nakagomi O, Iizuka M. Measles virus in Crohn's disease. *Lancet*, 1995;345:660
- 43 Fisher NC, Yee L, Nightingale P, McEwan R, Gibson JA. Measles virus serology in Crohn's disease. *Gut*, 1997;41:66-69
- 44 Miyamoto H, Tanaka T, Kitamoto N, Fukuda Y, Shimoyama T. Detection of immunoreactive antigen, with a monoclonal antibody to measles virus, in tissue from a patient with Crohn's disease. *J Gastroenterol*, 1995;30:28-33
- 45 Chiodini RJ, van Kruiningen HJ, Merkal RS, Thayer WR, Jr., Coutu JA. Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease. *J Clin Microbiol*, 1984;20: 966-971
- 46 Chiodini RJ, van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of Mycobacteria in inflammatory bowel disease. I. An unclassified Mycobacterium species isolated from patients with Crohn's disease. *Dig Dis Sci*, 1984;29:1073-1079
- 47 Moss MT, Sanderson JD, Tizard MLV, Hermon-Taylor J, El-Zaatari FAK, Markesich DC *et al*. Polymerase chain reaction detection of Mycobacterium paratuberculosis and Mycobacterium avium subsp silvaticum in long term cultures from Crohn's disease and control tissues. *Gut*, 1992;33:1209-1213
- 48 Lisby G, Andersen J, Engbaek K, Binder V. Mycobacterium paratuberculosis in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction. *Scand J Gastroenterol*, 1994;29:923-929
- 49 Thayer WR, Jr., Coutu JA, Chiodini RJ, van Kruiningen HJ, Merkal RS. Possible role of mycobacteria in inflammatory bowel disease. II. Mycobacterial antibodies in Crohn's disease. *Dig Dis Sci*, 1984;29:1080-1085
- 50 Suenaga K, Yokoyama Y, Okazaki K, Yamamoto Y. Mycobacteria in the intestine of Japanese patients with inflammatory bowel disease. *Am J Gastroenterol*, 1995;90:76-80
- 51 Al-Shamali M, Khan I, Al-Nakib B, Al-Hassan F, Mustafa AS. A multiplex polymerase chain reaction assay for the detection of Mycobacterium paratuberculosis DNA in Crohn's disease tissue. *Scand J Gastroenterol*, 1997;32:819-823
- 52 Afdhal NH, Long A, Lennon J, Crowe J, O'Donoghue DP. Controlled trial of antimycobacterial therapy in Crohn's disease. Clofazimine versus

- placebo. *Dig Dis Sci*, 1991;36:449-453
- 53 Thayer WR. The use of antimycobacterial agents in Crohn's disease. *J Clin Gastroenterol*, 1992;15:5-7
- 54 Prantera C, Kohn A, Mangiarotti R, Andreoli A, Luzi C. Antimycobacterial therapy in Crohn's disease: results of a controlled, double-blind trial with a multiple antibiotic regimen. *Am J Gastroenterol*, 1994;89:513-518
- 55 Swift GL, Srivastava ED, Stone R, Pullan RD, Rhodes J, Wilkinson S. Controlled trial of anti-tuberculous chemotherapy for two years in Crohn's disease. *Gut*, 1994;35:363-368
- 56 Nakamura Y, Labarthe DR. A case-control study of ulcerative colitis with relation to smoking habits and alcohol consumption in Japan. *Am J Epidemiol*, 1994;140:902-911
- 57 Boyko EJ, Koepsell TD, Perera DR, Inui TS. Risk of ulcerative colitis among former and current cigarette smokers. *N Engl J Med*, 1987;316:707-710
- 58 Lindberg E, Tysk C, Andersson K, Jarnerot G. Smoking and inflammatory bowel disease. A case control study. *Gut*, 1988;29:352-357
- 59 Franchesi S, Panza E, La Vecchia C, Parazzini F, Decarli A, Bianchi Porro G. Nonspecific inflammatory bowel disease and smoking. *Am J Epidemiol*, 1987;125:445-452
- 60 Calkins BM. A meta-analysis of the role of smoking in inflammatory bowel disease. *Dig Dis Sci*, 1989;34:1841-1854
- 61 Persson PG, Ahlbom A, Hellers G. Inflammatory bowel disease and tobacco smoke: a case-control study. *Gut*, 1990;31:1377-1381
- 62 Lashner BA, Hanauer SB, Silverstein MD. Testing nicotine gum for ulcerative colitis patients. Experience with single-patient trials. *Dig Dis Sci*, 1990;35:827-832
- 63 Pullan RD, Rhodes J, Ganesh S, Mani V, Morris JS, Williams GT et al. Transdermal nicotine for active ulcerative colitis. *N Engl J Med*, 1994;330:811-815
- 64 Thomas GAO, Rhodes J, Mani V, Williams GT, Newcombe RG, Russell MAH et al. Transdermal nicotine as maintenance therapy for ulcerative colitis. *N Engl J Med*, 1995;332:988-992
- 65 Silverstein MD, Lashner BA, Hanauer SB, Evans AA, Kirsner JB. Cigarette smoking in Crohn's disease. *Am J Gastroenterol*, 1989;84:31-33
- 66 Russel MGVM, Volovics A, Schoon EJ, v Wijk EHI, Logan RF, Shivananda S et al. Inflammatory bowel disease: is there any relationship between smoking status and disease presentation Results of the European Collaborative Study on inflammatory bowel disease. In: Russel MGVM Ph.D thesis. Incidence, risk factors and quality of life in IBD: Inflammatory bowel disease registry South Limburg. Maastricht: University Maastricht, 1997:117-129
- 67 Russel MG, Nieman FH, Bergers JM, Stockbrugger RW. Cigarette smoking and quality of life in patients with inflammatory bowel disease. South Limburg IBD Study Group. *Eur J Gastroenterol Hepatol*, 1996;8:1075-1081
- 68 Elson CO, McCabe RP. The immunology of inflammatory bowel disease. In: Kirsner JB, Shorter RG, eds. Inflammatory bowel disease. Baltimore: Williams & Wilkins, 1995:203-251
- 69 Mombaerts P, Mizoguchi E, Grusby MJ, Glimcher LH, Bhan AK, Tonegawa S. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell*, 1993;75:274-282
- 70 Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*, 1993;75:253-261
- 71 Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 1993;75:263-274
- 72 Warren JR. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet*, 1983;1:1273-1275
- 73 Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet*, 1983;1:1273-1275
- 74 Cahill RJ, Foltz CJ, Fox JG, Dangler CA, Powrie F, Schauer DB. Inflammatory bowel disease: an immunity-mediated condition triggered by bacterial infection with *Helicobacter hepaticus*. *Infect Immun*, 1997;65:3126-3131
- 75 Shomer NH, Dangler CA, Schrenzel MD, Fox JG. *Helicobacter bilis*-induced inflammatory bowel disease in scid mice with defined flora. *Infect Immun*, 1997;65:4858-4864
- 76 Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am J Gastroenterol*, 1997;92(suppl 12):S5-S11S
- 77 May GR, Sutherland LR, Meddings JB. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology*, 1993;104:1627-1632
- 78 Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med*, 1986;105:883-885
- 79 Hilsden RJ, Meddings JB, Sutherland LR. Intestinal permeability changes in response to acetylsalicylic acid in relatives of patients with Crohn's disease. *Gastroenterology*, 1996;110:1395-1403
- 80 Podolsky DK, Isselbacher KJ. Glycoprotein composition of colonic mucosa. Specific alterations in ulcerative colitis. *Gastroenterology*, 1984;87:881-998
- 81 Rhodes JM. Colonic mucus and mucosal glycoproteins: the key to colitis and cancer. *Gut*, 1989;30:1660-1666
- 82 Clamp JR, Fraser G, Read AE. Study of the carbohydrate content of mucus glycoproteins from normal and diseased colons. *Clin Sci (Colch)*, 1981;61:229-234
- 83 Teahon K, Smethurst P, Levi AJ, Menzies IS, Bjarnason I. Intestinal permeability in patients with Crohn's disease and their first degree relatives. *Gut*, 1992;33:320-323
- 84 Ainsworth M, Eriksen J, Rasmussen JW, Schaffalitzky de Muckadell OB. Intestinal permeability of <sup>51</sup>Cr-labelled ethylenediaminetetraacetic acid in patients with Crohn's disease and their healthy relatives. *Scand J Gastroenterol*, 1989;24:993-998
- 85 Ruttenberg D, Young GO, Wright JP, Isaacs S. PEG-400 excretion in patients with Crohn's disease, their first-degree relatives, and healthy volunteers. *Dig Dis Sci*, 1992;37:705-708
- 86 Munkholm P, Langholz E, Hollander D, Thornberg K, Orholm M, Katz KD, Binder V. Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. *Gut*, 1994;35:68-72
- 87 Abreu-Martin MT, Targan SR. Regulation of T cells in the intestinal mucosa. *Curr Opin Gastroenterol*, 1996;12:569-576
- 88 Greenwald BD, James SP. Immunology of inflammatory bowel disease. *Curr Opin Gastroenterol*, 1997;13:293-301
- 89 Engleman EG, Benike CJ, Grumet FC, Evans RL. Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. *J Immunol*, 1981;127:2124-2129
- 90 Meuer SC, Schlossman SF, Reinherz EL. Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc Natl Acad Sci USA*, 1982;79:4395-4399
- 91 Schieferdecker HL, Ullrich R, Hirsland H, Zeitz M. T cell differentiation antigens on lymphocytes in the human intestinal lamina propria. *J Immunol*, 1992;149:2816-2822
- 92 Qiao L, Schurmann G, Betzler M, Meuer SC. Activation and signaling status of human lamina propria T-lymphocytes. *Gastroenterology*, 1991;101:1529-1536
- 93 Targan SR, Deem RL, Liu M, Wang S, Nel A. Definition of a lamina propria T cell responsive state. Enhanced cytokine responsiveness of T cells stimulated through the CD2 pathway. *J Immunol*, 1995;154:664-675
- 94 Selby WS, Janossy G, Bofill M, Jewell DP. Intestinal lymphocyte subpopulations in inflammatory bowel disease: an analysis by immunohistological and cell isolation techniques. *Gut*, 1984;25:32-40
- 95 James SP, Fiocchi C, Graeff AS, Strober W. Immunoregulatory function of lamina propria T cells in Crohn's disease. *Gastroenterology*, 1985;88:1143-1150
- 96 Moore K, Walters MT, Jones DB, Garvey E, Harvey J, Cawley MI et al. An immunohistological study of CD4+ lymphocyte subsets within inflammatory lesions with special reference to rheumatoid arthritis and inflammatory bowel disease. *Immunology*, 1988;65:457-463
- 97 Pallone F, Fais S, Squarcia O, Biancone L, Pozzilli P, Boirivant M. Activation of peripheral blood and intestinal lamina propria lymphocytes in Crohn's disease. In vivo state of activation and in vitro response to stimulation as defined by the expression of early activation antigens. *Gut*, 1987;28:745-753
- 98 Lee HB, Kim JH, Yim CY, Kim DG, Ahn DS. Differences in immunophenotyping of mucosal lymphocytes between ulcerative colitis and Crohn's disease. *Korean J Intern Med*, 1997;12:7-15
- 99 Choy MY, Walker-Smith JA, Williams CB, MacDonald TT. Differential expression of CD25 (interleukin-2 receptor) on lamina propria T cells and macrophages in the intestinal lesions in Crohn's disease and ulcerative colitis. *Gut*, 1990;31:1365-1370
- 100 Brynskov J, Tvede N. Plasma interleukin-2 and a soluble/shed interleukin-2 receptor in serum of patients with Crohn's disease. Effect of cyclosporin. *Gut*, 1990;31:795-799
- 101 Mahida YR, Gallagher A, Kurlak L, Hawkey CJ. Plasma and tissue interleukin-2 receptor levels in inflammatory bowel disease. *Clin Exp Immunol*, 1990;82:75-80
- 102 Mueller C, Knoflach P, Zielinski CC. T-cell activation in Crohn's disease. Increased levels of soluble interleukin-2 receptor in serum and

- in supernatants of stimulated peripheral blood mononuclear cells. *Gastroenterology*, 1990;98:639-646
- 103 Mullin GE, Maycon ZR, Braun-Elwert LEA. Inflammatory bowel disease mucosal biopsies have specialized lymphokine mRNA profiles. *Inflamm Bowel Dis*, 1996;2:16-26
- 104 Weiner HL. Oral tolerance: immune mechanisms and treatment of autoimmune disease. *Immunol Today*, 1997;18:335-343
- 105 Strober W, Kelsall B, Fuss I, Marth T, Ludviksson B, Ehrhardt R *et al*. Reciprocal IFN- $\gamma$  and TGF- $\beta$  responses regulate the occurrence of mucosal inflammation. *Immunol Today*, 1997;18:61-64
- 106 MacDonal TT, Spencer J. Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med*, 1988;167:1341-1349
- 107 Fiocchi C, Podolsky DK. Cytokines and growth factors in inflammatory bowel disease. In: Kirsner JB, Shorter RG, eds. *Inflammatory bowel disease*. Baltimore: Williams & Wilkins, 1995:252-280
- 108 Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today*, 1996;17:138-146
- 109 Casini-Raggi V, Kam L, Chong YJT, Fiocchi C, Pizarro TT, Cominelli F. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. *J Immunol*, 1995;154:2434-2440
- 110 Ferretti M, Casini-Raggi V, Pizarro TT, Eisenberg SP, Nast CC, Cominelli F. Neutralization of endogenous IL-1 receptor antagonist exacerbates and prolongs inflammation in rabbit immune colitis. *J Clin Invest*, 1994;94:449-453
- 111 Melani L, Hirsch E, Guanzon M, Pizarro TT, Hirsh D, Cominelli F. Deletion of the IL-1 receptor antagonist (IL-1ra) gene increases susceptibility to experimental colitis in mice [Abstract]. *Gastroenterology*, 1997;112:A1040
- 112 Holt LC, Bocker U, Damiao A, Murphy M, Haskill JS, Sartor RB. Decreased ratio of IL-1ra/IL-1 mRNA is the result of inflammation rather than a unique IBD-related abnormality [Abstract]. *Gastroenterology*, 1997;112:A998
- 113 Kojouharoff G, Hans W, Obermeier F, Mannel DN, Andus T, Scholmerich J *et al*. Neutralization of tumor necrosis factor (TNF) but not of IL-1 reduces inflammation in chronic dextran sulphate sodium-induced colitis in mice. *Clin Exp Immunol*, 1997;107:353-358
- 114 Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effect of interleukin 4: suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci USA*, 1989;86:3803-3807
- 115 Kucharzik T, Stoll R, Luger N, Domschke W. Circulating antiinflammatory cytokine IL-10 in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol*, 1995;100:452-456
- 116 Kucharzik T, Luger N, Adolf M, Domschke W, Stoll R. Synergistic effect of immunoregulatory cytokines on peripheral blood monocytes from patients with inflammatory bowel disease. *Dig Dis Sci*, 1997;42:805-812
- 117 Kucharzik T, Luger N, Weigelt H, Adolf M, Domschke W, Stoll R. Immunoregulatory properties of IL-13 in patients with inflammatory bowel disease; comparison with IL-4 and IL-10. *Clin Exp Immunol*, 1996;104:483-490
- 118 Broberger O, Perlman P. Autoantibodies in human ulcerative colitis. *J Exp Med*, 1959;110:657-674
- 119 Broberger O, Perlman P. In vitro studies of ulcerative colitis. I. Reactions of patients serum with human fetal colon cells in tissue cultures. *J Exp Med*, 1963;117:705-715
- 120 Saxon A, Shanahan F, Landers C, Ganz T, Targan S. A distinct subset of antineutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. *J Allergy Clin Immunol*, 1990;86:202-210
- 121 Yang H, Rotter JJ, Toyoda H, Landers C, Tyran D, McElree CK *et al*. Ulcerative colitis: a genetically heterogeneous disorder defined by genetic (HLA Class II) and subclinical (Antineutrophil cytoplasmic antibodies) markers. *J Clin Invest*, 1993;92:1080-1084
- 122 Duerr RH, Neigut DA. Molecularly defined HLA-DR2 alleles in ulcerative colitis and an anti-neutrophil cytoplasmic antibody-positive subgroup. *Gastroenterology*, 1995;108:423-427
- 123 Heresbach D, Alizadeh M, Reumaux D, Colombel JF, Delamaire M, Danze PM *et al*. Are HLA-DR or TAP genes genetic markers of severity in ulcerative colitis. *J Autoimmun*, 1996;9:777-784
- 124 Abad E, Tural C, Mirapeix E, Cuxart A. Relationship between ANCA and clinical activity in inflammatory bowel disease: variation in prevalence of ANCA and evidence of heterogeneity. *J Autoimmun*, 1997;10:175-180
- 125 Das KM, Dasgupta A, Mandal A, Geng X. Autoimmunity to cytoskeletal protein, a clue to the pathogenetic mechanism for ulcerative colitis. *J Immunol*, 1993;150:2487-2493
- 126 Takahasi F, Shan Hs, Wise LS, Das KM. Circulating antibodies against human colonic extract enriched with a 40 kDa protein in patients with ulcerative colitis. *Gut*, 1990;31:1016-1020
- 127 Halstensen TS, Das KM, Brandtzaeg P. Epithelial deposits of immunoglobulin G1 and activated complement colocalise with the M(r) 40 kD putative autoantigen in ulcerative colitis. *Gut*, 1993;34:650-657
- 128 Grisham B. Oxidants and free radicals in inflammatory bowel disease. *Lancet*, 1994;344:859-861
- 129 Emerit J, Pelletier S, Tosoni-Verlignue D, Mollet M. Phase II trial of copper zinc superoxide dismutase (CuZnSOD) in treatment of Crohn's disease. *Free Radic Biol Med*, 1989;7:145-149
- 130 Keshavarzian A, Morgan G, Sedghi S, Gordon JH, Doria M. Role of reactive oxygen metabolites in experimental colitis. *Gut*, 1990;31:786-790
- 131 Keshavarzian A, Haydek, Zabihi R, Doria M, MDA, Sorenson JR. Agents capable of eliminating reactive oxygen species. Catalase, ER-2721, or Cu(II)2(3,5-DIPS)4 decrease experimental colitis. *Dig Dis*, 1992;37:1866-1873
- 132 Yamada T, Volkmer C, Crisham B. Antioxidant properties of 5-ASA: potential mechanism for its anti-inflammatory activity. *Can J Gastroenterol*, 1990;4:295-302
- 133 Morales VM, Snapper SB, Blumberg RS. Probing the gastrointestinal immune function using transgenic and knockout technology. *Curr Opin Gastroenterol*, 1996;12:577-583
- 134 Podolsky DK. Lessons from genetic models of inflammatory bowel disease. *Acta Gastroenterol Belg*, 1997;60:163-165
- 135 MacDeemott RP. Etiology and pathogenesis of inflammatory bowel disease. *Curr Opin Gastroenterol*, 1997;13:303-306
- 136 Bregenholt S, Delbro D, Claesson MH. T-cell transfer and cytokine/TCR gene deletion models in the study of inflammatory bowel disease. *APMIS*, 1997;105:655-662
- 137 Dieleman LA, Pena AS, Meuwissen SGM, van Rees EP. Role of animal models for the pathogenesis and treatment of inflammatory bowel disease. *Scan J Gastroenterol*, 1997;223(Suppl): 99-104
- 138 Sartor RB, ath HC, Sellon RK. Microbial factors in chronic intestinal inflammation. *Curr Opin Gastroenterol*, 1996;12:337-333
- 139 Pena As, Crusius JBA, Oudkerk Pool M, Casanova MG, Pals G, meuwissen SGM *et al* Genetics and epidemiology may contribute to understanding the pathogenesis of IBD—a new approach is now indicated. *Can J Gastroenterol*, 1993;7:71-75
- 140 atsangi J, Jewell DP, Rosenberg WMC, Bell JJ. Genetics of inflammatory bowel disease. *Gut*, 1994;35:696-700
- 141 Ekbo M A. Epidemiology of inflammatory bowel disease. *Curr Opin Gastroenterol*, 1997;13:289-292
- 142 Satsangi J, Jewell DP. Genetic markers in inflammatory bowel disease. *Curr Opinion Gastroenterol*, 1996;12:322-326
- 143 Yang HY, Rotter JJ. Genetic aspects of idiopathic inflammatory bowel disease. In: Kirsner JB, Shorter G, eds. *Inflammatory bowel disease*. Baltimore: Williams & Wilkins, 1995:301-331
- 144 Orholm M, Iselius L, Sorensen TLA, Munkholm P, Langholz E, Binder V. Investigation of inheritance of idiopathic inflammatory bowel diseases by complex segregation analysis. *BMJ*, 1993;306:20-24
- 145 Hugot JP, Laurent-Puig p, Gower-Rousseau C, Caillat-Zucman S, Beaugerie, Dupas JL *et al*. Linkage analyses of chromosome 61oci, including HLA, in familial aggregations of rhn's disease. *Am J Med Genet*, 1994;52:207-213
- 146 Tysk C, Lindberg E, Jamerot G, Floderus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut*, 1988;29:990-996
- 147 Thompson Np, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ*, 1996;312:95-96
- 148 Breslin NP, Todd A, Kilgallen C, O'Morain . Monozygotic twins with Crohn's disease and ulcerative colitis: a unique case report. *Gut*, 1997;41:557-560
- 149 Kuster W, Pascoe L, Purmann J, Funk S, Majewski F. The genetics of Crohn disease: complex segregation analysis of a family study with 265 patients with Crohn disease and, 187 relatives. *Am J Med Genet*, 1989;32:105-108
- 150 Mayberri JF, Rhodes J, Newcombe RG. Familial prevalence of inflammatory bowel disease in relatives of patients with Crohn's disease. *B Med J*, 1980;280:84
- 151 Weterman IT, Pena AS. Familial incidence of Crohn's disease in The Netherlands and a review of the literature. *Gastroenterology*, 1984; 86:449-452
- 152 Bennett RA, Rubin PH, Present DH. Frequency of inflammatory bowel disease in offspring of couples both presenting with

- inflammatory bowel disease. *Gastroenterology*, 1991; 100: 1638-1643
- 153 Pena AS. Genetic aspects In: Jamerot G,ennard-Jones J, Truelove S, eds. Inflammatory bowel diseases. *Lancaster: MTP Press*, 1992: 37-49
- 154 Samuels AD, Weese JL, Beman PM, Kirshner JB. An epidemiologic and demographic study of inflammatory bowel disease in black patients. *Dig Dis Sci*, 1974;19:156-160
- 155 Brahme F, Lindstrom C, Wenckert A. Crohn's disease in a defined population. *Gastroenterology*, 1975;69:342-351
- 156 Novis BH, Marks IN, Bank S, Louw JH. Incidence of Crohn's disease at Grootte Schuur Hospital during 1970.1974. *S Afr Med J*, 1975;49: 693-697
- 157 Pinchbeck BR, Kirdeikis J, Thomson ABR. Effects of religious affiliation and education status on the prevalence of inflammatory bowel disease in northern Alberta. *Can J Gastroenterol*, 1988;2(suppl. A):95-100
- 158 Odes Hs, Fraser D, Krugliak P, Fenyvcs D, Fraser GM, Sperber AD. Inflammatory bowel disease in the Bedouin Arabs of southern Israel: rarity of diagnosis and clinical features. *Gut*, 1991;32:1024-1026
- 159 Odes Hs, Fraser D, Krawiec J. Ulcerative colitis in the Jewish population of Southern Israel 1961-1985:epidemiological and clinical study. *Gut*, 1987;28:1630-1636
- 160 Gilat T, Grossman A, Fireman Z, Rozen P. Inflammatory bowel disease in Jews. In: McConnell R, Rozen P, Langman M, Gilat T, eds. The genetics and epidemiology of inflammatory bowel disease. *Basel: Karger*, 1986:135-140
- 161 Roth M-P, Petersen GM, McElree C, Feldman E, Rotter JI. Geographic origins of Jewish patients with inflammatory bowel disease. *Gastroenterology*, 1989;97:900-904
- 162 Zlotogora J, Zimmerman J, Rachmilewitz D. Crohn's disease in Ashkenazi Jews. *Gastroenterology*, 1990;99:286-290
- 163 Pena As Genetics of inflammatory bowel disease. Research and Clinical Forums. *Wells Medical Ltd*, 1993;15:13-18
- 164 Hugot JP, Laurent-Puig P, Gower-Rousseau C, Olson JM, Lee JC, Beaugerie L *et al.* Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature*, 1996;379:821-823
- 165 Ohmen JD, Yang HY, Yamamoto KK, Zhao HY, Ma YH, Bentley LG *et al.* Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohn's disease, but not in ulcerative colitis. *Hum Mol Gen*, 1996;5:1679-1683
- 166 Satsangi J, Parkes M, Louis E, Hashimoto L, Kato N, Welsh K *et al.* Two stage genome wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nature Genetics*, 1996;14:199-202
- 167 Tsuchiya M, Yoshida T, Asakura H, Hibi T, Ono A. HLA antigens and ulcerative colitis in Japan. *Digestion*, 1977;15:286-294
- 168 Hiwatashi N, Kikuchi T, Masamune O, Ouchi E, Watanabe H, Goto Y. HLA antigens in inflammatory bowel disease. *Tohoku J Exp Med*, 1980;131:381-385
- 169 Asakura H, Tsuchiya M, Aiso S, Watanabe M, Kobayashi K, Hibi T *et al.* Association of the human lymphocyte DRz antigen with Japanese ulcerative colitis. *Gastroenterology*, 1982;82:413-418
- 170 Sugimura K, Asakura H, Mizuki M, Hibi T, Yagita A *et al.* Analysis of genes within the HLA region affecting susceptibility to ulcerative colitis. *Hum Immunol*, 1993;36:112-118
- 171 Futami S, Aoyama N, Honsako Y, Tamura T, Morimoto S, Nakashima T *et al.* HLA-DRB1-1502 allele, subtype of R15, is associated with susceptibility to ulcerative colitis and its progression. *Dig Dis Sci*, 1995;40:814-818
- 172 Asquith P, Mackintosh P, Stokes PL, Holmes GKT, Cooke WT. Histocompatibility antigens in patients with inflammatory bowel disease. *Lancet*, 1974;1:113-115
- 173 Papasteriades C, Spiliadis C, Emmanouilidis A, Papadimitriou C, conomidou J, Manousos O. Histocompatibility antigens (HLA-A,B) and ulcerative colitis in a Greek population. *Digestion*, 1986;33:229-232
- 174 Guo Z, Zhang PY, Wei K, Wang ZR, Ge KL, uo Q *et al.* Association of HLA with ulcerative colitis. *Genetics Dis (Chi)*, 1988; 5:38-39
- 175 Wu L, Huang XQ, Wang XL, Huang NX, Zhang XL, Liu QY. Relation of HLA-A, B to ulcerative colitis. *Chin J Digest (Chi)*, 1994;14:244-245
- 176 Kobayashi K, Atoh M, Yagita A, Konoeda Y, Inoko H, Ando A *et al.* rohn's disease in the Japanese is associated with the HLA-DRw53. *Exp Clin Immunogenet*, 1990;7:101-108
- 177 Masuda H, Nakamura Y, Yanaka T, Hayakawa S. Distinct relationship between HLA-DR genes and intractability of ulcerative colitis. *Am J Gastroenterol*, 1994;89:1957-1962
- 178 Sugimura K, Assakura H, Hibi T, Ysuij T, Inoko H. Molecular analysis of genes responsible for the susceptibility to ulcerative colitis within the HLA region [Abstract]. *Gastroenterology*, 1991; 100: A619
- 179 Toyoda H, Wang SJ, Yang HY, Redford A, Magalong D, Tyan D *et al.* Distinct associations of HLA class II genes with inflammatory bowel disease. *Gastroenterology*, 1993;104:741-748
- 180 Farrant JM, Bunce M, Artlett C *et al.* HLA DR2 is a susceptibility marker for UC in British patients irrespective of ANCA positivity [Abstract]. *Gastroenterology*, 1994;106:A679
- 181 Andus T, Caesar I, Vogl D, Scholmerich J, Gross V. Association of HLA-DR15, pANCA and IL-1 receptor antagonist allele 2 with ulcerative colitis [Abstract]. *Gastroenterology*, 1995;108:A770
- 182 De la Concha EG, Fernandez-Arquero M, Santa-Cruz S *et al.* Positive and negative associations of distinct HLA-DR2 subtypes with ulcerative colitis (UC). *Clin Exp Immunol*, 1997;108:392-395
- 183 Bouma G, Oudkerk Pool M, rusius JBA, Schreuder GMTh, Hellemans HPR, Meijer UGA *et al.* Evidence for genetic heterogeneity in IBD. HLA genes in the predisposition to suffer from ulcerative colitis and Crohn's disease. *Clin Exp Immunol*, 1997;109:175-179
- 184 Zetterquist H, Broome U, Einarsson K, Olerup O. HLA class II genes in primary sclerosing cholangitis and chronic inflammatory bowel disease: no HLA-Rw52a association in Swedish patients with sclerosing cholangitis. *Gut*, 1992;33:942-946
- 185 Mehal WZ, Lo YMD, Wordsworth BP, Neuberger JM, Hubscher SC, Fleming KA *et al.* HLA DR4 is a marker for rapid disease progression in primary sclerosing cholangitis. *Gastroenterology*, 1994;106: 160-167
- 186 Satsangi J, Welsh KI, Bunce M, Julier, Farrant JM, Bell JI *et al.* ontribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. *Lancet*, 1996;347:1212-1217
- 187 Forcione DG, Sands B, Isselbacher KJ, Rustgi A, Podolsky K, Pillai S. An increased risk of Crohn's disease in individuals who inherit the HLA class II DRB3\* 0301 allele. *Proc Natl Acad Sci USA*, 1996;93: 5094-5098
- 188 Cottone M, Bunce M, Taylor CJ, Ting A, Jewell DP. Ulcerative colitis and HLA-phenotype. *Gut*, 1985;26:952-954
- 189 Roussomoustakaki M, Satsangi J, Welsh K, Louis E, Fanning G, Targan S *et al.* Genetic markers may predict disease behavior in patients with ulcerative colitis. *Gastroenterology*, 1997;112:1845-1863
- 190 Kobayashi, Atoh M, Konoeda Y, Yagita A, noko H, Sekiguchi S. HLA-DR, DQ and T cell antigen receptor constant beta genes in Japanese patients with ulcerative colitis. *Clin Exp Immunol*, 1990;80: 400-403
- 191 Purrmann J, Bertrams J, napp M, leveland S, Hengels KJ, Gema R *et al.* Gene and haplotype frequencies of HLA antigens in 269 patients with Crohn's disease. *Scand J Gastroenterol*, 1990; 25:981-985
- 192 Jacoby RK, Jayson MIV. HL-A 27 in rohn's disease. *Ann Rheum Dis* 1974;33:422-424
- 193 Thorsby E, Lie SO. Relationship between HL-A system and susceptibility to diseases. *Transplant Proc*, 1971;3:1305-1307
- 194 Kuhl P, Sibrowski W, Bohm BO, Bender SW, Kalmar G, Loliger C. HLA antigen frequencies in familial Crohn's disease. *Beitr Infusionsther*, 1990;26:283-286
- 195 Purrmann J, Zeidler H, Bertrams J, Juli E, Cleveland S, Berges W *et al.* HLA antigens in ankylosing spondylitis associated with Crohn's disease. Increased frequency of the HLA phenotype B27,B44. *J Rheumatol*, 1988;15:1658-1661
- 196 can den Berg-Loonen EM, Dekker-Saeyns BJ, Meuwissen SGM, Nijenhuis LE, Engelfriet CP. Histocompatibility antigens and other genetic markers in ankylosing spondylitis and inflammatory bowel diseases. *J Immunogenet*, 1977;4:167-175
- 197 Lewkonja RM, Woodrow JC, McConnell RB, Price Evans DA. HL-A antigens in inflammatory bowel disease. *Lancet*, 1974;4:167-175
- 198 Bergman L, Lindblom JB, Safwenberg J, Krause U. HL-A frequencies in Crohn's disease and ulcerative colitis. *Tissue Antigens*, 1976; 7:145-150
- 199 Pena As, Biemond I, Kuiper G, Weterman IT, van Leeuwen A, Schreuder I *et al.* HLA antigen distribution and HLA haplotype segregation in Crohn's disease. *Tissue Antigens*, 1980;16:56-61
- 200 Woodrow JC, Lewkonja RM, McConnell RB, berg-Loonen EM, Meuwissen SG, Dekker-Saeyns J *et al.* HLA antigens in inflammatory bowel disease. *Tissue Antigens*, 1978;11:147-152
- 201 Danze PM, Colombel JF, Jacquot S, Lose, MN, Heresbach D, Atego S *et al.* Association of HLA class II genes with susceptibility to Crohn's disease. *Gut*, 1996;39:69-72

- 202 Fujita K, Naito S, Okabe N, Yao T. Immunological studies in Crohn's disease. I. Associations with HLA systems in the Japanese. *J Clin Lab Immunol*, 1984;14:99-102
- 203 Mataka H, Okabe N, Naito S, Yao T. An HLA study on 149 Japanese patients with Crohn's disease. *Gastroenterol Jpn*, 1992;27:496-501
- 204 Nakajima A, Matsushashi N, Kodama T, Yazaki Y, Takazoe M, Kimura A. HLA-linked susceptibility and resistance genes in Crohn's disease. *Gastroenterology*, 1995;109:1462-1467
- 205 Caruso C, Oliva L, Palmenri P, Cottone M. B cell alloantigens in Sicilian patients with Crohn's disease. *Tissue Antigens*, 1983;21:170-172
- 206 Reinshagen M, Loeliger C, Kuehnl P, Weiss U, Manfras BJ, Adler G *et al*. HLA class II gene frequencies in Crohn's disease: a population based analysis in Germany. *Gut*, 1996;38:538-542
- 207 Wassmuth R, Keller Y, Thomson G *et al*. HLA DPBI alleles provide protection against Crohn's disease in Caucasians. *Eur J Gastroenterol Hepato*, 1994;6:405-411
- 208 Schwartz SE, Siegelbaum Sp, Fazio TL, Hubbell, Henry B. Regional enteritis: evidence for genetic transmission by HLA typing. *Ann Intern Med*, 1980;93:424-427
- 209 Achord JL, Gumm CH, Jackson JF. Regional enteritis and HLA concordance in multiple siblings. *Dig Dis Sci*, 1982;27:330-332
- 210 Shohat T, Cantor RM, Tyan D, McElree C, Rotter JI. Evidence for linkage of familial occurrence of inflammatory bowel disease [Abstract]. *Am J Med Genet*, 1989;45:248
- 211 Colombel JF, Guillemot F, van Gossom AV, Dufosse F, Cortot A, Dupont E *et al*. Familial Crohn's disease in multiple siblings: no linkages to the HLA system. *Gastroenterol Clin Biol*, 1989;13:676-678
- 212 Naom I, Lee J, Ford D, Bowman SJ, Lanchbury JS, Harris I *et al*. Analysis of the contribution of HLA genes to genetic predisposition in inflammatory bowel disease. *Am J Hum Genet*, 1996;59:226-233
- 213 Spies T, Morton CC, Nedospasov SA, Fiers W, Pious D, Strominger JL. Genes for the tumor necrosis factors alpha and beta are linked to the human major histocompatibility complex. *Proc Natl Acad Sci USA*, 1986;83:8699-8702
- 214 French MAH, Dawkins RL. Central MHC genes, IgA deficiency and autoimmune disease. *Immunol Today*, 1990;11:271-274
- 215 Partanen J, Koskimies S. Low degree of DNA polymorphism in the HLA-linked lymphotoxin (tumor necrosis factor beta) gene. *Scand J Immunol*, 1988;28:313
- 216 Messer G, Spengler U, Jung MC, Honold G, Blomer K, Pape GR *et al*. Polymorphic structure of the tumor necrosis factor (TNF) locus: a NcoI polymorphism in the first intron of the human TNF-beta gene correlates with a variant amino acid in position 26 and a reduced level of TNF-beta production. *J Exp Med*, 1991;173:209-219
- 217 Ferencik S, Lindemann M, Horsthemke B, Grosse-Wilde H. A new restriction fragment length polymorphism of the human TNF-beta gene detected by AspHI digest. *Eur J Immunogenet*, 1992;19:425-430
- 218 Hamann A, Mantzoros C, Vidal-Puig A, Flier JS. Genetic variability in the TNF-alpha promoter is not associated with type II diabetes mellitus (NIDDM). *Biochem Biophys Res Com*, 1995;211:833-836
- 219 Wilson AG, di Giovine FS, Blakemore AIF, Duff GW. Single base polymorphism in the human tumor necrosis factor alpha gene detectable by NcoI restriction of PCR product. *Hum Mol Gen*, 1992;1:353
- 220 D'Alfonso S, Momiagliano Richiardi P. A polymorphic variation in a putative regulation box of the TNFA promoter region. *Immunogenetics*, 1994;39:150-154
- 221 Brinkman BMN, Kaijzel EL, Huizinga TWJ, Giphart MJ, Breedveld FC, Verweij CL. Detection of a C-insertion polymorphism in the human tumor necrosis factor alpha (TNFA) gene. *Hum Genet*, 1995;96:493
- 222 Udalova IA, Nedospasov SA, Webb GC, Chaplin DD, Turetskaya RL. Highly informative typing of the human TNF locus using six adjacent polymorphic markers. *Genomics*, 1993;16:180-186
- 223 Greenberg SJ, Fujihara K, Selkirk SM, Yu F, Du TL, Glenister N *et al*. Novel compound tetra, dinucleotide microsatellite polymorphism in the tumor necrosis factor/lymphotoxin locus. *Clin Diagn Lab Immunol*, 1997;4:79-84
- 224 Mansfield JC, Holden H, Tarlow JK, Di-Giovine FS, McDowell TL, Wilson AG *et al*. Novel genetic association between ulcerative colitis and the anti-inflammatory cytokine interleukin-1 receptor antagonist. *Gastroenterology*, 1994;106:637-642
- 225 Louis E, Satsangi J, Roussomoustakaki M, Parkes M, Fanning G, Welsk K *et al*. Cytokine gene polymorphisms in inflammatory bowel disease. *Gut*, 1996;39:705-710
- 226 Plevy SE, Targan SR, Yang H, Fernandez D, Rotter JI, Toyoda H. Tumor necrosis factor microsatellites define a Crohn's disease-associated haplotype on chromosome 6. *Gastroenterology*, 1996;110:1053-1060
- 227 Crusius JBA, Bing X, Mulder CJJ, Mearin ML, Pena AS. Relevance of haplotypes in the TNF region in celiac disease. *Eur Cytok Network*, 1994;2:168
- 228 Nicklin MJH, Weith A, Duff GW. A physical map of the region encompassing the human interleukin-1-a, interleukin-1-b, and interleukin-1 receptor antagonist genes. *Genomics*, 1994;19:382-384
- 229 McDowell TL, Symons JA, Ploski R, Forre O, Duff GW. A polymorphism in the 5' region of the interleukin-1 alpha gene is associated with juvenile chronic arthritis (JCA). *Br J Rheumatol*, 1993;32(Suppl. 1):162
- 230 Bailly S, di Giovine FS, Duff GW. Polymorphic tandem repeat region in interleukin1a intron 6. *Hum Genet*, 1993;91:85-86
- 231 Bailly S, di Giovine FS, Blakemore AI, Duff GW. Genetic polymorphism of human interleukin-1a. *Eur J Immunol*, 1993;23:1240-1245
- 232 di Giovine FS, Takhsh E, Blakemore AIF, Duff GW. Single base polymorphism at 511 in human interleukin-1b gene (IL1b). *Hum Mol Genet*, 1992;1:450
- 233 Pociot F, Melvig J, Wogensen L, Worsaae H, Nerup J. A TaqI polymorphism in the human interleukin-1b (IL-1b) gene correlates with IL-1b secretion in vitro. *Eur J Clin Invest*, 1992;22:396-402
- 234 Tarlow JK, Blakemore AIF, Lennard A, Solari R, Hughes HN, Steinkasserer A *et al*. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86 bp tandem repeat. *Hum Genet*, 1993;91:403-404
- 235 Bioque G, Bouna G, Crusius JBA, Kostense PJ, Meuwissen SGM, Pena AS. Evidence for genetic heterogeneity in IBD. 1. The interleukin-1 receptor antagonist in the predisposition to suffer from ulcerative colitis. *Eur J Gastroenterol Hepatol*, 1996;8:105-110
- 236 Bioque G, Crusius JBA, Koutroubakis I, Bouma G, Kostense PJ, Meuwissen SGM *et al*. Allelic polymorphism in IL-1b and IL-1 receptor antagonist (IL-1RA) genes in inflammatory bowel disease. *Clin Exp Immunol*, 1995;102:379-383
- 237 Nishiyama T, Mitsuyama K, Toyonaga A, Sasaki E, Tanikawa K. Colonic mucosal interleukin 1 receptor antagonist in inflammatory bowel disease. *Digestion*, 1994;55:368-373
- 238 Brett PM, Yasuda N, Yiannakou JY, Herbst F, Ellis HJ, Vaughan R *et al*. Genetic and immunological markers in pouchitis. *Eur J Gastroenterol Hepatol*, 1996;8:951-955
- 239 Hacker UT, Gomolka M, Keller E, Eigler A, Folwaczny C, Fricke H *et al*. Lack of association between an interleukin-1 receptor antagonist gene polymorphism and ulcerative colitis. *Gut*, 1997;41:651-657
- 240 Andus T, Daig R, Vogl D *et al*. Imbalance of the interleukin 1 system in colonic mucosa-association with intestinal inflammation and interleukin 1 receptor agonist genotype 2. *Gut*, 1997;41:651-657
- 241 Yang H, Vora DK, Targan SR, Toyoda H, Beaudet AL, Rotter JI. Intercellular adhesion molecule 1 gene associations with immunologic subsets of inflammatory bowel disease. *Gastroenterology*, 1995;109:440-448
- 242 Elmgreen J, Sorensen H, Berkowicz A. Polymorphism of complement C3 in chronic inflammatory bowel disease. *Acta Med Scand*, 1984;215:375-378
- 243 Randolph LM, Toyoda H, McElree CK, Shanahan F, Targan SR, Rotter JI. Lack of an association between polymorphisms of the T-cell receptor alpha-chain and ulcerative colitis. *Gastroenterology*, 1989;98:1115-1120
- 244 gth polymorphism analysis of T-cell receptor genes in inflammatory bowel disease. *Scand J Gastroenterol*, 1989;24:381-384
- 245 Posnett DN, Schmelkin I, Burton DA, August A, McGrath H, Mayer LF. T cell antigen receptor V gene usage. Increases in V beta 8+ T cells in Crohn's disease. *J Clin Invest*, 1990;85:1770-1776
- 246 Lowes JR, Chahal H, Zewde M, Allan RN, Ibbotson JP. T cell receptor V beta gene usage in mesenteric lymph node mononuclear cell populations from patients with Crohn's disease. *Gut*, 1993;34(Suppl. 1):S23
- 247 Heresbach D, Alizadeh M, Bretagne JF, Dabadie A, Colombel JF, Pagenault M *et al*. TAP genes transporter polymorphism in inflammatory bowel disease. *Scand J Gastroenterol*, 1997;32:1022-1027
- 248 Fiocchi C. The immune system in inflammatory bowel disease. *Acta Gastroenterol Belg*, 1997;60:156-162

# High expression of human augments of liver regeneration in *E. coli*

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**Subject headings** augments, liver regeneration; gene expression; DNA; plasmid

## INTRODUCTION

Heat-stable hepatocyte stimulatory activity has been described in the liver of weanling rats and pigs. This growth factor is called hepatocyte stimulator substance (HSS)<sup>[1]</sup>. Hagiya *et al*<sup>[2]</sup> reported the complete amino acid sequence of a 30KDa band from their purified product of rat liver and the cloning and sequence analysis of its cDNA. They called it augments of liver regeneration (ALR). Our previous study<sup>[3]</sup> demonstrated that human ALR had been cloned and sequenced. To further study the bioactivity of human ALR (hALR), we constructed a highly expressed vector pBV-hALR in *E. coli* and about 20% of somatic protein of rhALR was expressed.

## MATERIALS AND METHODS

### Materials

Enzymes such as EcoRI, BamHI and Hind III were purchased from Promega. pBV220 plasmid and *E. coli* JM109 were stored and pGEM-hALR plasmid was constructed in our laboratory<sup>[3]</sup>.

### Construction of expressed vector

hALR cDNA fragment was obtained from low-melting gel after electrophoresis of pGEM-hALR plasmid DNA which was digested with EcoRI and BamHI. pBV220 vector DNA was also digested with EcoRI and then incubated at 75°C for 10min. hALR cDNA fragment with compatible cohesive termini and pBV220 DNA fragment were connected in bacteriophage T4 DNA ligation system at 14°C overnight. The reaction contained T4 DNA ligase 1 μL (3u), buffer 10×1 μL, 120 μg hALR cDNA fragment and 100 μg pBV220 DNA fragment. Five μL of ligation mixtures was added to 200μL competent *E. coli* JM109. Transfer appropriate volume of transformed cell onto LB plate agar

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Received 1998-04-06

containing ampicillin (100 mg/L) at 37°C overnight. Bacterial colonies that contain hALR plasmids by digestion were identified on plasmid DNA with restriction enzymes (EcoRI, BamHI) and agarose gel electrophoresis.

### Expression in *E. coli*

LB medium containing ampicillin (100 μg/ml) was inoculated with one colony which contain hALR plasmid to 0. D600 = 0.4 - 0.5 at 30°C and the temperature of the culture was regulated to 42°C, and the incubation was continued for 5 hours.

### Separation and purification of granule

It refers to reference<sup>[4]</sup>.

## RESULTS

### pBV-hALR expression vector

hALR cDNA fragment was inserted into pBV200 vector (Figure 1) and 6 positive colonies were detected and identified by Hind III. pBV200 vector showed 3 fragments 2727bp, 783bp and 125bp and pBV-hALR vector also showed 3 fragments, 2727 bp, 1183 bp and 125 bp, but the second fragment appeared differently (Figure 2).

### Expression in *E. coli*

Induced by temperature, the hALR gene was highly expressed in *E. coli*, and SDS-PAGE showed that 20% of somatic protein of rhALR was expressed (Figure 3). Most of rhALR protein existed as inclusion bodies in *E. coli*. After isolation and washing the purity of granule reached 70% (Figure 3).

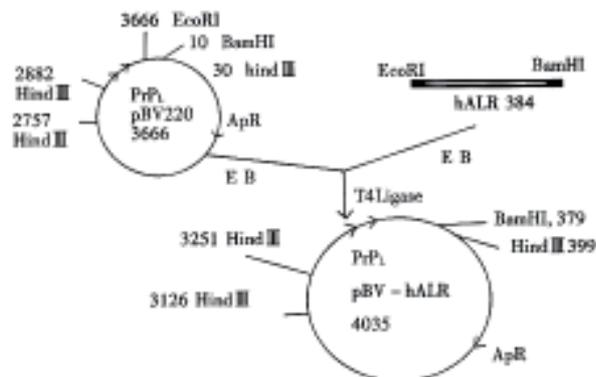
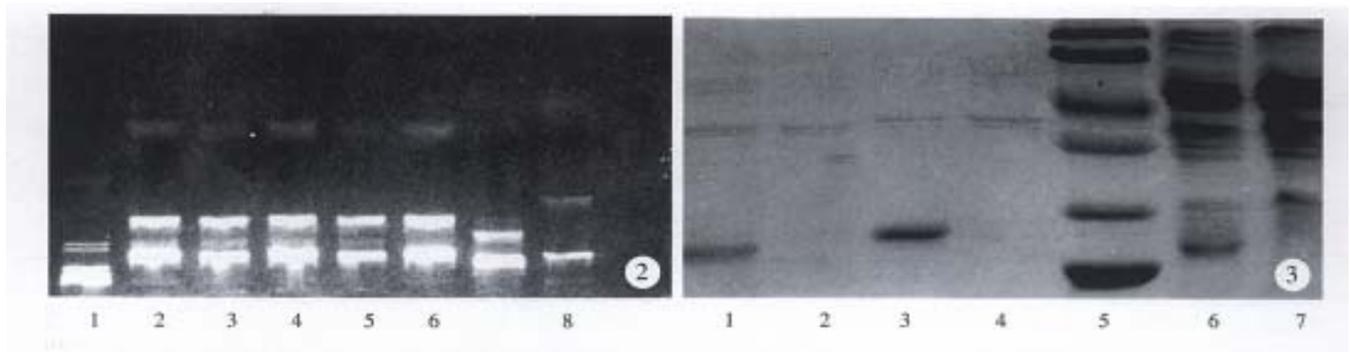


Figure 1 Construction pBV-hALR vector.



**Figure 2** Detection pBV-hALR vector by Hind III.

1  $\gamma$ /Hind III DNA marker 2-6 pBV-hALR vector digested by Hind III 8 pBV-220 vector digested by Hind III

**Figure 3** SDS-PAGE profile of expressed rhALR.

1 Precipitation of *E. coli* JM109 with pBV-hALR after sonication 2 Precipitation of *E. coli* JM109 with pBV-220 after sonication  
 3 Precipitation of *E. coli* DH5 $\alpha$  with pBV-hALR after sonication 4 Precipitation of *E. coli* DH5 $\alpha$  with pET after sonication  
 5 Protein standard ( $M_r$  14 400, 21 500, 31 000, 45 000, 66 2000, 97000)  
 6 *E. coli* JM109 with pBV-hALR 7 *E. coli* JM109 with pBV-220

## DISCUSSION

Since LaBreeque<sup>[1]</sup> reported a kind of heat-stable, liver specific stimulator in weanling liver extraction, the gene has been cloned for about 20 years, but in vain. In 1994 Hagiya<sup>[2]</sup> found a similar stimulator to HSS (ALR). The recombinant ALR eventually produced by the gene derived from the purified rat cytosol retained the hepatotropic potency as the native peptide, with no effect on the cultured hepatocytes. ALR is not only expressed in liver tissue but also in thymus. With the availability of the hALR gene and its rhALR product we think a series of questions can be explained about its specificity, heat-stable quality and mechanisms. Its

potential clinical implication for the treatment of liver diseases, including fulminant liver failure, is worth further studies.

## REFERENCES

- 1 Labrecque DR, Peshch IA. Preparation and partial characterization of hepatic regenerative stimulator, substance (SS) from rat liver. *J Physiol*, 1975;248(2): 273-278
- 2 Hagiya M, Francavilla A, Polimene L, Ihara I, Sakai H, Seki T *et al*. Cloning and sequence analysis of the rat augments of liver regeneration (ALR) gene: expression of biologically active recombinant ALR and demonstration of tissue distribution. *Proc Natl Acad Sci USA*, 1994;91(4): 8142-8146
- 3 Yi XR, Tong MH, Kong XP, Yang LP, Li RB, Zhang YJ. Molecular cloning and sequence analysis of rat and human augments of liver regeneration. *World Chin J Digest*. In press
- 4 Lu SD. Current protocols for molecular biology. Beijing: Advanced Education Press, 1993

# Gastrin as an autocrine growth factor in colorectal carcinoma: implications for therapy

Graham S. Baldwin and Arthur Shulkes

**Subject headings** Autocrine loop; colorectal neoplasms; gastrin; gastrin receptor; progastrin

anti-growth factor antibodies suitable for radioimmunoassay.

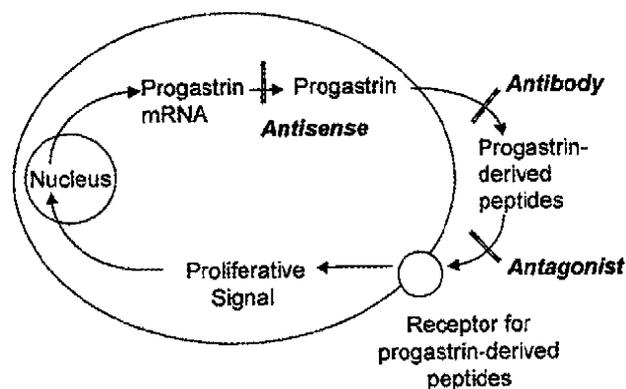
There is now considerable experimental support for the hypothesis that progastrin-derived peptides stimulate proliferation of the normal colonic mucosa<sup>[1]</sup>, and act as autocrine growth factors in colorectal carcinoma (CRC). In a previous review<sup>[2]</sup> we summarized the evidence for the presence of progastrin-derived peptides and their receptors in CRC, and presented a model in which amidated and non-amidated progastrin-derived peptides stimulate proliferation of CRC cells via distinct receptor classes. In this editorial we will consider the various strategies available to interfere with the individual components of the autocrine loop, and the potential of the strategies to yield novel diagnostic and therapeutic agents.

## AUTOCRINE LOOPS

In the autocrine model (Figure 1) a cell synthesises its own growth factor, which is released into the surrounding medium. Binding of the growth factor to cell surface receptors then results in the transmission of a mitogenic signal to the cell nucleus, with a consequent increase in cell proliferation. In principle, autocrine loops could be disrupted in at least 3 ways:

1. By reduction of growth factor mRNA with introduction of antisense RNA or oligonucleotides,
2. By reduction of the concentration of extracellular growth factor by treatment with specific antibodies, or
3. By blockade of growth factor receptors with selective antagonists.

In addition the presence of elevated concentrations of tumour-derived growth factors in the sera of patients with CRC may offer a sensitive method of tumour detection, via the development of



**Figure 1** An autocrine growth loop involving progastrin-derived peptides.

## ANTISENSE mRNA EXPRESSION

Antisense experiments have provided clear evidence for the involvement of progastrin-derived peptides in an autocrine loop in some cell lines of colonic origin. Expression of antisense gastrin mRNA reduced *in vitro* growth of the CRC cell lines Colo 320 and HCT 116<sup>[3]</sup>, and of the conditionally immortalized mouse colon cell line YAMC<sup>[4]</sup>. The ability of HCT 116 cells to grow as tumours in nude mice was also reduced by antisense gastrin mRNA expression<sup>[3]</sup>. In control experiments *in vitro* and *in vivo* growth of the CRC cell line Colo 205A, which expressed negligible amounts of gastrin mRNA prior to transfection, was unaffected by expression of antisense gastrin mRNA<sup>[3]</sup>. However the inherent difficulty of selectively targeting antisense constructs to tumour cells may delay development of related clinical therapies.

## ANTIBODIES

### Diagnosis

The question of whether or not CRCs produce progastrin-derived peptides has been controversial,

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Received 1998-10-28

at least partly because of the large number of potential products of the gastrin gene<sup>[2]</sup>. Progastrin is processed to amidated gastrin via a number of intermediates which include glycine-extended gastrins. Some early reports were confined to measurement of amidated forms of gastrin only, and the variable extent of posttranslational processing of progastrin in peptide-producing tumours may explain some of the negative findings reported in the literature. Progastrin or progastrin-derived peptides are now detected in 80%-100% of CRCs<sup>[2]</sup>. The concentrations of progastrin-derived peptides in the serum of patients with CRC are also elevated between 2.3-fold (*H. pylori* negative) and 5.2-fold (*H. pylori* positive)<sup>[5]</sup>. The availability of a panel of antibodies recognizing different regions of intact progastrin, and of antibodies selective for individual progastrin-derived peptides, may permit the early diagnosis of CRC by radioimmunoassay of serum samples. In this context a large prospective study has recently indicated that hypergastrinaemia was associated with a 3.9-fold increase in the risk of later development of CRC<sup>[6]</sup>.

### Therapy

Antibodies against progastrin-derived peptides may also be useful for treatment of CRC. The proliferation of some, but not all, CRC cell lines was inhibited by antibodies recognizing the C-terminal amidated tetrapeptide of gastrin<sup>[2]</sup>. On the other hand, proliferation of the mouse colon cell line YAMC was inhibited by antibodies recognizing glycine-extended, but not amidated, gastrins<sup>[4]</sup>. A promising approach to future therapy has been provided by the observation that preimmunization of rats with Gastrimmune (a conjugate of amino acids 1-9 of gastrin17 and diphtheria toxoid which recognises both gastrin17 and gastrin17-gly) reduced the *in vivo* growth of the rat CRC cell line DHDK12, either alone or in conjunction with 5-fluorouracil and leucovorin<sup>[7]</sup>.

## ANTAGONISTS

### Gastrin/CCK receptor antagonists

At least four receptors exist for the gastrin/CCK family of peptides<sup>2</sup>. The CCK-A and gastrin/CCK-B receptors are specific for amidated peptides, while the glycine-extended gastrin receptor is selective for non-amidated forms of gastrin. The low affinity gastrin/CCK-C receptor binds amidated and non-amidated forms of gastrin with equal affinity. While the nonselective antagonists proglumide and benzotript inhibit the binding to gastrin/CCK-A, B and C receptors, antagonists selective for either-A or -B receptors have also been

developed<sup>[2]</sup>.

The non-selective antagonists proglumide and benzotript inhibit proliferation of many gastrointestinal carcinoma cell lines both *in vitro* and *in vivo*<sup>[8]</sup>. Comparison of the inhibitory potencies of proglumide, benzotript and other selective gastrin/CCK receptor antagonists with receptor affinities suggests that the gastrin/CCK-C receptor is the probable target<sup>[9]</sup>. However a clinical trial of proglumide in patients with gastric carcinoma did not reveal any benefits, perhaps because the concentrations achieved were not sufficient to saturate gastrin/CCK receptors. Gastrin/CCK-B receptor antagonists have also been shown to inhibit the growth of some CRC cell lines *in vitro*, and of primary human CRCs *in vitro* and *in vivo*, but have not yet been subjected to clinical trials. However the observation that most CRCs do not express gastrin/CCK-B receptors indicates that it will be unlikely that gastrin/CCK-B receptorselective antagonists will be a general treatment for CRC<sup>[2]</sup>.

### Non-steroidal anti-inflammatory drugs

Epidemiological studies have revealed that non-steroidal anti-inflammatory drugs (NSAIDs), and in particular aspirin, reduce by approximately 50% the risk of CRC and other cancers of the gastrointestinal tract<sup>[10]</sup>. The NSAID sulindac also reduces the size and number of colorectal polyps in patients with familial adenomatous polyposis, and inhibits the development of chemically-induced CRC in rodents. Although selective antagonists have indicated that the inducible isozyme cyclooxygenase-2 is one of the targets for the inhibitory effects of NSAIDs on CRC growth *in vivo*, several lines of evidence suggest that other targets may contribute to the anti-proliferative effects *in vitro*<sup>[10]</sup>.

The gastrin/CCK-C receptor may be one such alternative target. All of a panel of 17 NSAIDs tested inhibited the binding of gastrin to the gastrin/CCK-C receptor with affinities which correlated well with their potencies as inhibitors of the proliferation of CRC cell lines<sup>[11]</sup>. The most potent antagonist of gastrin binding to date is sulindac sulphide, which has an IC<sub>50</sub> value of 40μM, and more potent antagonists of the gastrin/CCK-C receptor may well be of use in the treatment of CRC.

## CONCLUSIONS

This editorial has summarized several promising avenues for future research into the effects of progastrin-derived peptides as autocrine growth factors in CRC. In particular the development of

antibodies against progastrin-derived peptides, and of antagonists selective for progastrin-derived peptide receptors, may provide new opportunities for diagnosis and therapy of CRC.

## REFERENCES

- 1 Wang TC, Koh TJ, Varro A, Cahill RJ, Dangler CA, Fox JG, Dockray GJ. Processing and proliferative effects of human progastrin in transgenic mice. *J Clin Invest*, 1996;98:1918-1929
- 2 Baldwin GS and Shulkes A. Gastrin, gastrin receptors and colorectal carcinoma. *Gut*, 1998;42:581-584
- 3 Singh P, Owlia A, Varro A, Dai B, Rajaraman S, Wood T. Gastrin gene expression is required for the proliferation and tumorigenicity of human colon cancer cells. *Cancer Res*, 1996;56:4111-4115
- 4 Hollande F, Imdahl A, Mantamadiotis T, Ciccosto GD, Shulkes A, Baldwin GS. Glycineextended gastrin acts as an autocrine growth factor in a non-transformed colon cell line. *Gastroenterology*, 1997;113:1576-1588
- 5 Ciccosto GD, McLeish A, Hardy KJ, Shulkes A. Expression, processing and secretion of gastrin in patients with colorectal carcinoma. *Gastroenterology*, 1995; 109:1142-1153
- 6 Thorburn CM, Friedman GD, Dickinson, CJ, Vogelmann, JH, Orentreich N and Parsonnet J. Gastrin and colorectal carcinoma: A prospective study. *Gastroenterology*, 1998;115:275-280
- 7 Watson SA, Michaeli D, Grimes S, Morris TM, Robinson G, Varro A *et al.* Gastrimmune raises antibodies that neutralize amidated and glycine-extended gastrin-17 and inhibit the growth of colon cancer. *Cancer Res*, 1996;56:880-885
- 8 Hoosein NM, Kiener PA, Curry RC, Rovati LC, McGilbra DK, Brattain MG. Antiproliferative effects of gastrin receptor antagonists and antibodies to gastrin on human colon carcinoma cell lines. *Cancer Res*, 1988;48:7179-7183
- 9 Baldwin GS. Antiproliferative gastrin/cholecystokinin receptor antagonists target the 78-kda gastrin-binding protein. *Proc Natl Acad Sci USA*, 1994;91:7593-7597
- 10 Gupta RA and DuBois RN. Aspirin, NSAIDs and colon cancer prevention: Mechanisms. *Gastroenterology*, 1998;114:1095-1100
- 11 Baldwin GS, Murphy VI, Yang Z and Hashimoto T. Binding of non-steroidal antiinflammatory drugs to the  $\alpha$ -subunit of the trifunctional protein of long chain fatty acid oxidation. *J Pharmacol Exp Ther*, 1998;286:1110-1114

# Immune response against HBsAg vaccine \*

Maria Cristina Honorati and Andrea Facchini

**Subject headings** rHBsAg; hepatitis B; vaccines; vaccination

Hepatitis B virus (HBV) is a hepadnavirus which can cause systemic infection and hepatocellular damage in humans. Infection is acquired through contact with the blood of a person carrying HBV. The passive administration of anti-HBV surface antigen (HBsAg) antibodies protects against a subsequent infection and vaccination with HBsAg has proved an effective means of protection against HBV infection.

HBsAg purified from the plasma of HBsAg-carriers has been used for many years as an effective vaccine but its major limitation is the availability of the raw material and the cost. Since the late 1980s, genetic engineering has allowed the development of yeast-derived recombinant, very safe and efficient DNA vaccines<sup>[1]</sup> and compulsory vaccination was introduced in many countries to progressively eradicate HBV infection. Recombinant vaccines contain only the sequence 1-226 of HBsAg (rHBsAg), representing the small nonglycosylated HBs protein (S-domain), with the exclusion of the preS1 and preS2 domains (Figure 1).

A serum level of at least 10 mIU/ml of anti-HBs antibodies reached after vaccination has been proposed to be the lowest limit for protection<sup>[2]</sup>. Several studies were conducted to examine the duration of protection after immunization with recombinant vaccines, but these were related to seroconversion in selected clusters of vaccine recipients, introducing parameters (age, gender, smoking, obesity, etc.) which are known to influence the immune response<sup>[3,4]</sup>. Recently, we and others proposed a mathematical model to calculate the time after the vaccination with rHBsAg for the loss of protection due to an antibody fall to below 10 mIU/ml<sup>[5,6]</sup>. This model, which will be available on the web-6, showed that the decay

in the level of specific immunoglobulins reached after last booster dose is well described by the log time-log titre linear function. In practice, anti-HBs level falls quickly after the peak and then slowly, and the time to reach very low levels depends on the serum level reached at the end of the vaccination protocol.

The analysis of specificity of raised circulating antibodies after inoculation with rHBsAg showed a binding region for one B-cell epitope represented by a peptide located within the HBsAg sequence 110-168 (Table 1). The same specificity has been described after immunization with plasma-derived vaccines or natural infection. This epitope represents the «a» determinant of HBsAg, that may be considered the conserved sequence among different HBV subtypes (adr, adw, ayr, ayw) and is able to stimulate cross-protection after immunisation<sup>[7]</sup>. This dominant B-cell epitope is conformational, discontinuous, characterized by disulphide bonds and corresponds to a hydrophilic, external region of HBsAg. Although antibodies directed against this region protect against HBV infection, an escape mutant of HBV has been isolated from an infant vaccinated with rHBsAg and later became positive for markers of viral replication. The mutant presents an amino acid substitution of glycine to arginine at position 147<sup>[8]</sup>. This point mutation is stable, allows the attachment of the virus to hepatocytes and represents a new specificity not cross-reacting with normal virus subtypes. The mutation of one amino acid can be responsible for different conformations of «a» determinant, with varying abilities to elicit and bind anti-HBs antibodies.

Although the immunogenicity and protective effect of rHBsAg are comparable with that of HBV natural infection, differences have been described in IgG subclass distribution after infection or vaccination. The serological response to HBsAg has been well characterized in patients with acute or chronic hepatitis B, who presented circulating immunocomplexes containing mostly IgG1 and IgG4 antibodies, or in convalescent sera where specific antibodies were predominantly IgG1 and IgG3. The analysis of IgG subclass distribution induced by vaccination with rHBsAg shows that the IgG2 subclass is mainly synthesized with IgG1. The presence of IgG2 with IgG1 subclass following

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\*Supported in part by Grants from the Italian Ministry of Health, University of Bologna (ex60%) and Ricerca Corrente, Istituti Ortopedici Rizzoli, Bologna, Italy.

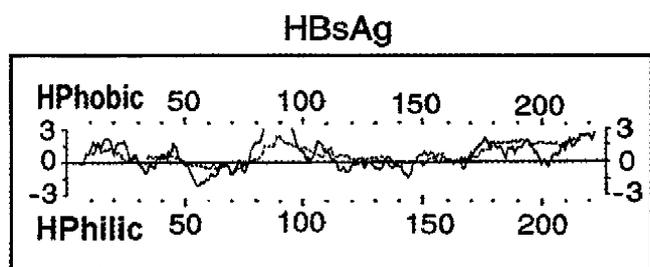
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Received 1998-11-16

vaccination against HBV was also described using plasma-derived vaccines, where HBsAg is glycosylated. Although the synthesis of IgG2 subclass antibodies is generally associated with polysaccharide antigens, the absence of glycosylated residues in the rHBsAg cannot explain the switch to IgG2 production. A hypothesis to explain a comparable immunogenicity from both vaccines is that the network of cytokines secreted by T lymphocytes recognising natural or recombinant HBsAg, could induce the B lymphocyte switch to this IgG subclass synthesis<sup>[9]</sup>.

**Table 1 B and T cells epitopes of HBsAg**

Sequence	Determinants	Cell subsets	HLA restriction
110-168 <sup>[6]</sup>	Conformational «a» determinant	B	Not HLA restricted
21-28 <sup>[11]</sup>	Minimal epitope	CD4 T	Class II
124-147 <sup>[11]</sup>	Immunodominant zone	CD4 T	Class II
165-172 <sup>[11]</sup>	Minimal epitope	CD4 T	Class II
215-223 <sup>[11]</sup>	Minimal epitope	CD4 T	Class II
80-98 <sup>[11]</sup>	Containing predicted epitope	CD4 T	Class II
171-179 <sup>[16]</sup>	Minimal epitope	CD8 T	Class I



**Figure 1** Structure of the HBsAg molecule. The hydrophathy of the sequence 1-226 is predicted according to Kyte and Doolittle 1982, *J Mol Biol* (—) and Engelman *et al.* 1986, *Annu Rev Biophys Biophys Chem* (---).

Anti-HBS production is the consequence of T cell-mediated immunity stimulated by the vaccination, which also plays a fundamental role in preventing HBV infection. The use of genetic engineering to synthesize recombinant HBV envelope protein increased investigation into the immunising activity of HBsAg. Some early studies *in vitro* disclosed that CD4+T cells help HBsAg specific B cell for the synthesis of anti-HBs antibodies in subjects vaccinated with rHBsAg<sup>[10,11]</sup>.

The isolation of T cell clones specific for HBsAg from subjects vaccinated with recombinant vaccine provided an important tool for investigating the immunizing activity of this protein. It has been observed that the response of antigen-specific T lymphocytes to HBsAg depends on the sequence of

the antigen molecule. In an attempt to identify the sequences recognized by HBsAg-primed T cells from vaccine recipients, synthetic peptides representing short sequences of HBsAg protein have been used *in vitro* to investigate specific cellular proliferation. Recently, immunodominant HBsAg epitopes have been confirmed or identified analysing the proliferation of CD4+T cell clones (helper/inducer) by peptides spanning the whole HBsAg protein<sup>[12]</sup>. The clones belong to the Th<sub>0</sub>/Th<sub>2</sub> subset, expressing and secreting *in vitro* interleukin 4, 5 and interferon- $\gamma$ . Three minimal T-cell epitopes have been described: sequences 21 - 28, 165-172 and 215-223. There is an evidence that T-cell epitopes are also present within the 124-147 sequence, corresponding to that zone on HBsAg containing the «a» determinant. The difficulty in identifying a minimal epitope within this sequence could be due to the low level of immunogenicity of this HBsAg region. We cannot exclude the presence of other important epitopes with in the most hydrophobic region of HBsAg, that is very difficult to study because of this characteristic, which prevents the solubility of peptides in culture media (Table 1). These findings are in agreement with the Berzofsky algorithm<sup>[13]</sup>, which predicts for different protein zones the probability of representing Thepitopes on the basis of the sequential and conformational structure. The pathway of exogenous antigen processing needs the cooperation of antigen presenting cells (APC) and the recognition of short aminoacid sequences (epitopes) by Th cells in association with HLA class II determinants. Recently it has been shown that:

- ① DR molecules are mostly involved in the presentation of immunodominant HBsAg peptides, but at least DP determinants can participate in antigen presentation; and
- ② an epitope can be efficiently presented by different MHC loci, suggesting that a short sequence of HBsAg can be immunogenically dominant, because of its specificity for Th cells from subjects presenting different HLA class II alleles<sup>[12]</sup>. On the other hand, studies on the contribution of HLA class II determinants to the regulation of antibody production suggested that MHC loci are involved in regulation of the immunological response after rHBsAg immunization.<sup>[14]</sup>

*In vitro* studies on CD8+T cells responsible for cytotoxic activity are hampered by the difficulty of utilising target cells expressing appropriate HLA class II determinants for the association with the antigen. Although the cytotoxic activity of CD8+T cells stimulated by HBsAg is not well known, one minimal essential epitope on HBsAg has been

described in sequence 172-180<sup>[15]</sup> corresponding to a peptide candidate for the binding to HLA-A2 determinant and this sequence has been described to activate T CD8+ lymphocytes isolated from subjects sharing this HLA class II determinant. It is also noteworthy that this antigen can evoke cytotoxic activity by subsets of CD4+T cells expressing the CD56 phenotype<sup>[16]</sup>. We do not exclude that different short sequences of S protein could induce an immunological response involving both CD4+ and CD8+T lymphocytes. Double pathway for the antigen processing, utilising endosomal and cytosolic cellular districts, has been described for the pre S2 protein sequence 120-134 of HBV, which can be presented by HLA class I and class II determinants, leading to activation of CD4+ (helper/inducer) and CD8+ (cytotoxic) T cells<sup>[17]</sup>. Identification of dominant epitopes on HBsAg specific B, CD4+ and CD8+T cells may clarify the effective role of rHBsAg in inducing a protective cellular and humoral response against HBV infection. The major histocompatibility complex (MHC) controls the immune response to protein antigen and allelic variants of MHC can influence the lack of response to the immunisation in a small percentage of vaccine recipients (5%-10%).

More recently some authors have analyzed the possibility of utilising HBsAg-encoding plasmid DNA as vaccine. Good findings have been obtained in vivo following i.m. injection of the plasmid into mice<sup>[18]</sup>. This route of administration induced muscular cells to synthesize endogenous HBsAg followed by the association of peptides with HLA class I determinants and the secretion of the whole molecule. In the future, DNA vaccine could be considered an alternative method to obtain an active immunization to several pathogens if this approach proves to be as effective and safe as recombinant protein.

## REFERENCES

- 1 Harford N, Cabezon T, Colau B, Delisse AM, Rutgers T, De Wilde M. Construction and characterization of a *Saccharomyces cerevisiae* strain (RIT4376) expressing hepatitis B surface antigen. *Postgrad Med J*, 1978;63(2):65-70
- 2 Hollinger FB. Hepatitis B vaccines-to switch or not to switch. *J Am Med Assoc*, 1987;257:2634-2636
- 3 Honorati MC, Mariani E, Dolzani P, Facchini A. Biological parameters influencing the immunological response to plasma derived and recombinant hepatitis B vaccines. *Ann Ist Super Sanita*, 1996;32(3):369-374
- 4 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-40S
- 5 Gesemann M, Scheiermann N. Quantification of hepatitis B vaccine-induced antibodies as a predictor of anti-HBs persistence. *Vaccine*, 1995;13(5):443-447
- 6 Honorati MC, Palareti A, Dolzani P, Busachi P, Rizzoli R, Facchini A. A mathematical model predicting anti-HBs decay after vaccination against hepatitis B. *Clin Exp Immunol*, in press
- 7 Neurath AR. Chemical synthesis of hepatitis B vaccines. In Zuckerman AJ, ed. Recent developments in prophylactic immunization. London: Kluwer Academic, 1989;210-242
- 8 Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E *et al*. Vaccine-induced escape mutant of hepatitis B virus. *The Lancet*, 1990;336:325-329
- 9 Honorati MC, Borzi RM, Dolzani P, Toneguzzi S, Facchini A. Distribution of IgG subclasses after anti-hepatitis B virus immunization with a recombinant vaccine. *Int J Clin Lab Res*, 1997;27:202-206
- 10 Degrossi A, Mariani E, Honorati MC, Roda P, Miniero R, Capelli M *et al*. Cellular response and anti-HBs synthesis *in vitro* after vaccination with yeast-derived recombinant hepatitis B vaccine. *Vaccine*, 1992;10(9):617-622
- 11 Ducos J, Bianchi M, Mondain AM, Pageaux G, Conge AM, Poncet R, Vendrell JP *et al*. Hepatitis B virus (HBV) specific *in vitro* antibody production by peripheral blood mononuclear cells (PBMC) after vaccination by recombinant hepatitis B surface antigen (rHBsAg). *Clin Exp Immunol*, 1996;103(1):15-18
- 12 Honorati MC, Dolzani P, Mariani E, Piacentini A, Lisignoli G, Ferrari C *et al*. Epitope specificity of Th<sub>0</sub>/Th<sub>2</sub> CD4+T-lymphocyte clones induced by vaccination with rHBsAg vaccine. *Gastroenterology*, 1997;112(6):2017-2027
- 13 Margalit H, Spouge JL, Cornette JL, Cease KB, Delisi C, Berzofsky JA. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. *J Immunol*, 1987;138:2213-2229
- 14 Mineta M, Tanimura M, Tana T, Yssel H, Kashiwagi S, Sasazuki T. Contribution of HLA class I and Class II alleles to the regulation of antibody production to hepatitis B surface antigen in humans. *Int Immunol*, 1996;8(4):525-531
- 15 Nayersina R, Fowler P, Guilhot S, Missale G, Cerny A, Schlicht HJ *et al*. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J Immunol*, 1993;150:4659-4671
- 16 Barnaba V, Franco A, Paroli M, Benvenuto R, De Petrillo G, Burgio VL, *et al*. Selective expansion of cytotoxic T lymphocytes with a CD4+CD56+ surface phenotype and a T helper type 1 profile of cytokine secretion in the liver of patients chronically infected with hepatitis B virus. *J Immunol*, 1994;152:3074-3087
- 17 Barnaba V, Franco A, Alberti A, Balsano C, Benvenuto R, Balsano F. Recognition of hepatitis B virus envelope proteins by liver-infiltrating T lymphocytes in chronic HBV infection. *J Immunol*, 1989;143(8):2650-2655
- 18 Bohm W, Mertens T, Schirmbeck R, Reimann J. Routes of plasmid DNA vaccination that prime murine humoral and cellular immune response. *Vaccine*, 1998;16(9-10):949-954

# The role of adhesion molecules in gastric ulcer healing

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**Subject headings** stomach ulcer; adhesion molecules; vascular cell surface adhesion molecule-1; platelet endothelial cell adhesion molecule

Gastric ulcer is a deep necrotic lesion involving the entire mucosal depth and the muscularis mucosae. Ulcer healing is an active and complicated process of filling the mucosal defect with proliferating and migrating epithelial cells and connective tissue components, so as to reconstruct the mucosal architecture. In this process, the concerted interaction of a variety of tissues and cellular systems are required, including those of soluble mediators, formed blood elements, extracellular matrix (ECM), and parenchymal cells. In fact, healing of an ulcer follows a specific time sequence and can be temporally categorized into three processes which occur in a sequential order: (I) inflammation; (II) tissue formation; and (III) tissue remodeling. This editorial will mainly discuss the involvement of adhesion molecules in the phase of tissue formation, a fundamentally important process in ulcer healing.

The critical steps of tissue formation during the repair of the gut mucosal injuries include the reconstitution of the epithelial mucosal barrier and the development of new microvasculature by angiogenesis. The healing of the gut is initiated by restitution. Enterocytes at the edge of the wound lose their differentiated characteristics and migrate across the region of denuded basement membrane in mucosa, while stimulation of angiogenesis during initial wound healing facilitates the reconstruction of original appearing mucosa and submucosa. These processes could be initiated by a variety of soluble chemotactic cytokines and growth factors secreted from damaged cells.

Epithelial restitution is a fundamental protective mechanism that allows the gastrointestinal mucosa to reestablish functional and structural integrity following superficial injury. The initial step and progression of epithelial cell migration depends on the adherence between

epithelial cells and establishment of stable cell-substratum adhesion to generate friction and forward movement. The transformation from an attached to a motile cell requires disruption of cell junctional proteins such as E-cadherin/ catenin complex and the modulation of expression, affinity and binding specificity of ECM adhesion receptors (e.g., integrins). Integrins have been implicated in the regulation of cell migration in a variety of systems<sup>[1]</sup>. Knock-out of  $\beta 1$  integrin gene in embryonic stem cells has been shown to inhibit cell migration and adhesion<sup>[2]</sup>. The up-regulation of the functional activity of integrins  $\alpha 2\beta 1$  and  $\alpha 3\beta 1$ , which are receptors for laminin and collagen, is required for cell attachment and migration. Epithelial cadherin is the prime mediator of cell-cell adhesion in epithelial cells. Perturbation of E-cadherin/ catenin mediated adhesion is associated with epithelial migration and restitution following ulceration of the gastrointestinal tract<sup>[3]</sup>. Epidermal growth factor has been shown to induce rapid tyrosine phosphorylation of  $\beta$ -catenin and  $\gamma$ -catenin. This is associated with scattering and dispersion of epithelial cells and disruption of E-cadherin from the cell-cell junctions<sup>[4]</sup>.

Angiogenesis is another crucial factor for ulcer healing and tissue regeneration<sup>[5]</sup>, which is a process of new blood vessel formation from pre-existed vessels. The importance of angiogenesis in gastroduodenal ulcer healing has been extensively studied. For instance, stimulation of angiogenesis in granulation tissues has been shown to dramatically accelerate the healing of experimental duodenal ulcer in rats<sup>[6]</sup>. Furthermore, chronic indomethacin administration inhibits angiogenesis in granulation tissues and delays healing of experimental gastric ulcers in animals<sup>[7]</sup>. Blood vessels are especially important during tissue injury. When there is inflammation, blood delivers nutrients, growth factors, and immunocytes to the site of injury, whereas waste products are removed from there. In addition, regulation of blood flow in the gastrointestinal mucosa is important for the maintenance of the integrity of gastric mucosa and protection against further mucosal injury. The formation of new vessels in ulcer healing is a dynamic process that is controlled by many diverse, sometimes complex factors acting together in a local environment. Again, a wide variety of growth factors are involved in the regulation of adhesion

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Received 1998-10-19

molecule expression in a concerted manner during the process of vessel assembly. The principal cell type involved in the process of angiogenesis is the microvascular endothelial cell.

Angiogenesis begins with proteolysis of the basement membrane. Proteolysis is necessary to induce microvascular endothelial cell invasion and tube formation. Activation of both endothelial cells and lymphocytes or monocytes is required for their secretion of proteases. Their activation, however, is dependent on adhesion molecules interaction. During the course of inflammation, local endothelial cells are exposed to various cytokines that induce a series of endothelial surface adhesion molecules. One of these inducible molecules, vascular cell surface adhesion molecule-1 (VCAM-1), a member of the immunoglobulin (Ig) supergene family, is the counter-receptor for very late antigen-4 (VLA-4), a surface protein present on lymphocytes and monocytes. VCAM-1/VLA-4 interaction induces the increased expression of a series of proteases required for proteolysis, and therefore endothelial cells could get a hold on the basement membrane, where they proliferate and extend.

Following proteolysis, tube formation occurs. A series of adhesion molecules come to play in tube formation. Early events of tube formation are mediated by platelet endothelial cell adhesion molecule (PECAM-1/PECAM-1) interactions. As a result of the cell to cell contact, a tube-like structure is formed. Antibodies directed against PECAM-1 can inhibit tube formation *in vitro*<sup>[8]</sup>. Integrin  $\alpha\beta 3$  is another adhesion molecule found only on the tips of the endothelial cells in sprouting vessels. Its immunoreactivity is absent in mature and quiescent vessels. Both  $\beta 1$  and  $\beta 3$  integrins are involved in the attachment between cells and their substrates. But the presence of the latter adhesion molecule was also thought to induce gelatinase expression on the surface of the endothelial cells so as to enhance migration and proliferation<sup>[9]</sup>. When the tube-like structure is stabilized with  $\beta 1$  and  $\beta 3$  integrins, tight junction formation occurs, which correlates with the junction-associated molecules assembly and organization<sup>[10]</sup>.

Epithelial restitution, as well as angiogenesis,

two of the fundamental components of the ulcer healing process, are characterized by complex alterations in adhesion between cells and the ECM. Growth and motility factors involved in mucosal repair of the gastrointestinal tract seem to modulate these interactions in a coordinated fashion in order to reestablish functional and structural integrity of the mucosa. The mechanisms that regulate the production of these adhesion molecules await further exploration and clarification, as do the differences between the consequences of different types of mucosal injury. It is clear, however, that the modulation of the cell migration, and angiogenesis by adhesion molecules may be the fruitful targets for future pharmacological intervention in gastrointestinal wound healing.

**Acknowledgement:** This review is supported in part by the RGC grant of the Hong Kong Research Grant Council, awarded to C.H. Cho.

## REFERENCES

- 1 Nigam AK, Savage FJ, Boulos PB, Stamp GWH, Liu D, Pignatelli M. Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer. *Br J Cancer*, 1993;68:507-514
- 2 Fassler R, Pfaff M, Murphy J, Noegel AA, Johansson S, Timpl R, Albrecht R. Lack of  $\beta 1$  integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. *J Cell Biol*, 1995;128:979-988
- 3 Hanby AM, Chinery R, Poulosom R, Pignatelli M. Down-regulation of E-cadherin in the reparative epithelium of the gastrointestinal tract. *Am J Pathol*, 1996;148:723-729
- 4 Shiozaki H, Kadowaki T, Doki Y, Inoue M, Tamura S, Oka H, Iwazawa T, Matsui S, Shimaya K, Takeichi M. Effect of epidermal growth factor on cadherin-mediated adhesion in a human esophageal cancer cell line. *Br J Cancer*, 1995;71:250-258
- 5 Tarnawski A, Stachura J, Krause WJ, Douglass TG, Gergely H. Quality of gastric ulcer healing: a new, emerging concept. *J Clin Gastroenterol*, 1991;13:S42-47
- 6 Folkman J, Szabo S, Stovroff M, McNeil P, Li W, Shing Y. Duodenal ulcer. Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. *Ann Surg*, 1991;241:414-425
- 7 Schmassmann A, Tarnawski A, Peskar BM, Varga L, Flogerzi B, Halter F. Influence of acid and angiogenesis on kinetics of gastric ulcer healing in rats: interaction with indomethacin. *Am J Physiol*, 1995;268:G276-285
- 8 Madri JA, Bell L, Merwin JR. Modulation of vascular cell behavior by transforming growth factors beta. *Mol Reprod Dev*, 1992;32:121-126
- 9 Seftor REB, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Rouslahti E, Hendrix MJC. Role of the  $\alpha\beta 3$  integrin in human melanoma cell invasion. *Proc Natl Acad Sci USA*, 1992;89:1557-1561
- 10 Merwin JR, Anderson J, Kocher O, van Itallie C, Madri JA. Transforming growth factor  $\beta 1$  modulates extracellular matrix organization and cell-cell junctional complex formation during *in vitro* angiogenesis. *J Cell Physiol*, 1990;142:117-128

# Phytochemical malabsorption: clinical significance

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Subject headings phytochemicals;vitamins;minerals; gastrointestinal malabsorption

## CLINICAL SIGNIFICANCE OF PHYTOCHEMICALS

Until recent years, nutritionists have focused primarily on macronutrients and micronutrients in foods. Appreciation is now increasing that many food components, of plant origin ('phytochemicals') in particular, have the potential to affect human biology. Phytochemicals by definition are important components of food that may not be essential in the classical sense, and may not even be required to sustain life as vitamins or minerals do, but are likely to contribute to optimal health.

A problem in considering the place of phytochemicals in human health is that they are numerous, alongside a few known essential nutrients. Therefore, their net interactive effect ultimately requires a study of food itself and food patterns, so that food component intake may need to be subject to sophisticated mathematical modelling. However, the advent of advanced informatics may help resolve this dilemma.

Examples of phytochemicals, along with some of their food sources are listed according to their chemical structure in Table 1. The possible roles of these phytochemicals in the treatment of health conditions are rapidly unfolding<sup>[1]</sup>, and the use of an index of preferred phytochemical intake has been suggested<sup>[2]</sup>. Yet limited information is available on the bioavailability of most of these compounds. With complex factors influencing the absorption and transport of phytochemicals, it is not easy to predict their bioavailability, let alone consider the implications of gastrointestinal malabsorption.

## BIOLOGICAL OCCURRENCE AND RELEVANCE OF PHYTOCHEMICALS

The presence and the physiological concentration of phytochemicals in biological tissues or fluids, especially blood and urine, create the opportunity for biomarkers of the consumption of

phytochemical-containing foods<sup>[4-6]</sup> (Table 2). Equally, measurements of phytochemicals reflect bioavailability, including absorption.

**Table 1 Selected phytochemicals and their possible roles in health<sup>[3]</sup>**

Phytochemicals	Some important food sources	Possible roles in health
Carotenoids	Orange pigmented and green leafy vegetables, e.g. carrots, tomatoes, spinach	Antioxidants Antimutagen Anticarcinogen Immuno-enhancement
Flavonoids, isoflavonoids and saponins	Green and yellow leafy vegetables, e.g. parsley, celery, soy bean and soy products	Antioxidants Anticarcinogen Oestrogenic Immuno-modulating
Polyphenols	Cranberry, raspberries, blackberries Rosemary, oregano, thyme	Antibacterial Reduce urinary tract infection
Catechins	Green tea	Antimutagen Anticarcinogen
Allyl thiosulfinates	Garlic, onions, leeks	Anticarcinogen Antibacterial Cholesterol lowering
Isolthiocyanates and indoles	Cruciferous vegetables, e.g. broccoli, cabbage	Antimutagen
Phytosterols, e.g. $\beta$ -sitosterol	Pumpkin seeds	Reduce symptoms of prostate enlargement

This list is not exhaustive for phytochemicals.

**Table 2 Occurrence of phytochemicals in human blood and tissues**

Phytochemicals	Where can we find them in the body
Carotenoids	Serum (five major carotenoids)
Lutein/zeaxanthin	Skin
$\beta$ -cryptoxanthin	Adipose tissues
Lycopene	Lens and macula (lutein/zeaxanthin)
$\alpha$ -carotene	Various tissues like prostate (lycopene)
$\beta$ -carotene	
Flavonoids	Serum
Quercetin, kaempferol	Urine
Isoflavones	Serum
Genistein, daidzein	Urine
Catechins epigallocatechin gallate	Serum
Allyl thiosulfinates	Blood, serum, red blood cells
organosulfides	Adipose tissue
vinyl dithiols	Liver
	Kidney
	Breath
Tocotrienols	Sikin

For the moment, the presence of phytochemicals in tissues, is presumptive evidence of functional

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Received 1998-10-20

significance, especially where there is a plausible mechanism of action (e.g. carotenoids as antioxidants for serum lipoproteins). Observations like this generate a number of exciting hypotheses for further research.

### CLINICAL SIGNIFICANCE OF PHYTOCHEMICAL MALABSORPTION

Given the putative health benefits of phytochemicals, gastrointestinal malabsorption may well contribute to a loss of their protective effects. This could result in a number of clinical disorders which could be referred to as 'phytochemical deficiency disorders'<sup>[1]</sup>. Candidate disorders are:

a. The menopause as a 'phytoestrogen deficiency disorder'<sup>[7]</sup>

b. Cardiovascular disease because of the role of certain phytochemicals as antioxidants, others as regulators of endothelial function and others as modulators of myocardial function

c. Colorectal cancer in relation to a range of phytochemical intakes from various fruits and vegetables, and whole grain cereals<sup>[8]</sup>

d. Prostatic disease in relation to lycopene and isoflavones<sup>[9,10]</sup>

e. Maculopathy on account of the contribution to macular function of lutein and zeaxanthin<sup>[11]</sup>

Two cases of short bowel syndrome where carotenoids were undetectable in serum illustrate the potential for these orders (Case reports 1 and 2). In each case, it was possible to increase serum carotenoid concentrations by vegetable juice supplements.

#### Case report 1 Mrs BW (b. 1956)

Vaginal cancer (1990) treated with radiotherapy

Rectovaginal fistula - colostomy (1992)

Short bowel syndrome (1995)

Vegetable juice/soup		No	Yes
Serum concentration (nmol/L)	Reference range	Jan 96	May 97
Lutein/zeaxanthin	80-850	102	202
β-cryptoxanthin	175-1350	Not detectable	12
Lycopene	69-650	Not detectable	33
α-carotene	15-300	Not detectable	Not detectable
β-carotene	45-900	Not detectable	93

#### Case report 2. Mrs CM (b. 1956)

Severe road traffic accident (1976) → ruptured bowel, bowel resections

Short bowel syndrome

V-8ceTM (glass/day)	0	1×2	1×1	
Serum concentration (nmol/L)	Reference range	Nov 95	May 96	Nov 96
Lutein/zeaxanthin	80-850	Not detectable	243	17
β-cryptoxanthin	175-1350	Not detectable	18	Not detectable
Lycopene	69-650	Not detectable	32	11
α-carotene	15-300	Not detectable	Not detectable	Not detectable
β-carotene	45-900	Not detectable	10	8

### CONCLUSIONS

With anergent evidence for physiological roles of phytochemicals and for their potential for disease protection, the use of foods, which are good phytochemical sources, to prevent and manage disease will be encouraged. The malabsorption of phytochemicals is likely to be one of many pathways to so-called "phytochemical deficiency disorders".

### REFERENCES

- 1 Wahlqvist ML. Nutritional deficiencies underpin some clinical disorders. *Current Therapeutics*, 1997 December/1998 January: 34-35
- 2 Wahlqvist ML, Wattanapenpaiboon N, Kouris-Blazos A, Mohandoss P, Savage GS. Dietary reference values for phytochemicals. *Proc Nutr Soc Aust*, 1998 (in press)
- 3 Wahlqvist ML, Wattanapenpaiboon N, Kannar D, Dalais F, Kouris-Blazos A. Phytochemical deficiency disorders: inadequate intake of protective foods. *Current Therapeutics*, 1998 July: 53-60
- 4 Forman MR, Lanza E, Yong LC. The correlation between two dietary assessments of carotenoid intake and plasma carotenoid concentrations: application of a carotenoid food-composition database. *Am J Clin Nutr*, 1993;58: 519-524
- 5 Hutchins AM, Slavin JL, Lampe JW. Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. *J Am Diet Assoc*, 1995;95:545-551
- 6 Taucher J, Hansel A, Jordan A, Lindinger W. Analysis of compounds in human breath after ingestion of garlic using proton-transfer-reaction mass spectrometry. *J Agric Food Chem*, 1996;44:3778-3782
- 7 Wahlqvist ML, Dalais FS. Phytoestrogens: emerging multifaceted plant compounds. *Med J Aust*, 1997;167:119-120
- 8 Steinmetz KA, Potter JD. Vegetables, fruits and cancer. *Epidemiol Cancer Cause Control*, 1991;2:325-357
- 9 Giovannucci EL, Ascherio A, Rimm EB. Intake of carotenoids and retinol in relationship to risk of prostate cancer. *J Natl Cancer Inst*, 1995;87:1767-1776
- 10 Evans BA, Griffiths K, Morton MS. Inhibition of 5α-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. *J Endocrinol*, 1995;147:295-302
- 11 Landrum JT, Bone RA, Kilburn MD. The macular pigment: a possible role in protection from age related macular degeneration. *Adv Pharmacol*, 1997;38:537-556

# Clinical research advances in primary liver cancer

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**Subject headings** liver neoplasms/surgery; hepatectomy; liver neoplasms/therapy

Primary liver cancer (PLC) is one of the most common cancers in China. According to the statistics of our country, primary liver cancer claims 20-40 lives per 100 000 people annually, with 19.98 per 100 000 in cities and 23.59 per 100 000 in rural areas, ranking as the second and the first leading cause of cancer death respectively. Of all the newly enrolled cases in the world each year, 45% are found in the mainland of China. In the southeast areas of high incidence, the situation is even worse that the tumor tends to occur in a younger age group.

In China, the research in the diagnosis and treatment of primary liver cancer has undergone four stages: ① in the 1950s, the anatomical study of the liver lay a solid foundation for liver resection. ② In the 1960s and 1970s, as the detection of AFP and other liver cancer markers were widely used, the ability of early diagnosis was greatly improved with a better therapeutic effect. ③ In the 1980s, the introduction of some new techniques, such as CT, MRI, DSA, Doppler ultrasonography, etc., some new methods, such as hepatic artery chemoembolization (TACE), percutaneous intra-tumor ethanol injection (PEI), hepatic artery ligation (HAL) plus catheterized chemotherapy and target therapy, and some new concepts, such as curative local resection, reoperation of the recurrent liver cancer, two-stage resection, the combined surgical management of liver cancer complicated with biliary duct thrombi, splenomegaly, portal hypertension and comprehensive treatment further enhanced the development of liver cancer surgery. ④ In the 1990s, the concept of comprehensive therapy focusing mainly on surgery, the biotherapy strategy based on the rapidly developing molecular biology research and the study of liver transplantation for liver cancer are paid close attention.

The progress of diagnosis and treatment of primary liver cancer in recent years can be summarized as follows.

## EARLY DETECTION OF LIVER CANCER AND THE CHANGE OF THE CONCEPT OF SMALL LIVER CANCER

The methods for early detection of liver cancer include: ① men aged more than 35 years, with a history of hepatitis, and positive HBV or HCV, associated with cirrhosis or chronic hepatitis, should be recognized as a high risk population. Periodical monitoring of this population is a key step to find early liver cancers. ② AFP and B-US screening are, at present, the most sensitive, convenient and economical methods for detecting early liver cancers. ③ For the patients with low level AFP, AFP variant detection is helpful. As to the patients with negative AFP, other liver cancer markers can be used for the early diagnosis. ④ In combination with CT, MRI, CTA or DSA, B-US is of great benefit in the early diagnosis of liver cancer for both the nature of determination and localization ⑤ Fine needle aspiration for cytological assay or the diffusion way of the ethanol injected under ultrasonograph is also helpful for the establishment of the diagnosis.

The criteria for small liver cancer have not been standardized so far. In China, single nodular mass with the diameter or the sum of the diameters of two adjacent nodules less than 5cm was once regarded as small liver cancer. However, with the development of imaging tools, the sensitivity is greatly increased. The liver cancers with the diameter less than 5cm are no longer regarded as small liver cancers clinically. The research in molecular pathology also showed that the great majority of liver cancers presented their biological features at the borderline of 3cm in diameter. Liver cancers with the diameter less than 3cm present the features of an early one, such as, growing largely in the swelling mode, encapsulated, low incidence of vascular invasion and intra hepatic metastasis, diploid type of DNA contents and relatively slow growth. In contrast, the tumor with the diameter more than 3 cm has the capability of invasive growth and dissemination, the features of malignant biology and does not belong to the classical small liver cancer. In addition, as most of the liver cancer is homogeneous, tumor with more than two nodules has a higher probability of intra-hepatic metastasis. The concept that mononodular tumor with 3cm or less in diameter is a small liver cancer may be more suitable for the present liver cancer management and research. The early detection of liver cancer may significantly improve the efficacy of surgical

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Received 1998-10-18

interventions. In our group<sup>[1]</sup>, there was no surgical death in the 709 cases with the tumor diameter less than 5cm. The overall 1-year, 3-year and 5-year survival rates were 89.9%, 85.0% and 79.8%. Among them, of the 241 cases with 3cm or less in diameter, the overall one-year, 3-year and 5-year survival rates were 95.5%, 91.7% and 85.3% respectively. However, of the massive-sized liver cancers resected at the same period, they were 62.5%, 42.6% and 27.5% respectively.

#### THE HEPATECTOMY OF PRIMARY LIVER CANCER

At present, hepatectomy is still the treatment of choice for primary liver cancer. From 1960 to 1996<sup>[3]</sup>, we performed 3932 cases of hepatectomy for liver cancer. Anatomical or extended hepatectomy accounted for 55.4% and local curative resection was 44.6%. The total mortality rate was 0.76% postoperatively, with 8.84% before 1977, 0.43% between 1978 and 1989 and 0.35% after 1990. The total 5-year survival rate was 36.1%, with 16.0% before 1977, 30.6% between 1978 and 1989 and 48.6% since 1990. One patient has survived more than 33 years. Comparing the survival rate listed above, we can tell that the efficacy of surgical intervention has been elevated significantly and rapidly since 1978. The main reasons are: the ability of early diagnosis was elevated; the renewal of some surgical concepts; the improvement of surgical techniques and perioperative management; and the development of comprehensive therapy postoperatively.

The pathological data from this group showed that 86.5% liver cancers were concomitant with cirrhosis or chronic hepatitis. The anatomical or extended hepatectomy might lead to a severe decompensated liver function. Therefore, the modality of liver resection drifted from an extended resection to an irregularly radical local one. Under the circumstance of chronic hepatitis or cirrhosis, the radical local resection not only increases the resectability, but also significantly decreases the surgical mortality rate and attains the same long-time therapeutic effects as the extended resection, or even better. The patients used to be subjected to conservative therapy when one or more complications presented, such as, jaundice, severe portal hypertension and esophageal varices with or without hemorrhage. With the accumulation of clinical practice, obstructive jaundiced patients resulting from involvement of hepatic hilus or cancerous thrombi invasion of biliary tract could undergo hepatectomy and the removal of biliary duct thrombi if hepatic cellular jaundice and other contra-indications could be ruled and most often, the jaundice disappeared gradually. To the patients with splenomegaly, hypersplenism and

esophageal varices with or without hemorrhage, the hepatectomy can also be performed with splenectomy plus ligation of varices or portocaval shunts.

#### COMPREHENSIVE THERAPY FOR PRIMARY LIVER CANCER<sup>[4]</sup>

The comprehensive therapy was mainly focused on advanced liver cancers that were unresectable. But now, the concept of the comprehensive therapy for liver cancer is extended and includes ① the pre- and post-operative comprehensive therapy for resectable liver cancer to prevent the recurrence; ② palliative removal of incurably resected tumors followed by anti-cancer therapy in order to make the tumor shrink and prolong the survival time with tumor burden; and ③ comprehensive therapy for non-surgical patients, with the hope of two-stage resection and long-time survival with tumor burden.

Comprehensive therapy includes surgical and non-surgical interventions. The former are hepatectomy, hepatic artery ligation (HAL), operative hepatic artery embolization (OHAE), drug delivery system (DDS), operative ethanol injection, microwave consolidation, laser gasification, freezing, etc. The latter are transcatheter arterial chemo-embolization (TACE), B-US directed percutaneous ethanol injection (PEI) or other drugs, radioisotopes and bio-agents, biotherapy, radiotherapy and traditional Chinese medicine.

Comprehensive therapy is so called with contrast to single method. Rational multimodality comprehensive therapy is superior to single method in terms of effects. The tetralogy method of surgical comprehensive therapy, which is the combination of HAL, OHAE, DDS and radiotherapy performed, in 603 advanced liver cancers in our hospital<sup>[2]</sup> showed that the rate of two-stage resection and one-year, 3-year and 5-year survival rate were significantly higher than that of single procedure (HAL or OHAE). The incidence of recurrence was only 7.4% in the 27 cases treated with comprehensive immuno-chemotherapy (cytokines plus low dose chemotherapy) after resection, while in control group, it was 32.0%. In 86 cases operated upon, DDS chemotherapy was administered and the incidence of total one-year recurrence was 34.9%, while in hepatic artery group ( $n=39$ ), portal vein group ( $n=26$ ) and hepatic artery combining portal vein group were 33.3%, 34.6% and 23.6%, respectively. Non-surgical comprehensive therapy is indicative to almost all the unresectable liver cancer patients, with the methods of TACE and intra-tumor drug injection the most popular. In a series of 8 000 TACE cases, the 3-year survival rate was 13.9%.

The drugs was selected in the B-US directed local drug injection were absolute ethanol,  $^{32}\text{P}$  radioisotope, OK432, TNF- $\alpha$  and IL-2. The 2-year survival rate in 700 patients receiving PEI was 80.0%, with a total of 3000 times treatment. In another group, 113 patients received TACE in combination with PEI, the tumors shrank in most patients (91.2%) at miscellaneous degrees, and the total 2-year survival rate was 81.6%. Among them, 11 of the 71 patients with monofocal large tumors received two<sup>a2</sup>stage resection after the tumors had shrunk and the two-stage resection rate was 14.28%.

Comprehensive therapy was not simply a random combination of miscellaneous methods. If it was not properly combined, the therapeutic effects would be compromised. The design of the protocol should be case-specific. The model of the comprehensive therapy is multiple in literature. We propose two principles: ① attentions should be paid to the complimentary effects of each method. ② Avoiding the counteraction of the effects or the accumulation of side effects. At the same time, the toxic and negative effects of each method and its possible damage to the liver function should be paid enough attention. In addition, we stress the effects of traditional Chinese medicine in comprehensive therapy.

#### TWO-STAGE RESECTION OF PRIMARY LIVER CANCER<sup>[5]</sup>

In 1978, we reported a two-stage resection of a massive-sized liver cancer shrunk after HAL procedure. From then on, many reports followed and this procedure became a promising model for the unresectable large liver cancers. Surgical and non-surgical comprehensive therapies lead to the shrinkage of massive-sized liver cancers. At present, the documented methods for massive liver cancer shrinkage are: surgical comprehensive methods, such as HAL, OHAE and DDS, and non-surgical procedures, such as TACE, PEI, target therapy and radiotherapy. A rational combination of these methods makes some unresectable tumors resectable if they were successively employed. From 1974 to 1994, 649 patients received this therapy and 73 cases of them had their tumors resected with a resectability rate of 11.1% and no operative death. The 5-year survival rate was 61.5% postoperatively, with the longest survival being 17 years. The pathological data in this group showed that although the tumor shrank due to the comprehensive therapy, it was essential to remove the tumor because of the remnant living tumor cells. At present, though the reported two-stage resectability rates of liver cancer varies, the rates are still very low. The main causes of the low resectability rate are ① no general

accepted criteria for tumor resectability. We propose that the two-stage resection is indicative only to the certainly unresectable tumors, otherwise, the one-staged removal is the first choice; ② rational employment of comprehensive therapy is crucial for the tumor shrinkage and ③ the unresectable liver cancers are recommended for non-surgical comprehensive therapy, such as TAE, PEI and guided chemoimmunotherapy as primary choice.

#### PROPHYLAXIS AND MANAGEMENT OF RECURRENCE<sup>[6]</sup>

The five-year recurrence rate in massive-sized liver cancers is 80%, while in small liver cancers, 40%-50%. The recurrence is most often found in liver, with few cases in bone, lung and brain or in abdominal cavity for the liver cancers ruptured before operation. The postoperative anti-recurrent comprehensive therapy, the early detection of the recurrent lesions and early management of the recurrent lesions are important steps to improve the therapeutic effects. Periodical postoperative follow-up is key to the early detection. We examine them with B-US, AFP and chest roentgenogram every 2-3 months. The patients with negative AFP are subjected to detection of other markers. The CT, MRI or CTA examinations are recommended to those highly suspected of recurrence. On the whole, the recurrence and metastases could be detected at subclinical stage. Comprehensive therapy is helpful in preventing the recurrence of liver cancer. The earliest recurrence happens within 2 months postoperatively, with the peak recurrence rate at 1-2 years. The recurrence after 5 years is rarely seen. Therefore, the anti-recurrent procedure should be given periodically in the 5 years after operation. Fine surgical manipulation to avoid medical dissemination, portal chemotherapy and suction of cancerous thrombi are all essential for prevention of recurrence. TACE, DDS, radiotherapy, immunotherapy and traditional Chinese medicine are given with detailed planning according to the condition of different patients. In recent years, we have employed immunochemotherapy, cytokines such as IFNs, TNF- $\alpha$ , TIL, CTL and some of them work well in anti-recurrence.

Reoperation is the treatment of choice for recurrent liver cancer. In this group, 123 patients received reoperation. The 1-year, 3-year, 5-year and 10-year survival rates after first resection were 99.2%, 71.4%, 53.2% and 19.1%. The 1-year, 3-year, 5-year survival rates after second resection were 83.5%, 38.2% and 19.6% while they were 94.7%, 44.9% and 25.0% after third resection. The reoperation of liver cancer is an effective

method for the improvement of 5-year survival rate and the establishment of reoperation concept has changed the idea that once the liver cancer recurred, it reached an advanced stage and was not fit for another operation. TACE and intra-tumor drug injection are indicative to those with poor liver function, hidden or multifocal lesions. In recent years, we performed PEI therapy in 109 recurrent cases with 0.7cm-15.2cm in diameter, averaging 4.6cm and the 1-year, 3-year and 5-year survival rates were 85.9%, 44.0% and 19.0% respectively. This procedure is easily performed and has the characteristic of faint side effects and damage.

#### CONSIDERATIONS FOR FURTHER IMPROVEMENT OF THE OUTCOME OF PRIMARY LIVER CANCER<sup>®</sup>

At present, several factors influence the prognosis of liver cancer clinically. They are ① whether small liver cancers detected early enough; ② pathological features of the liver cancer; ③ the curative degree of the resection; and ④ the efficacy of the anti-recurrent comprehensive therapy and the resectability of the recurrent lesions. For unresectable cases, the therapeutic effects depend on whether the comprehensive treatment is indicated and sensitive which will directly affect the survival and the two-stage resectability. To all of the liver cancer patients, the tolerance of the liver function to the long, successive traumatic therapy are the basis of therapeutic effects. Further improvement of therapeutic effects on liver cancer counts on the progress in basic liver cancer research. Recently, there were many progresses in the malignant biological features of liver cancer and new methods of biotherapy. The former includes clonal origin of liver cancer, oncogenes and enzymes related to the recurrence and metastasis of liver cancer and their mechanisms, glycoproteins and glycolipids research, the mechanism of the down-regulated immunity in liver cancer hosts and immune escape of liver cancer, induced differentiation of liver cancer, etc. The latter includes regimens that inhibit the recurrence and metastasis of liver cancer and angiogenesis inhibition therapy of liver cancer, specific active and passive immunotherapy, etc. Gene therapy and tumor vaccine technique are also developing rapidly.

It is controversial to the indications of liver transplantation on liver cancer. In advanced primary liver cancer, the recurrence after

transplantation is unavoidable due to the vascular invasion and distal metastasis as well as immunosuppressive agents used. On the contrary, the therapeutic effects of liver transplantation on small liver cancer combined with severe cirrhosis are corroborated. Comparing the therapeutic effects of hepatectomy and liver transplantation (60 cases each). Bismuth<sup>[9]</sup> concluded that the 3-year survival rates were almost the same, while the 3-year tumor-free survival rate was higher in liver transplantation group than in hepatectomy group. As to the small liver cancer (mononodular or binodular, with the diameter less than 3 cm), the results of liver transplantation were even better. Selby *et al*<sup>[10]</sup> showed that the overall 5-year survival rate in 105 unresectable cases of different stages that received liver transplantation was 36%, of whom the 5-year survival rate for one to three stage was up to 52.1%, while in stage four, it declined to 11%. They concluded that liver transplantation was fit for liver cancer in early stages ( $\leq 2$ cm, no vascular invasion and no distal metastasis). However, we still could not regard liver transplantation as a routine therapeutic method due to high incidence of liver cancer, liver donation shortage and high cost.

With the accumulation of 30 years of clinical study, especially the research work during the past decade, we extended our knowledge in its biological characteristics, its clinical features and its diagnosis and treatments. The great efforts should be made for further improving the overall therapeutic results of liver cancer.

#### REFERENCES

- 1 Cong WM, Wu MC, Chen H. Clinical pathological features of small liver cancer: 93 case report. *Chin J Oncol*, 1993;15(5):372-374
- 2 Wu MC, Chen H, Yao XP. Surgical management of primary liver cancer. *Chin J Surg*, 1996;34(12):707-710
- 3 Cong WM, Wu MC, Chen H. Effects of DNA content analysis on the clonal origin and its clinical importance in the recurrent hepatocellular carcinoma. *J Clin Hepatobiliary Dis*, 1993;9(1):3-5
- 4 Yang JM, Wu MC. Surgical comprehensive treatment model for advanced liver cancer. *Chin J Surg*, 1996;34(9):537-539
- 5 Chen H, Wu MC, Shen F. Histologic assessment on resected hepatocellular carcinoma specimens following preoperation transcatheter hepatic arterial chemo-embolization. *Reg Cancer Treat*, 1992;3-4:121-125
- 6 Chen H, Wu MC, Zhou WP. Reoperation of recurrent liver cancer experiences with 72 cases. *J Hepatobiliary Surg*, 1993;1(1):5-8
- 7 Wu MC, Zhang BH. Current status and future of liver surgery in China. *Chin J Surg*, 1996;34(9):515-517
- 8 Guo YJ, Wu MC, Chen H. Effective tumor vaccine generated by fusion of hepatoma with activated B cells. *Science*, 1994;263:518-520
- 9 Bismuth H. Indication of liver transplantation. Supplement to proceedings of 1996 Shanghai international symposium on liver cancer and hepatitis. Mar. 5-7, 1996, Shanghai, China
- 10 Selby R, Kadry K, Carr B. Liver transplantation for hepatocellular carcinoma. *World J Surg*, 1995;19(1):53-58

# Strengthen international academic cooperation and exchanges: prospects in the 21st century: Summary of the First World Chinese Congress of Digestion

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**Subject headings** academic cooperation; digestive neoplasms; digestive diseases

The First World Chinese Congress of Digestion was held in Beijing from October 20 to 22, 1998 in the beautiful capital city of Beijing. The specific aim of this meeting is to summarize and exchange the experience of modern and traditional digestive medicine, and to enhance academic exchanges and cooperation among the Chinese and all other scientists in the world. The congress includes extensive topics, such as the telomerase activity in hepatic carcinoma tissues, acupuncture and moxibustion expression of cellular factor related genes in ulcer colitis in rats antifibrosis by tetrandrine, and *Helicobacter pylori*, gastric carcinoma etc. More than one thousand participants from 12 countries, including the United States, United Kingdom, Australia, Japan, Canada, Israel, South Korea, etc attended this magnificent meeting. Nineteen hundred and eighty-six abstracts were submitted to the meeting and from which, seven hundred abstracts were accepted. Some distinguished experts were invited to make special lectures at the plenary sessions (WJG, Supplement 2, 1998) and 81 doctors and researchers made presentation at the symposia mainly on the following areas.

## DIGESTIVE NEOPLASMS

### *Esophageal cancer*

Alteration of *p19* mRNA expression in esophageal cancer tissue from patients at high incidence area in northern China was reported by Qi *et al*, from Henan Medical University. RT-PCR was used to measure the expression of *p19ARF*, *p53* and *p21* in 19 pairs of frozen normal esophageal and tumor

samples. The cycle number for each pair of primers was fine-tuned to limit the amplification to a linear range. PCR products were then resolved on 2% agarose gel. The density and area of each band was measured using image-pro-plus 1.3 software. The relative expression level of each gene in tumor and normal tissues was calculated using the housekeeping gene GAPDH as an internal control. In the total of 19 tumor samples, 8 (42%) had at least a 3-fold decrease in *p19 ARF* but with no decrease in *p53* expression, 5 (26%) had significantly decreased expression of *p53* but had normal expression of *p19ARF*, only two sample (11%) had decreased level in both *p19ARF* and *p53* expression. The results suggest a negative correlation between the alterations of these two genes in the esophageal tumor. The relative expression level of *p21* in *p19ARF* negative sample ( $0.78 \pm 0.16$ ) was about half of that in *p19ARF* positive samples ( $1.63 \pm 0.22$ ). The results support the hypothesis that *p19* inactivation contributes to esophageal tumor progression and follows the same pathway as *p53* and *p21*. The cyclin-dependent kinase inhibitor *p16* and *p15* play important roles in the regulation of the cell cycle, and have been found to have tumor suppressing roles in a variety of types of cancer. It has been shown that *p16* aberrant methylation and *p15* homozygous deletions were frequently involved in human esophageal squamous cell carcinoma (ESCC). This study examined the impact of such molecular alterations on the expression of these genes. Jiao *et al* (Henan Medical University) measured the mRNA level of both genes in 21 frozen ESCC specimens using semiquantitative RT-PCR. Nineteen cases were observed at a low basal level of *p16* expression ( $0.11 \pm 0.07$ , expression units normalized by housekeeping glyceraldehyde-3-phosphate dehydrogenase gene as internal standard) in the normal epithelia adjacent to the cancer tissues. Among the 19 cases, only 5 showed a significant elevation of *p16* expression (>3.2 folds) in the tumor, whereas the remaining 14 showed either a slight increased (1-2 folds), or decreased *p16* expression compared to normal, whereas 11 had

Editorial Board of World Journal of Gastroenterology, Beijing 100023, China

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**Received** 1998-10-24

only a slight increase (1-2 folds), or decreased *p16* expression compared to normal. In the 5 cases where *p15* was already activated ( $P>0.5$ ) in the adjacent normal epithelium, 4 of them had similar or a slightly lower expression level, but one had a great decrease in *p15* expression (<1% of the normal level). For intact *p16* and *p15* genes, which encode cell cycle regulators, significant increase of their expression on expected in the cancer cells as a response to accelerated cellular proliferation. The findings from Bai et al (Henan Medical University) indicate that the occurrence of hMSH2 protein expression is associated with the cell cycles and related to PCNA expression, implying that the hMSH2 protein is expressed as a guardian in DNA-synthesizing cells.

To observe the morphological changes of the cell apoptosis of esophageal carcinoma cell line EC8712 induced by arsenic trioxide, Shen *et al*, Shantou University Medical College studied the morphology on apoptosis of esophageal carcinoma cell line induced by arsenic trioxide EC8712 cells routinely cultured in 199 medium and acted under  $3\mu\text{mol As}_2\text{O}_3$  harvested after 72 hours, and then HE-stained, TUNEL labeled and examined by transmitting electron microscope and flow cytometry. When adding  $\text{As}_2\text{O}_3$  ( $3\mu\text{mol}$ ) to EC8712 cells for 72 hours, many apoptotic cells appeared. Under light microscope, two kinds of apoptotic cells were seen after H-staining. One was condensed, small-sized and rounded in shape with nucleus dented. The other cells had eosin stained cytoplasm, chromatin agglutinated and the nucleolus existed. The nuclei of the apoptotic cells were positive by labelling with TUNEL kit. The apoptotic peak was identified by flow cytometry. Under electron microscope, two kinds of apoptotic modes were also seen. At early stage of apoptosis, the chromatin of the nucleus agglutinated to pieces and the organelles in the cytoplasm preserved complete. At the second stage, following the change of chromatin, the nucleus became round and full with small pieces of chromatin stickled to the nuclear membrane, while the large clumps of chromatin made the nucleus look like a wheel or a crescent mass. Most of the apoptotic cells showed disintegration of nuclear membrane, from which the chromatin flew out. At the final stage, the apoptotic cells showed degeneration or necrosis. Apoptotic bodies were easily seen which were dense, piece-like or ball-like, naked or capsulated. In the other mode of apoptosis, pyknotic cells showed cell shrinkage, cytoplasm condensation, high electron density and gradual solidification of cell nucleus. CT scan in esophageal carcinoma is

reliable and accurate. The tumor center should be taken on simulation in order to encompass the whole tumor by the 80%-90% isodose curves. He *et al* (Lingi Cancer Hospital, Shandong) investigates the protective effect of radiated auto-blood transfusion on radiation. The incidence of acute radioactive esophagitis was 12.1% in study group and 60.6% in control group ( $P<0.01$ ). The average dose of radiotherapy causing acute radioactive esophagitis was  $4050\pm 609$  (cGY) in control group ( $P<0.01$ ). Significant change of IL-2 and T-cell subgroups was seen in study group. Low dose radiation can stimulate the body's immune function, through which the threshold dose of radiotherapy increases without damaging normal tissues.

### **Stomach neoplasm**

To diagnose the alteration of oncogenes and tumor suppressor genes in gastric carcinomas, Cen *et al* (Affiliated Hospital of Qiannan Medical College) researched on *P53* gene mutation of biopsy samples from stomach cancer patients. The mutation rate of *P53* in exon 5-8 was 60.0% (18/30). Point mutation of *P53* was found at both early and advanced tumors. In contrast amplification of oncogenes and loss of tumor suppressor genes were correlated with poorly differentiated and metastatic tumors.

The induction of apoptosis by cisplatin (CDDP) in gastric carcinoma cell line for demonstration of a human gastric carcinoma with CDDP was investigated by Xin *et al* (Fourth Military Medical University). A human gastric carcinoma cell line SGC-7901 was cultured in full medium with various doses of cisplatin for different hours. The treated cells were examined under light microscope and transmission electron microscope (TEM). Cell cycle analysis was performed in flow cytometry (FCM). After treatment with CDDP, the cells became smaller and condensed. Their chromatin changed to periphery of the nucleus. Apoptotic bodies were observed. There were apoptotic peaks in cell cycle analysis on FCM. There were apoptotic peaks in cell were 9.3% and 14.9% after being treated. CDDP might induce apoptosis in the gastric carcinoma cell line SGC-7901 that led to the death of the cancer cell. The data of Beijing Medical University (Cao *et al*) show that antisense RNA to *bcl-2*, not only can induce apoptosis, but also reverse the biological behavior of MGC-803 cells. This would be a potential application to the gene therapy for stomach cancers. Preoperative cimetidine application can restore NK cells, which may be beneficial to reducing recurrence and metastasis (Li *et al*, Hebei Medical University). Ma *et al* (Third

Military Medical University) detected the occurrence and development of precancerous lesion of residual gastric mucosa and their relationship to gastric cancer in the gastric stump. The conventional Billroth gastrectomy is closely associated with the lesion of the residual gastric mucosa. The common manifestations of gland atrophy and proliferative lesions of the residual gastric mucosa are the important bases of the precancerous lesion, which should be paid more attention to in clinical practice. Shen *et al* (Nantong Medical College) observed the therapeutic effect of compound Shenqitang decoction on gastric adenocarcinoma after gastrectomy, and studied its inhibitory effect on gastric adenocarcinoma induced by MNNG in Wistar rats. The results shown that it has good therapeutic results in combined operative treatment of gastric carcinoma. Colonic neoplasms periphera blood (PB) T-lymphocyte subsets and natural killer cytotoxicity (NKCC) were measured in 43 patients with colorectal carcinoma (CRC) pre- and post-operatively by using the APAAP and LDH release methods respectively. The CRC patients were still in immunodepressive state in the first 2 weeks after operation, and the immunotherapy can improve the preoperative cellular immunofunction, and shorten the perioperative immunodepressive period (Liu *et al*, Taian Central Hospital). Xiao *et al* (Central Hospital in Jiangnan Oil Field) explored the therapeutic effect of chemoembolization in hepatic metastases in colorectal carcinoma. Forty patients underwent chemoembolization of metastatic liver lesion from colorectal carcinoma. Selective angiography of the hepatic artery was performed to identify the feeding vessels of the metastatic lesion. The injected chemoemulsum consisted of 100 mg 5-fluorouracil, 10mg mitomycin c and 10 ml lipiodol ultra-fluid in a total volume of 30 ml. Gel foam embolization was then followed until stagnation of blood flow was achieved. Patients were evaluated for response, overall survival, and side effects. Overall median survival time from date of first chemoembolization was ten months. Median survival time of cirrhotic patients with class A and B by Child-Pugh classification was 24 and 3 months, respectively. The difference was significant ( $P < 0.01$ ). Patients with metastatic disease confined to the liver did better than those who also had extrahepatic disease, with median survivals of 14 and 3 months, respectively ( $P < 0.02$ ). The median survival of patients with hypervascular metastases was longer than that of patients with hypovascular metastases. The most common side effects were transient fever, abdominal pain and fatigue. Three

patients died within one month from the procedure. The therapeutic effect of systemic chemotherapy in hepatic metastases of large intestinal carcinoma was not satisfactory and there were more side effects, whereas the therapeutic effect of selective chemoembolization was promising and there were fewer side effects. Selective chemoembolization may be an effective first-line therapy in hepatic metastases of large intestinal carcinoma. Gao *et al* (The Harrison International Peace Hospital) reported that after abdomino-perineal resection of rectal cancer (Miles), greater omentum was cut off and retroperitoneal tunnel was performed. According to the ways of greater omentum into pelvis, the tunnels had three ways (left, middle and right). Left way reaches pelvis through retroperitoneal tunnel in descending colon side dish, 6 cases; middle way reaches pelvis through retroperitoneal tunnel behind colon and on the left of spine, 35 cases; and right way reaches pelvis through retroperitoneal tunnel in ascending colon side ditch, 9 cases. Middle way is the best, which has a short tunnel, is situated below abdominal incision, convenient and easy on practice. Packing in presacral space with the pedicle greater omentum transplantation has better effects on promoting the primary healing of the perineal wound, with extensively clinical application value.

#### **Liver and pancreatic neoplasm**

Hepatic arterial branch supplying hepatocellular carcinoma (HCC) has a lower impedance than the branch not supplying HCC (Wang JG & Pan LL, Shantou Central Hospital). To study the blood AFPmRNA in the patients with distant metastasis of human HCC using nested reverse transcriptase polymerase chain reaction (nested RT-PCR) and its significance, 93 blood samples from human HCC were examined by nested RT-PCR to find out AFPmRNA by Liu *et al* (Second Military Medical University). AFPmRNA was detected in 21 blood samples from 72 human HCC (40.28%) without distant metastasis. AFPmRNA (100%) was detected in all HCC patients with distant metastasis. AFPmRNA can be used as a distant metastasis marker of HCC. Portal vein chemotherapy combined with 0.25MPa HBO can significantly reduce the tissue impairment and oxygen radical after resection of HCC. These findings may indicate that hyperbaric oxygenation plays a positive role in combined treatment for HCC (Li *et al*, Fujian Medical University). Blood AFPmRNA and AFP detection is useful in predicting relapse or distant metastasis after surgery in HCC patients (Zhang *et al*, Second Military Medical University). Para-HCC

specimens (24 cases, Group A) and noncancer cirrhosis (33 cases, Group B) were all tested by *in situ* terminal end labeling (ISEL), HBsAg immunohistochemistry and HE analysis. ISEL (+) intensity was divided into 4 grades. The results were compared between the two groups. The positivity rates and positive intensity of ISEL in Group A were significantly higher than that in Group B ( $P < 0.01$ ). The positive cell nuclei tended to scatter just near the septa and portal tracts. The majority of Group A are of static portal cirrhosis, while Group B also included cirrhosis of chronic active hepatitis and chronic severe hepatitis. Inflammatory cell infiltration was more evident in Group B than in Group A ( $P < 0.05$ ). The HBsAg(+) rates of both Groups A and B are very high. There were no correlation among ISEL and HBsAg, proliferation and dysphasia of hepatocyte. About half of the hepatocytes in one case of Group A underwent apoptosis identified by both ISEL and HE (Lian *et al*, Shantou University Medical College). The detection of TGF- $\beta$ 1 and PCNA expressions in primary hepatocarcinoma tissues may be useful in identifying and judging the tumor-differentiation and prognosis (He *et al*, China Medical University). The ras oncogene and p53 anti-oncogene expressions of 55 pancreatic paraffin-embedded specimens, including 32 carcinomas, were studied by immunohistochemistry ABC method. Twelve specimens taken from the normal pancreatic tissue near the tumor transaction margin, 7 specimens of pancreatitis and 4 specimens of normal pancreas were compared.

The positive expression rate of ras oncogene and p53 anti-oncogene was 71.9% and 28.1% in 32 cases of pancreatic carcinomas and it is higher than that of the pancreatitis and normal pancreatic tissue near the tumor transected margin ( $P < 0.05$ ). The ras and p53 gene expression was not significantly related to sex, age, site, size and incipient symptoms ( $P > 0.05$ ). The p53 anti-oncogene expression was related to tumor staging and grading ( $P < 0.05$ ). The tumor mass with negative p53 gene expression usually had a higher respectability ( $P < 0.05$ ), and its positive expression usually associates with lymph node metastasis ( $P < 0.05$ ), and worse prognosis. It also provided an important guidance to choose the methods of treatment. Sun *et al* (Guiyang Medical College) suggested that the ras and p53 gene expression could be used to evaluate the pancreatic cancerous biological behaviour, and it might aid the diagnosis and treatment for pancreatic carcinomas.

## GASTROINTESTINAL DISEASES

The poor living condition is the sources of the *Hp* infection, and it is the main pathogenetic factor of PU and upper gastrointestinal tumor. Kang *et al* (People's Hospital of Liulin County) investigated and summarized incidence of upper gastrointestinal diseases in Liulin County. A total of 3142 patients were had *Hp* tested by urease and pathological tests. Gastric ulcer was found in 287 cases, duodenal ulcer in 245 cases, and esophageal cancer in 241 cases. The rate of *Hp* infection was 98% in gastric ulcer, duodenal ulcer and cancer of the stomach. The ratio of GU to DU was 1.17:1, including 672 PU cases, and 414 cases of malignant tumor. The incidence of PU, esophagus cancer and stomach cancer was found to be increasing. The incidence of GU in males was much higher than that in other areas reported, possibly due to living conditions and dietary habits. One hundred patients with chronic atrophic gastritis were treated with Wuji capsule. Of them, 42 were mild, 38 moderate and 20 severe atrophic gastritis and 41 and 13 accompanied with intestinal metaplasia (IM) and degree I dysphasia (Dys), respectively. The clinical manifestations were stomach pain (86 patients), fullness of abdomen (72), anorexia (90), eructation (34) and bitterness of the mouth (20). After treatment for three months the improvement of patient's symptoms, atrophy of gastric mucosa, IM and Dys were annualized. After 3 months with Wuji capsule treatment, 7 patients were recovered, 48 very effective, 34 improved, and 11 ineffective. The total efficacy was 89%, and 5 unchanged. Of the 38 patients with moderate atrophic gastritis, 17 developed mild atrophic gastritis, 14 superficial gastritis, 3 became normal and 4 unchanged. Of the 20 severe atrophic gastritis, 10 turned into moderate atrophic gastritis, 8 superficial gastritis and 2 had no changes. Of the 26 patients with mild IM, IM disappeared in 20, and 6 had no changes. Of the 8 patients with moderate IM, IM disappeared in 1, 4 changed into mild IM, and 3 had no changes. Of the 7 patients with severe IM, 2 changed into moderate IM, 2 mild IM, and 3 had no changes. Of the 13 patients with degree I Dys, Dys disappeared in 7, and 6 had no changes. Hao *et al* (China Medical University) investigated the effects of different kinds of Bupleurum and Citrus on gastrointestinal motility. Choosing the two main varieties of Bupleurum and Citrus, B. Chinese DC. (B. Cdc), B. Scorzoneraefolium Wild (B.sW) and Citrus aurantium (Ca), Citrus sinensis (Cs), as the test drugs, we compared the effects of 4 drugs. Different mixtures of Bupleurum and Citrus and different dosage of the mixtures on mice gastrointestinal motility with Blue Dextran 2000 as a

marker in the gastrointestinal tract. B. cDC. and Ca had obvious enhancing effects on the gastric emptying function and small intestinal propulsion function, while the effect of B.sW and Cs had no difference with negative control group ( $P > 0.05$ ). The effects of the gastrointestinal motility proved to be more significant than single drug and the mixture of the above two herbs decocted respectively. Li *et al* (China Medical University) investigate the influences on gastric emptying and small intestine transportation of 6 formula compositions combined with *Atractylodis ovatae rhizoma* (AOR), *Magnoliae Cortex* (MC), *Arecae Pericarpium* (AP), *Amomi Semen/Seu Fructus* (ASSF), *Galli Gigerii Endothelium* (GGE), *Massa Medicata Fermentata* (MMF), *Hordei Frutus Germinalus* (HFG) and *Carateggi Endocarpium et Semen* (ACES). The decoctions of ASSF, GGE, MMF and HFG, MC, AP, MMF and HFG; MC, AP, ASF, GGE, MMF, HFG; and the decoction of MC, AP, MMF, HFG, ACES and APR can improve the gastric emptying function. The decoction of ASSF, GGE, MMF and HFG can also promote the small intestinal transportation function. Zhang *et al* (Youhong Chinese Medicine, Huinong) analysed the therapeutic effect of Jieyu Yuyang San, Xiaqi Xiaoshi Yutong San, Yangyin Yuyang Zhentong Wan on three kinds of peptic ulcer. After the whole course treatment, 382 affective ulcer patients were recovered, 120 had evident effect, 204 improved, and 14 ineffective, with a total effective rate of 98%. A total of 450 dietary ulcer patients were recovered, 240 very effective, 108 improved, and 32 ineffective, with a total effective rate of 96.2%; and 301 mixed ulcer patients were recovered, 209 very effective, 107 improved, and 33 ineffective with a total effective rate of 95%. Symptoms disappeared in 2121 patients, and 79 patients ineffective with a cure rate of 77.6%. Before treatment, the degree I, II, III peptic ulcer was found in 720, 830 and 650 patients and 14, 32 and 33, respectively after treatment. Of 157 cases of liver cirrhotic ascites, 57 cases had upper gastrointestinal bleeding, and 100 cases had no bleeding. Complications uncluded hypersplenism, gastric ulcer, spontaneous peritonitis, hepatic coma and poor renal function. Complications in positive bleeding group were compared with negative bleeding group as controls. The positive rates of poor renal function and hepatic coma in positive bleeding group were significantly increased as compared to that in controls ( $P < 0.01$ ). The positive rates of complications with upper gastrointestinal bleeding in ascitic liver cirrhosis were higher than non-bleeding. In order to

investigate the cause and the position of bleeding with cirrhotic ascities and search for therapeutic methods, emergency endoscopy was performed (Li *et al*, Hainan Provincial People's Hospital). Assessment of disease outcome in a large inception cohort of patients with IBD showed that the majority had symptomatic improvement over a four-year period after diagnosis and mortality from IBD related causes was low. In Europe, with present medical treatment, medium-term outcome of IBD appears favorable. The plasma concentration of nitrite/nitrate (stable end products of NO, standing for NO) and molitin of 18 patients with UC and 11 control subjects were respectively measured with Cadmium-reduction chromatography and development process (Greiss) and RIA (Radioimmuno assay). The concentration of plasma NO and MTL in UC groups were significantly higher than the controls ( $P < 0.01$ ,  $P < 0.05$ , respectively). The concentration change of plasma NO in UC group significantly correlated with the change of MTL ( $P < 0.05$ ); but there was no significant correlation in the control group ( $P < 0.02$ ). Nitric oxide and molitin were both involved in the pathophysiologic process of ulcerative colitis. Moreover, there may be some positive interactions between NO and MTL in the pathogenesis of UC (Wu *et al*, Fujian Medical University). Hu *et al* (461st Hospital of PLA) observed the therapeutic effects of ulcerative colitis managed by integrated traditional Chinese medicine (TCM) and western therapy and compared with conventional interventions solely. The results were investigated 2 weeks afterward 39 cases cured (81.3%), 8 improved (16.7%) and 1 case ineffective (2%), i.e. 98% total effective rate in Group I. Comparatively 27 cases cured immediately (64.3%), 9 improved (21.4%) and 6 ineffectiveness (14.3%). With a total effective rate of 86.9% in Group II. Significant differences were found statistically between the two groups ( $P < 0.01$ ). CD and UC are two forms of intestinal inflammation with possible common genetic predisposition and may be part of a spectrum, rather than two distinct disease. Induction may be non-specific. Genetic susceptibility and uptake of bacterial products perpetuate inflammation. Genetic and environmental factors are critical, but neither alone is sufficient. Progression and resolution of CD and UC are dependent on the balance of pro- and anti-inflammatory mediators. Homeostasis or chronic inflammation depends on the balance between inflammatory luminal constituents and protective mucosal factors. Specific therapy directed at an immunoregulatory defect or an inciting agent could alter the disease course. Current

therapies, such as glucocorticoids and 5-aminosalicylic acid (5-ASA), inhibit concentrations of interdependent, soluble mediators of inflammation, which may amplify one another or have parallel effects. It remains, however, to define whether targeting multi-inflammatory actions or a single key pivotal process is a better therapeutic strategy. The type of new drugs being developed include conventional pharmaceuticals, receptor antagonists-agonists, enzyme inhibitors, bio-engineered compounds (monoclonal antibodies, chimerical-targeted toxins, receptor legends-soluble receptors), and gene therapy.

#### LIVER, BILIARY AND PANCREATIC DISEASES

In cold weather, upper gastrointestinal hemorrhage is common in the patients with liver cirrhosis. The mechanism is that the change of temperature affects the redistribution of blood of the human body. When the weather temperature drops, the effective blood circulatory volume of shallow tissues is reduced to some extent, while that of deep tissues is increased relatively. This will raise the pressure of portal vein system and its collateral circulation. Guo *et al* (Second Military University) analyzed the relationship between cr1 genetic density polymorphism on erythrocytes and ability of erythrocytes adhering tumor cells in different groups, such as normal people, patients with HBV infection, liver cirrhosis and liver cancer. In the same population, the ability of HH type erythrocyte adhering tumor cells was significantly higher than that of HL type erythrocytes. The ability of HL type erythrocytes adhering tumor cells was significantly higher than that in LL type erythrocytes. In the same cr1 genomic type population, the ability of erythrocytes adhering tumor cells of normal people was significantly higher than that of patients with HBV infection and patients with liver cirrhosis and liver cancer. Zhang *et al* (General Hospital of Jinan Command Area) established liver injury model induced by ConA in Kunming mice. ConA was administered to Kunming mice via tail vein. The model was dose dependent; the histopathological examinations of liver specimen showed the T lymphocytes infiltration in portal areas, spot necrosis and piecemeal necrosis. With the inhibition of T cell activation by cyclosporine A (CSA), liver injury and infiltration of lymphocytes were not seen. Huang *et al* (Fujian Medical University) explored the clinical significance of serum type III procollagen (PC III), laminin (LN), prolidase (PLD) and type IV collagen in patients with liver diseases. Serum levels of LN, PC III, IV-C and PLD were helpful in clinical diagnosis of

patients with liver cirrhosis and in judgment of developing tendency in patients with chronic HBV infection. Combined determination of PCIII and LN can elevate the specificity in the diagnosis of cirrhosis. The level of  $\gamma$  globulin can reflect the pathology of the liver, the level of serum cholinesterase can reflect the synthetic function of the liver and is negatively related to the damage of the liver (Zou *et al*, Chinese PLA 302 Hospital). Dan *et al* (Chinese PLA 302 Hospital) reported that isolates of HCV genotype 1b between China and Japan share high similarity in NS5A nucleotide sequence. Variation in the NS5A region between amino 2209 to 2248 failed to predict IFN response in Chinese patients infected with HCV genotype 1b. Xu *et al* (Shuang Ya Shan General Hospital) reported that estradiol and HCG are related to the formation of gallbladder cholesterol stone. Sixty rabbits were randomly divided into 6 groups, in 4 of which (groups E<sub>2</sub>, P, T, H) estradiol, progesterone, testosterone and HCG were administered separately, and normal saline and refined oil were given to the other groups (C<sub>1</sub>, C<sub>2</sub>) as control. The animals were sacrificed after 6 weeks. The blood, bile, gallbladder, bile duct, liver and gallstones were assayed. The gallstone formation rate was 90% in group E<sub>2</sub>, 50% in group H and 10% in group T. No gallstone was formed in group P, C<sub>1</sub> and C<sub>2</sub>. Most of the gallstones were found in female animals, only in 4 male rabbits of group E<sub>2</sub>. The composition of stone was mainly cholesterol (Wang *et al*, Anhui Medical University). Wang *et al* (Guiyang Second People's Hospital) studied the relationship between the estrogen, blood lipids and cholelithiasis. Serum estradiol (E<sub>2</sub>), progesterone (P), total cholesterol (TC), triglyceride (TG) were tested in 104 patients (Group A) confirmed to have cholecystolithiasis by B-mode ultrasonography and cholecystostomy and the results were compared with that of 54 normal persons (group B). Serum E<sub>2</sub> and P levels of the men in group A were remarkably higher than those in group B ( $P < 0.05-0.01$ ). Serum E<sub>2</sub> levels of the women of child-bearing age were not different between groups A and B ( $P > 0.05$ ), but P levels of group A were higher than that of group B. Serum levels of E<sub>2</sub> or P of menopause women in group A were all markedly higher than those of women in group B ( $P < 0.01$ ). The ratio of E<sub>2</sub>/P of women in group A was significantly lower than those in group B ( $P < 0.001$ ). Serum levels of TG, TC and the ratio of TG/TC in persons of group A (either men or women) were all higher than those in group B ( $P < 0.01$ ). Estrogen and lipid metabolism of

cholecystolithiasis patients are disordered. The role of oxygen free radical (OFR) and other inflammatory mediators in acute necrotized pancreatitis (ANP) was studied by Wang *et al* (Inner Mongolia Medical College). Oxygen free radicals were involved in the aggravation of ANP and were associated with the increased of serum endotoxin and PLA<sub>2</sub>. Those mediators were positively correlated with severe multiple organ damage. The results also suggested that IL-2 could inhibit the overexpression of OFR and endotoxin, and reduce the incidence of multiple organ damage in ANP. TNF $\alpha$  mRNA plays an important role in ANP progression and somatostatin and growth hormone may be the effectual treatment to prevent the development and progression of multiple organ dysfunction syndrome in acute necrotized pancreatitis (Zhang *et al*, Shanghai Medical University). Zhong *et al* (Zhongshan Medical University) suggested that patients with acute pancreatitis have significantly different changes of platelet formative property from acute hemorrhage and necrotized pancreatitis, which indicates the severity of the disease. Pt has no significant change, but platelet activity was increased after SS treatment. Qin *et al* (Luoyang Second People's Hospital) evaluated the curative effects of Octreotide (Oct) on acute pancreatitis. Oct was used to treat 38 cases of acute pancreatitis, and 59 patients were treated as the control group by non-octreotide. Before and after Oct was injected, serum amylase and pancreatic fluid amylase were analyzed quantitatively for the two groups, and the incidence of complications were also compared among these patients. Oct was found to ameliorate the clinical symptoms and signs and decrease the occurrence of complications.

#### HELICOBACTER PYLORI

*Helicobacter pylori* (*Hp*) is a gastric pathogen strongly implicated in the causation of gastritis, duodenal ulcer, gastric ulcer, gastric cancer and gastric lymphoma. Almost half of the world's population or 2 billion people are *Hp* infected, making it the commonest chronic infection in men, and an important global health problem. There are several striking differences in the pattern of *Hp* infection and gastroduodenal diseases between countries of the East and West, including: *Hp* presence and characteristics; disease patterns; and host differences. These differences do not occur on the basis of geographic boundaries, but are the outcome of genetic and environmental factors in the respective populations. Strategies for the management of *Hp* infection in Asia must take these

factors into account. These differences and their implications for clinical management and health care policies in Asian countries were presented by Dr. Yeoh Khay Guan (National University Hospital, Singapore). Xu *et al* (Harbin Medical University) demonstrated that CCK-8 could antagonize the effect of morphine which inhibited the potentiation of Ache on the electrical and mechanical activities of rat duodenum *in vitro*, whereas devazepide could reverse the anti-morphine effect of CCK-8. It is suggested that the antagonistic effect of CCK-8 on morphine should be mainly mediated by CCK-A receptor, thus providing a new clue for the clinical treatment of disturbances in intestinal movement function. *Hp* infection is closely related to DU occurrence and can lead to antral gastritis. Owing to *Hp* antral gastritis, antral D cells in patients with DU decrease in number and SS synthesis (Zheng *et al*, China Medical University). Han *et al* (Chinese PLA Institute of Genetic Diagnosis) cloned the 5'-end of *cagA* (854bp) into the expression vector pBV220 and transformed DH5 $\alpha$  with the plasmid pBV220/*fcagA*, in which a single *CagA* fragment (FCagA) was produced when the temperature reached 42°C. After being renatured, FcagA was purified by anion exchange and sephadex G-100 chromatography. The FcagA had a relative molecular weight of 38 000. With the prepared FCagA, colloidal gold, and immunogold, they established the dot immunogold filtration assay (DIGFA) to detect anti-*CagA* antibody in serum. FCagA had the similar antigenicity as *CagA*. The test of DIGFA took only a few minutes and could also be done for one or more persons with no need for special equipment. Compared with EIA, DIGFA had the sensitivity of 96.8%, and the specificity of 98.5%, when the sera of 262 cases were tested. One hundred and sixty-six patients completed all the study. The eradication rates were 84.2% in group A and 72.2% in group B ( $P < 0.05$ ). There was no significant difference in both the *Hp* eradication rate and healing rate of the ulcer patients between the two groups. More side effects occurred in group B than in group A, which still could be tolerated by the patients. The cost of group A is higher than that in group B (RMB 820.78 vs 418.04). Considering the effectiveness and cost, OCA therapy is more suitable for gastritis patients. For ulcer patients, RTA therapy is as effective as OCA therapy (Wang *et al*, Shanghai Zhongshan Hospital). Gastrin (Gas) and somatostatin (SS) of gastric mucosa and blood in patients with *Hp* positive group were significantly higher than *Hp* negative group and became normal after *Hp*

eradication. The SS contents in *Hp* positive group were significantly lower than *Hp* negative group and became normal after of the *Hp* eradication. On the other hand, Gas and SS contents of the mucosa significantly altered with chronic and active inflammations. Zhang *et al* (China Medical University) studied the sensitivity, specificity and clinical applicatia of  $^{14}\text{C}$  urea breathing thes of *Hp* infection. All the 150 cases (40 cases of chronic gastritis, 30 cases of gastric ulcer, 50 cases of duodenal ulcer, 20 cases of gastric carcinoma, 8 cases of polypous gastritis, 2 cases of portal hypertensive gasteopathy) were examined by fibrogastroscopy and confirmed by biopsy pathology, 15 cases of duodenal ulcer and 5 cases of gastric ulcer were treated with PPI therapy for 1 month, then a comparison between the pretreatment and posttreatment was made,  $^{14}\text{C}$  urea was calculated by scintillators. The results showed that the *Hp* infection rates of chronic gastritis duodenal ulcer and gastric ulcer and gastric carcinoma had no statistical difference with the method of  $^{14}\text{C}$ -UBT; there was no difference in  $^{14}\text{C}$ -

UBTY radioactivity value between chronic gastritis and duodenal ulcer; the incidence of chronic gastritis accompanied with gastric mucosal erosion atrophy and enterometaplasia was significantly higher than that of simple chronic gastritis ( $P < 0.05$ ); after one-month bactericidal treatment, the bactericidal rate reached 100%. In conclusion, this brief glimpse into the science and practice of gastroenterology in the next century offers us a mixed perspective, one of an ever-widening disparity between rising opportunities in the one hand, and restrained resources on the other. We are afraid that unless this serious dilemma will be resolved early in the next century, the practice of gastroenterology and the quality of health care will cut expenses by voluntarily reducing our dependence on technical procedures and expensive equipment, and by avoiding use of only marginally effective medications and surgical interventions. These, we believe, will be painful adjustments for the medical establishment, but they must be faced in the coming century.

# Coexistence of *Helicobacter pylori* spiral and coccoid forms in experimental mice

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**Subject headings** *Helicobacter pylori*, antibodies; ELISA; spiral form; coccoid form; mouse

## Abstract

**AIM** To infect mice with *Helicobacter pylori* and detect immune response against two form of *H. pylori*.

**METHODS** An isolate of *H. pylori* obtained from a patient with gastric cancer was used to infect mice. Fifty mice were divided into eight groups. Two groups served as negative control without any inoculation and internal negative control with 0.5M NaHCO<sub>3</sub> and brain heart infusion (BHI), respectively. Mice in each experimental group were first inoculated with 0.5M NaHCO<sub>3</sub> and then *H. pylori* suspension for 3 times at a 2-day interval. Mice from controls and infectious groups were sacrificed at a weekly interval postinfection. Gastric samples were trimmed, inoculated onto chocolate blood agar and then incubated in microaerophilic atmosphere at 37°C for 14 days. Sera were examined for immunoglobulins against *H. pylori* spiral and coccoid antigens by ELISA.

**RESULTS** After inoculation *H. pylori* was isolated in one mouse from one week postinfection. No *H. pylori* was detected in control mice. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with NaHCO<sub>3</sub> and BHI and 77% (23/30) mice infected with *H. pylori*. The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The mice showed immune responses to both *H. pylori* spiral and coccoid antigens one week after infection with *H. pylori*. The peak mean absorbances of antibodies against spiral and coccoid forms were four weeks postinfection

which showed 6 and 18 times higher than that of negative control group respectively ( $P < 0.01$ ).

**CONCLUSION** Spiral and coccoid forms of *H. pylori* coexist in experimental mice studied.

## INTRODUCTION

*Helicobacter pylori* colonizes stomach of human being and causes gastritis and peptic ulcer<sup>[1]</sup>. It has been reported that this organism exists in two forms, spiral form and coccoid form<sup>[2,3]</sup>. Many investigations are being performed on whether coccoid form is degenerative or viable. Hua and Ho<sup>[3]</sup> reported that similar to the exponential cultures, ageing coccoid form produces alkaline phosphatase, acid phosphatase, leucin arylamidase and naphthol-AS- $\beta$ -1-phosphohydrolase and remains genetically unchanged suggesting that it is highly likely to be viable. It was found that specialized attachment sites such as the "adhesion pedestal", "cup-like indentation" and "abutting adhesion" were seen in the interaction between coccoids and epithelial cells. These adherence patterns were similar to those observed with spiral form in gastric biopsy specimens in vivo, suggesting coccoid could be a differentiated infective form of *H. pylori*<sup>[4]</sup>. Therefore, this form was suspected to play a critical role in the transmission of *H. pylori* and could be one of the causes of recrudescence of *H. pylori* infection after antibiotic treatment. In this study we investigated mouse immune response against *H. pylori* after oral infection with the bacterium and demonstrated coexistence of spiral and coccoid forms of *H. pylori* in mouse.

## MATERIALS AND METHODS

### Animals

Female BALB/c mice weighing about 25g were obtained from the Laboratory Animal Center, National University of Singapore. Mice were 5 weeks old when they were sent to laboratory and maintained for one week to allow them to adapt to the new environment. Mice were fed with a

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Received 1998-11-09

commercial rodent diet and provided with sterile water.

### **Bacterial strain**

An isolate of *H. pylori* H132 obtained from a patient with gastric cancer was used for this study. Strain H132 was isolated on chocolate blood agar base No.2 medium with 5% horse blood at 6 days of incubation of biopsy at 37°C under microaerophilic environment. The bacterium was inoculated into brain heart infusion (BHI) broth supplemented with 10% horse serum and 0.4% yeast extract in a flask at 37°C for 2 days. The broth culture was centrifuged at 4000×g for 20min. The supernatant was discarded and fresh BHI broth supplemented with 10% horse serum and 0.4% yeast extract was added to the pellet. The suspension was mixed gently. The inoculum was incubated at 37°C for another 2 days. The concentration of spiral form was determined by spread plate method and bacterial counting chamber. In this experiment the concentration of *H. pylori* spiral form was about  $1-5 \times 10^8$  CFU/ml.

### **Animal experimental design**

Fifty mice were included in this experiment. They were divided into eight groups. Two groups with ten mice each. One of these 2 groups served as negative control without any inoculation while the second group of 10 mice was inoculated with 0.3ml of 5 mM NaHCO<sub>3</sub> and 0.3mL BHI serving as internal negative control. The remaining 30 mice were divided into six groups of 5 mice each. Mice in each experimental group were first inoculated with 0.3ml 0.5M NaHCO<sub>3</sub>. An hour following that, 0.3ml of *H. pylori* suspension was administered with a gastric gavage. The procedure was repeated 3 times at 2-day interval for these 30 mice.

Two mice from the controls and five mice from one infection group were sacrificed at weekly interval postinfection. Before being sacrificed, the mice were fasted for one day with free access to water. The mice were sacrificed by cervical dislocation. Stomachs were dissected for microbiological analyses. Five hundred microliters of blood samples were taken from the heart of sacrificed mice for immune response studies.

### **Microbiological analyses**

Gastric samples were examined within one hour. Samples of antrum were trimmed and inoculated on chocolate blood agars with antibiotics (vancomycin 6g/L, nalidixic acid 5 g/L, amphotericin 6 g/L and trimethoprim 10g/L) and without antibiotics. Plates were incubated in microaerophilic atmosphere

at 37°C for 14 days. Typical colonies were identified by standard methods<sup>[5]</sup>. Blood of mice was collected from heart and centrifuged at 4000×g for ten minutes. Sera were removed from clot and stored at -20°C. Sera were examined for immunoglobulins against *H. pylori* by ELISA.

### **ELISA**

Antigens of spiral and coccoid form of *H. pylori* were prepared by acid glycine extraction according to a modification method of Goodwin *et al*<sup>[6]</sup> as described by Vijayakumari *et al*<sup>[4]</sup>. Protein concentration was determined by the modified Lowry protein assay and the antigens were stored in 1ml aliquots at -20°C until use.

The stock antigen solution was diluted in carbonate buffer (90% 0.5M Na<sub>2</sub>CO<sub>3</sub> and 10% 0.5M NaHCO<sub>3</sub>, pH 9.6). Aliquot of 200μL of the diluted antigen preparation was added to each well of a microtitre plate (Nunc) to give 1μg of antigen per well. Plates were left for 24 hours at 4°C. Excess antigen was removed and each were replaced by 300μl of serum diluent (0.02% thimerosal, 0.05% Tween 20 and 1g/L gelatin in PBS) and kept at 4°C for at least 24 hours before used.

The sera to be tested were diluted 1:100 with serum diluent (0.02% thimerosal, 0.05% Tween 20 and 1g/L gelatin in PBS). A 100μl aliquot of each diluted test serum was added to each of the three wells of the microtitre plate. Plates were incubated at room temperature for 90 minutes. The plates were then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20.

The second antibody was horse radish peroxidase labelled goat anti-mouse immunoglobulins (Dako) which react with all mouse IgG subclasses, IgA and IgM. It was diluted to 1:4000 with PBS containing 0.02% thimerosal, 0.02% BSA and 0.1% gelatin. A 100μl of the diluted secondary antibody was added to each well of the plate that was subsequently incubated at room temperature for another 90 minutes. It was then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20 followed by washing two times with PBS containing 0.02% thimerosal only. A 100μl of substrate containing 40mg of o-phenylenediamine dihydrochloride (OPD, Sigma) and 40μl of 30% hydrogen peroxide (Merck) in 100ml phosphate citrate buffer (0.1M C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O and 0.2M Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O) at pH 5.0 was added to each well. Plate was left at room temperature in the dark for 15 minutes. A-50μl of 2.5M H<sub>2</sub>SO<sub>4</sub> was added to each well to stop reaction. The optical density (OD) of the reaction mixture was read immediately at wavelength of 490nm and 620nm reference filter using an ELISA

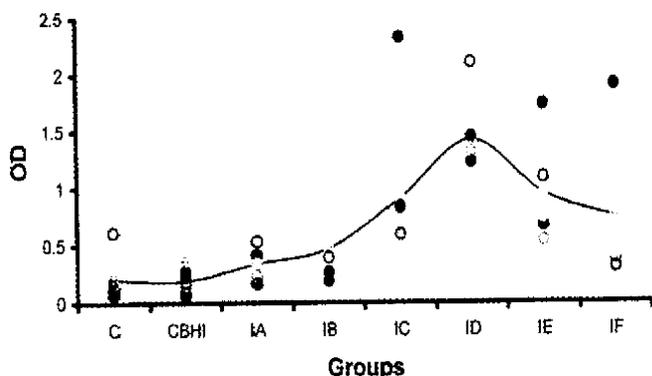
reader (Ceres 900 Bio-Ted).

## RESULTS

After inoculation *H. pylori* was isolated in only one mouse from one week postinfection. The isolate was identified by spiral morphology, Gram negative, urease positive and naphthol-AS-B1-phosphohydrolase, leucine arylamidase and alkaline and acid phosphatase in API ZYM test. No *H. pylori* was detected in control mice and mice inoculated with NaHCO<sub>3</sub> and BHI. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with NaHCO<sub>3</sub> and BHI and 77% (23/30) mice infected with *H. pylori*.

In macroscopic findings, no visible gastric erosion or ulceration was seen in either *H. pylori*-infected mice or control mice.

The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The distribution of OD values and the mean OD values of antibodies against *H. pylori* spiral antigens in different groups were shown in Figure 1. The sera of negative control group or the group inoculated with BHI gave very low absorbance except one mouse in negative control group with an OD of 0.616. The mice showed immune responses to *H. pylori* spiral antigens one week after infection with *H. pylori*. Two weeks postinfection, the mean OD value was doubled than that of negative control group ( $P < 0.05$ ). The peak mean absorbance was four weeks postinfection which showed six times higher than that of negative control group ( $P < 0.01$ ). However, mouse serum antibodies against *H. pylori* spiral antigens decreased gradually 5 weeks postinfection.

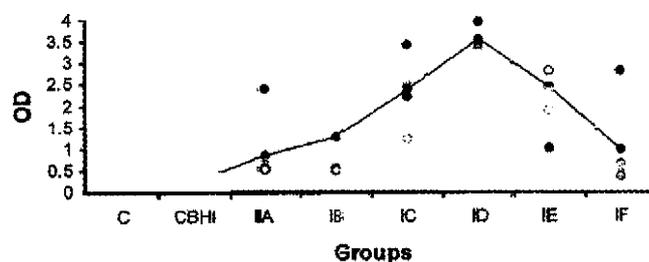


**Figure 1** Distribution of Mouse antibodies against *H. pylori* spiral antigens.

C: negative control. CBHI: mice inoculated with NaHCO<sub>3</sub> and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five week postinfection. IF: six weeks postinfection. The line represents mean OD value.

The mouse antibodies against *H. pylori* coccoid

antigens were also detected. The distribution of OD values and the mean OD values of mouse serum antibodies against *H. pylori* coccoid antigens in different groups were shown in Figure 2. One week postinfection, the antibodies against coccoid antigens could be detected ( $P < 0.05$ ) and were almost 4 times higher than that of control. Four weeks postinfection, mouse serum antibodies against *H. pylori* coccoid antigens in infection group were about 18 times higher than that of control group ( $P < 0.01$ ). The mouse serum antibodies against *H. pylori* coccoid antigens decreased gradually 5 weeks postinfection.



**Figure 2** Distribution of OD of mouse antibodies against *H. pylori* coccoid antigens.

C: negative control. CBHI: mice inoculated with NaHCO<sub>3</sub> and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five weeks postinfection. IF: six weeks postinfection. The line represents mean OD value.

## DISCUSSION

A number of animal models have been developed to provide information on pathogenesis, immunity and therapy for *H. pylori* infection. These include models in nonhuman primates<sup>[7,8]</sup>, gnotobiotic and conventional piglets<sup>[9,10]</sup>. However, there were contradictory reports in murine model study. Karita et al<sup>[11]</sup> detected colonization of *H. pylori* in the germfree athymic and euthymic mice up to 10 weeks after inoculation, but observed temporary colonization in conventional euthymic mice. Cantorna and Balish<sup>[12]</sup> tried in vain to colonize clinical strains of *H. pylori* in the alimentary tract of germfree rodents. Marchetti et al<sup>[13]</sup> were able to detect colonization of bacterium and gastric pathology in specific-pathogen-free mice up to 8 weeks following challenge with fresh clinical isolates whereas a laboratory strain failed to establish infection. Watanabe et al<sup>[14]</sup> successfully demonstrated the long-term infection with *H. pylori* induces adenocarcinoma in Mongolian

gerbils.

In the present study, attempts were made to colonized specific-pathogen-free mice with a fresh *H. pylori* isolate. *H. pylori* was isolated in one mouse. Culture of this fastidious micro-organism is always a challenge with regard to the proper conditions and interfering contaminants which may inhibit the growth of *H. pylori*. This may be the reason that in this study only one *H. pylori* strain was isolated from one mouse. Urease test was reported to be a highly effective detection method especially in the absence of other microflora as in human stomach or germfree animals<sup>[15,16]</sup>. However, this method of detection could be ineffective when used in conventional mice due to the presence of other urease-producing microflora which could lead to false positive results<sup>[17]</sup>. In this study, urease test was found positive in the gastric specimens of 50% (5/10) control mice, 70% (7/10) mice inoculated with NaHCO<sub>3</sub> and BHI and 77% (23/30) mice infected with *H. pylori*. The indiscrimination of urease test results among different experimental groups of mice made it difficult to determine whether positive urease tests were caused by *H. pylori* colonization or contaminating urease producers. The result is in agreement with report of Xia *et al*<sup>[17]</sup> that urease test may not be a suitable method of detection in conventional or specific-pathogen-free mice model.

The serum immune response of mice against *H. pylori* spiral and coccoid antigens was investigated. One week postinfection, the mouse antibodies against *H. pylori* increased. The peak mean OD values of antibodies were four weeks postinfection. Coincidentally, the profile of antibodies against coccoid antigens was similar. It was shown that four weeks postinfection, antibodies against coccoid antigens increased much higher than that of spiral antigens. Could there be more coccoid form than spiral form in the stomach of mice where the environment is not so favourable to the growth of *H. pylori*? Bhatia *et al*<sup>[18]</sup> observed the effect of the presence of *Lactobacillus acidophilus* or its metabolites on inhibition of *H. pylori* growth in *in vitro* culture. The presence of significant number of *Lactobacillus* in gastrointestinal tract of mice might affect *H. pylori* and promote it to convert to coccoid form. The detection of antibodies against *H. pylori* spiral and coccoid antigens is consistent with the observation in patients with gastroduodenal disease that both forms coexist in stomach and could involve in the outcomes of different gastric disorders<sup>[19]</sup>. It was reported that after antibiotic treatment for *H. pylori* infection, if failure happened the majority of patients were recrudescence<sup>[20]</sup>, i.e. the patients re-infected with same strains of *H. pylori*. This relapse occurred

between 5-50 months after treatment, while 4 weeks after treatment those patients showed eradication of *H. pylori* based on microbiological methods. One reason could be that two forms of *H. pylori* coexist in stomach. When the environmental condition is unfavourable to *H. pylori*, most of the cells might convert to coccoid form which is difficult to be detected by commonly used microbiological methods.

In this study we demonstrated coexistence of spiral and coccoid forms of *H. pylori* in experimental mice. This factor should be considered in clinical management since coccoid form might be viable and pathogenic as suggested by some investigators<sup>[3,4]</sup>.

## REFERENCES

- Sipponen P, Hyvarinen H. Role of *Helicobacter pylori* in the pathogenesis of gastritis, peptic ulcer and gastric cancer. *Scand J Gastroenterol*, 1993;28(suppl 196):3-6
- Sorberg M, Nilsson M, Hanberger H, Nilsson LE. Morphologic conversion of *Helicobacter pylori* from bacillary to coccoid form. *Eur J Clin Microbiol Infect Dis*, 1996;15(3):216-219
- Hua J, Ho B. Is the coccoid form of *Helicobacter pylori* viable. *Microbios*, 1996; 87(351):103-112
- Vijayakumari S, Khin MM, Jiang B, Ho B. The pathogenic role of the coccoid form of *Helicobacter pylori*. *Cytobios*, 1995;82(331):251-260
- Glupczynski Y. Culture of *Helicobacter pylori* from gastric biopsies and antibiogram susceptibility test. In: A. Lee and F. Megraud eds. *H. pylori: techniques for clinical diagnosis & basic research*. London: *WB Saunders Company LTD*, 1996:17-28
- Goodwin CS, Blincow E, Petersen S, Sanderson C, Cheng W, Marshall BJ *et al*. Enzyme-linked immunosorbent assay for *Campylobacter pyloridis*: Correlation with presence of *Campylobacter pyloridis* in the gastric mucosa. *J Infect Dis*, 1987;155(3):488-494
- Baskerville A, Newell DG. Natural occurring chronic gastritis and *Campylobacter pylori* infection in the rhesus monkey: a potential model for gastritis in man. *Gut*, 1988;29(4):465-472
- Drazek ES, Dubois A, Holmes PK. Characterization and presumptive identification of *Helicobacter pylori* isolates from rhesus monkeys. *J Clin Microbiol*, 1994;32(7):1799-1804
- Krakowka S, Morgan DR, Kraft WG, Leunk RD. Establishment of gastric *Campylobacter pylori* infection in the neonatal gnotobiotic piglet. *Infect Immun*, 1987;55(11):2789-2796
- Eaton KA, Morgan DR, Krokowka S. Persistence of *Helicobacter pylori* in conventional piglets. *J Infect Dis*, 1990;161(6):1299-1300
- Karita M, Li Q, Cantero D, Okita K. Establishment of a small animal model for human *Helicobacter pylori* infection using germ-free mouse. *Am J Gastroenterol*, 1994;89(2):208-213
- Cantorna MT, Balish E. Inability of human clinical strains of *Helicobacter pylori* to colonize the alimentary tract of germfree rodents. *Can J Microbiol*, 1990;36(4):237-241
- Marchetti M, Arico B, Burrone D, Figura N, Rappouli R, Ghiara P. Development of a mouse model of *Helicobacter pylori* infection that mimic human disease. *Science*, 1995;267(5204):1655-1658
- Watanabe T, Tada M, Nagai H, Sasaki S, Nakao M. *Helicobacter pylori* infection induces gastric cancer in mongolian gerbils. *Gastroenterology*, 1998;115(3):642-648
- Hazell SL, Borody TJ, Lee A. *Campylobacter pyloridis* gastritis I: detection of urease as a marker of bacterial colonization and gastritis. *Am J Gastroenterol*, 1987;82(4):292-296
- Eaton KA, Brooks CL, Morgan DR, Kraakowka S. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun*, 1991;59(7):2470-2475
- Xia HX, Keane CT, O'Morain CA. Pre-formed urease activity of *Helicobacter pylori* as determined by a viable cell count technique clinical implications. *J Med Microbiol*, 1994;40(6):435-439
- Bhatia SJ, Kochar N, Abraham P, Nair G, Mehta AP. *Lactobacillus acidophilus* inhibits growth of *Campylobacter pylori in vitro*. *J Clin Microbiol*, 1989; 27(10):2328-2330
- Chan WY, Hui PK, Leung KM, Chow J, Kwok F, Ng CS. Coccoid forms of *Helicobacter pylori* in the human stomach. *Am J Clin Pathol*, 1994;102(4):503-507
- Hua J, C Birac, Y Glupczynski, A Burette, H Lamouliatte, F Megraud. Differentiation of recrudescence and reinfection during relapse of *Helicobacter pylori*. *Am J Gastroenterol*, 1994;89(8):41

# A cross-sectional study on HGV infection in a rural population \*

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**Subject headings** GB virus-C; hepatitis G virus; non-A, non-B hepatitis; hepatitis B virus; hepatitis C virus; enzyme-linked immunoassay; polymerase chain reaction

## Abstract

**AIM** To determine the epidemiological characteristics and clinical significance of HGV infection, and to compare with HBV and HCV infections.

**METHODS** Anti-HGV, HBsAg, anti-HBs, anti-HBc and anti-HCV were detected by enzyme-linked immunoassays (EIA). Anti-HGV positive sera were further tested for HGV RNA by a nested reverse transcription polymerase chain reaction (RT-nPCR).

**RESULTS** The anti-HGV prevalence rate was 12.9% in the rural population. It was relatively low in children under 10 years of age, and then increased with age and peaked in the group of 50-59 years (29.2%). The Carrier rate of HBsAg was 12.6% in the population and quickly reached the highest (16.2%) in the 5-year age group. The prevalence rate of HBV infection was 64.9%, and rose to a high level in the group of 10 years, and maintained high till up to the top of 79.2% in the 50-59 age group. The HCV infection rate was 15.3%. No Anti-HCV positive cases were found in the group under 10 years of age. It was particularly high in the 20-40 age group, and reached the peak in the group of 30 years old. No significant differences were found in the infection rates of HBV, HCV and HGV between male and female. HGV infection was associated with the history of blood donation and the sexual transmission. The anti-HGV positive rate in

wives of husbands with HGV infection was 53.3%, significantly higher than that in those with anti-HGV negative husbands (7.8%). HGV coinfection with HBV or HCV had no influence on serum alanine aminotransferase (ALT). No ALT elevation was found in the group with HGV infection alone.

**CONCLUSION** The epidemiological characteristics of HGV infection are different from that of HBV and HCV. HGV is transmitted by blood and sex, and does not seem to cause liver damage.

## INTRODUCTION

Since the discovery of hepatitis C virus (HCV) in 1989, 90% of blood-borne non-A, non-B hepatitis cases, acute as well as chronic, are attributed to HCV. The remaining 10%-15% patients with non-A, non-B hepatitis have no evidence of HCV infection, indicating the existence of additional causative agents. GBV-C and HGV were newly discovered putative non-A to E hepatitis viruses reported by two groups of investigators<sup>[1,2]</sup>. However, the sequence homology analysis of the two viruses revealed that they are different isolates of the same virus and tentatively designated GBV-C/HGV. It is a positive single-stranded RNA virus, and has a similar genome organization as the flaviviruses, in particular, hepatitis C virus (HCV), and is classified in the same genus<sup>[3]</sup>. GBV-C/HGV is transmitted mainly through blood or blood products. This study was carried out in a rural population with a high proportion of plasma donors in Zhoukou Area, Henan Province of China, to determine the epidemiological characteristics and clinical significance of HGV infection, and to compare with that of HBV and HCV infection.

## MATERIALS AND METHODS

### Subjects

All 541 registered residents in the village of Zhoukou Area, Henan Province were investigated.

### Data collection

Every resident enrolled in this study received a

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\*Supported by the Ph. D Foundation of Ministry of Education of China.

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Received 1998-08-02

questionnaire including 17 items such as age, sex, hepatitis history, blood donation history, etc., and all questionnaires were filled out by the investigators. Then 3.5ml blood was drawn from each resident and the serum was immediately separated, and stored at  $-20^{\circ}\text{C}$  until tested.

### Laboratory tests

All residents were tested for serum alanine aminotransferase (ALT) levels by Reitman's method; antibody to hepatitis G virus (anti-HGV), hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) and antibody to hepatitis C virus (anti-HCV) by enzyme-linked immunoassays (EIA); and HGV RNA by RT-nPCR. The anti-HGV EIA kit was developed by our laboratory<sup>[4]</sup>; HBsAg, anti-HBs, anti-HBc and anti-HCV EIA kits were produced by Shanghai Ke Hua Co.. RT-nPCR kit for detection of HGV RNA was established by our laboratory<sup>[5]</sup>.

### Diagnostic Criteria

The diagnosis of HGV infection was made on the reactivity of anti-HGV in serum. HBV infection was diagnosed by the presence of one of HBV markers (HBVM) including HBsAg, anti-HBc and anti-HBs. Individuals previously inoculated with HBV vaccines were excluded from the study. HCV infection was diagnosed on the basis of anti-HCV positivity in serum.

### Statistical analysis

Frequency distributions and dichotomous variables were performed using the two-tailed Mantel-Haenszel chi-square test or the two-tailed Fisher's exact test (EPI-INFO software). Logistic regression analysis (SPSS statistical package) was applied to identify the independent variables associated with HGV, HBV or HCV infection. *P* value of less than 0.05 was considered to indicate statistical significance.

## RESULTS

### Age and sex distribution of HGV, HBV and HCV infections

The anti-HGV positive rate was 12.9% in the rural population. Forty-two of 70 anti-HGV positive individuals tested were also HGV RNA positive (60%). The anti-HGV prevalence rate was relatively low in children under 10 years, and then increased with age and peaked in the group of 50-59 years (29.2%). The HBsAg carrier rate was 12.6% in the population, and quickly reached the highest (16.2%) in the 5-year old group. The prevalence rate of HBV infection was 64.9% in the

population. It increased to a high level in the group of 10 years of age, and maintained high up to 79.2% in the 50-59 age group. The anti-HCV positive rate was 15.3% in the population. No anti-HCV positive cases were found in the group under 10 years of age. The anti-HGV prevalence was particularly high in the 20-40 age group, and reached the peak in the group of 30 years. It was 2.2%, 28.2%, 40% and 32.7% in the age groups of 10, 20, 30 and 40 years, respectively, and decreased quickly in the group above 50 years (Table 1).

No significant differences of HBV, HCV and HGV infection rates were found between male and female.

**Table 1** Age distribution of HGV, HBV and HCV infection in the rural population

Age group (yrs)	Cases tested	Anti-HGV(+)		HBsAg(+)		HBVM* (+)		Anti-HCV(+)	
		No.	%	No.	%	No.	%	No.	%
0-	52	1	1.9	7	13.5	25	48.1	0	0.0
5-	74	3	4.1	12	16.2	42	56.8	0	0.0
10-	92	10	10.9	13	14.1	62	67.4	2	2.2
20-	110	12	10.9	12	10.9	68	61.8	31	28.2
30-	70	12	17.1	10	14.3	47	67.1	28	40.0
40-	55	9	16.4	6	10.9	39	70.9	18	32.7
50-	48	14	29.2	10.4	5	38	79.2	2	4.2
60-	40	9	22.5	3	7.5	30	75.0	2	5.0
Total	541	70	12.9	68	12.6	351	64.9	83	15.3

\* One of HBsAg, anti-HBc and anti-HBs positive

### Epidemiological factors of HGV, HBV and HCV infections

Among 17 doubtful factors tested by single factor analysis, the blood donation history, anti-HBs, anti-HBc and HBVM (one of HBsAg, anti-HBs and anti-HBc) were related to HGV infection. The hepatitis history and age were risk factors for HBV infection, while the blood donation history, ALT level and HBsAg were associated with HCV infection. Multifactors were further analyzed using non-conditional logistic regression. Table 2 shows the risk factors correlated with HGV, HBV and HCV infections.

**Table 2** Non-condition logistic regression analysis of HGV, HBV and HCV infections

Markers	Related factors	B value	OR value	P value
Anti-HGV	Blood donation history	0.6759	1.97	<0.05
	Anti-HBc	0.7629	2.14	<0.05
HBsAg	Hepatitis history	1.1079	3.03	<0.05
	Blood donation history	-1.0001	0.37	<0.05
	Anti-HBs	-1.8481	0.16	<0.001
HBVM	Anti-HBc	2.3166	10.14	<0.001
	Hepatitis history	0.9554	2.59	<0.05
Anti-HCV	Blood donation history	5.0103	149.95	<0.001
	Frequency of plasma donation	2.6594	14.29	<0.05
	HBsAg	-2.7363	0.06	<0.01
	ALT level	1.1172	3.06	<0.05

Anti-HGV positive rate was not correlated to the duration and frequency of plasma donation, whereas anti-HCV positive rate was associated with them. The anti-HCV positive rate of individuals with plasma donation more than 1 year (32/37, 86.5%) was significantly higher than that of those with less than 1 year (45/69, 69.2%) or without plasma donation (6/433, 1.4%).

#### **Analyses of HGV, HBV and HCV infection between couples**

Eighty-three couples were divided into two groups: in one group, both wife and husband had blood donation, and in another group, only one or neither

of the couple had blood donations. The results showed that anti-HGV and HBVM positive rates in wives of husbands with anti-HGV or HBVM were significantly higher than in wives of anti-HGV or HBVM negative husbands ( $P<0.001$  and  $P<0.05$ , respectively). However, no significant correlation was found in HCV infection between wives and husbands (Table 3).

#### **Relationship between ALT and HGV, HBV and HCV infections**

The abnormal rate of ALT in the individuals with HCV infection (34.5%) was significantly higher than in those with HGV or HBV infection (0% and 6.6%).

**Table 3 Positive rates of anti-HGV, HBsAg, HBVM and anti-HCV of wives and husbands**

Husbands infection status	Wives anti-HGV positive rate(%)		Wives HBsAg (%)		Wives HBVM (%)		Wives anti-HCV (%)	
	A	B	A	B	A	B	A	B
+	40.0 (2/5)	53.3 (8/15)	0 (0/1)	11.1 (1/9)	60.0 (6/10)	72.0 (36/50)	68.8 (11/16)	11.1 (1/9)
-	8.3 (1/12)	7.8 (4/51)	17.6 (3/17)	8.9 (5/56)	62.5 (5/8)	40 (6/15)	100.0 (4/4)	7.4 (4/54)
Total	17.6 (3/17)	18.2 (12/66)	16.7 (3/18)	9.2 (6/65)	61.1 (11/18)	64.6 (42/65)	75.0 (15/20)	7.9 (5/63)
OR value	7.4	13.4	0	1.3	0.9	3.9		0
$\chi^2$	2.29	16.1	0.20	0.04	0.01	5.09	1.58	0.14
<i>P</i> value	<0.05	<0.001	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05

\*A: Both had blood donations; B: One or neither had blood donations

**Table 4 Relationship between ALT and HGV, HBV and HCV infections**

Group	No. tested	ALT abnormal rate (%)	<i>P</i> value
HBV-HCV-HGV+	11	0.0(0/11)	>0.05
HBV-HCV-HGV-	145	5.5(8/145)	
HBV+HCV-HGV+	46	8.7(4/46)	>0.05
HBV+HCV-HGV-	256	6.6(17/256)	
HBV-HCV+HGV+	5	60.0(3/5)	>0.05
HBV-HCV+HGV-	29	34.5(10/29)	
HBV+HCV+HGV+	8	50.0(4/8)	>0.05
HBV+HCV+HGV-	41	43.9(18/41)	

#### **DISCUSSION**

The enzyme-linked immunoassay (EIA) for detection of anti-HGV used in this study was established by our laboratory. The total coincidence rate between HGV RNA RT-nPCR and anti-HGV EIA kits was 94%, as reported previously<sup>[4]</sup>. So the

anti-HGV positive rate determined by anti-HGV EIA reflects the actual status of HGV infection in the population. The epidemiological characteristics and risk factors of HGV infection in the population appear to be different from that of HBV and HCV.

The anti-HGV positive rate was 12.9% in the

population, significantly higher than that in the general population of China<sup>[6]</sup>. The HBsAg carrier rate and the prevalence of HBVM in this rural population were 12.6% and 64.9%, respectively, which were similar to that of the general population in China reported by Liu *et al*<sup>[7]</sup>. However, the anti-HCV positive rate of this population (15.3%) was much higher as compared with the general population of the country (15.3% *vs* 3.2%)<sup>[8]</sup>. The high prevalence of HGV and HCV infections may be associated with the high proportion (19.6%) of plasma donors in the rural population.

The age distributions of HGV, HBV and HCV infections were different. The anti-HGV positive rate was relatively low in children under 10 years, and then increased with age and peaked in the group of 50-59 years (29.2%). However, the HBsAg carrier rate quickly reached the highest (16.2%) in the 5-year age group, and the prevalence rate of HBVM increased to a high level in the group of 10 years of age, and maintained high up to 79.2% in the 50-59 age group. HCV infection in the population had a special pattern of age distribution different from HGV and HBV. It mainly concentrated in groups of 20, 30 and 40 years of age, with the prevalence rates of 28.2%, 40.0% and 32.7%, respectively. No anti-HCV positive cases were found in the groups under 10 years of age. The high-prevalence rate of HCV infection in the groups of 20-40 years was related to the high proportion of plasma donors among them.

The anti-HGV positive rate in wives of husbands with HGV infection was 53.3%, significantly higher than that in those with HGV negative husbands (53.3% *vs* 7.8%). The same

phenomenon is also seen in HBV infection. The prevalence rate of HBVM in wives of husbands with HBV infection was significantly higher as compared with those of husbands without HBVM (72% *vs* 40%). Although the anti-HCV positive rate in Wives of husbands with HCV infection was relatively higher than that in those of anti-HCV negative husbands (11.1% *vs* 7.4%), but there was no statistical significance. The data demonstrated that the sexual transmission of HGV and HBV seems to be more important as compared with HCV.

The ALT abnormal rate in the individuals with HCV infection alone was significantly higher than that in those with HGV or HBV infection alone (34.5% *vs* 0% or 5.6%). It is interesting to note that the ALT levels in HBV patients with or without HGV coinfection had no difference<sup>[6]</sup>. It suggests that HGV, unlike HBV and HCV, may not cause the liver damage<sup>[2,9,10]</sup>.

## REFERENCES

- 1 Simons JN, Leary TP, Dawson GJ. Isolation of novel virus-like sequences associated with human hepatitis. *Nature Medicine*, 1995;6:564
- 2 Linnen J, Wages J, Zhang-Keck ZX. Molecular cloning and disease association of hepatitis G virus: A transfusion-transmissible agent. *Science*, 1996;27:505
- 3 Alter HJ, Bradley DW. Non-A, non-B hepatitis unrelated to the hepatitis C virus (non-ABC). *Semin Liver Dis*, 1995;15:110
- 4 Wang XT, Zhuang H, Li HM. Evaluation of peptide antigens for detection of antibodies to GBV-C. *Chin J Microbiol Immunol*, 1997;6:392
- 5 Ling BH, Zhuang H, Wang XT. Development and application of a reverse transcription nested polymerase chain reaction for detection of HGV RNA. *Chin J Hepatol*, 1998;6:21
- 6 Ling BH, Zhuang H, Li SH. HGV infection in different populations and patients with various liver diseases of China. *China Public Health*, 1998;14:145
- 7 Liu CB, Xu ZY, Cao HL. A study on seroepidemiology of hepatitis B virus infection. *Chin J Virol*, 1991;7(Suppl):8
- 8 Guo CS, Wei WJ. Prevention of hepatitis C. *Chin J Prev Med*, 1993;6:325
- 9 Alter HJ. The cloning and clinical implications of HGV and HGBV-C. *New Engl J Med*, 1996;23:1536
- 10 Masuko K, Mitsui T, Iwano K. Infection with hepatitis GB virus C in patients on maintenance hemodialysis. *New Engl J Med*, 1996;23:1485

# Persistence of hepatitis B vaccine immune protection and response to hepatitis B booster immunization\*

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**Subject headings** hepatitis B vaccines; immune protection persistence; booster; immunization

## Abstract

**AIM** To identify the persistence of immune protection of China-made, plasma-derived hepatitis B vaccine after infancy immunization and the time table of booster immunization.

**METHODS** A cross-sectional follow-up study and an experimental study on booster were used for the evaluation of the serological effect 7 years after vaccination and the antibody anamnestic response. Radioimmunoassay was used for the detection of hepatitis B virus markers.

**RESULTS** The protective anti-HBs positive rates of 1018 children, who were vaccinated according to the regimen of three doses of 10 µg hepatitis B vaccine in their infancy, declined from 75.0% during the first two years to 48.2% in the 7th year after the first dosage, however, the positive rates for HBsAg and anti-HBc always fluctuated at a low frequency. A total of 144 subjects aged 6 or 7 years, who were negative for both HBsAg and anti-HBc before booster, were selected from 1018 children of the follow-up study, and boosted with 1µg intradermally or 2µg hypodermically hepatitis B vaccines. Their anti-HBs GMT and anti-HBs positive rates were 190.6mIU/ml and 89.6% in the first month after booster, significantly higher than 14.7mIU/ml and 54.9% before booster ( $P < 0.01$ ), and declined back to 25.3mIU/ml and 75.5% in the

12th month; among 65 children with the anti-HBs negative before booster, 40 had a level of anti-HBs  $\geq 100$ mIU/ml one month after booster, suggesting retention of immune memory in most of them.

**CONCLUSION** No need for revaccination against hepatitis B in the 7th year after the initial immunization due to better persistence of immune protection of the vaccine and retention of immune memory to hepatitis B virus in the vast majority of the vaccinees.

## INTRODUCTION

Infant hepatitis B vaccine immunization integrated with EPI program has become a principal strategy for the hepatitis B control. Since the end of the 1980s, large-scale hepatitis B vaccination in infants has been implemented in the many areas of China<sup>[1]</sup>. The short-term effectiveness of hepatitis B vaccine has been confirmed in many studies<sup>[1-4]</sup>. The low-dose immunization has been recommended as a principal strategy to infancy vaccination of the rural areas<sup>[5]</sup>. However, it is necessary to answer the following questions in community-based hepatitis B prevention: what is the persistency of immune protection of China-made, plasmaderived hepatitis B vaccine, especially in the infancy should low-dose immunization be used? Is there antibody anamnestic reaction to hepatitis B surface antigen (HBsAg) in the vaccinees with vaccine-induced antibody negative-conversion When should the booster immunization be administered? In order to determine the duration of immune protection and the immune memory to HBsAg 7 years after the infancy vaccination, a follow-up study on the long-term effectiveness of hepatitis B vaccination and an experiment study of hepatitis B booster immunization were carried out in Longan County, a remote hepatitis B endemic rural area of China, from 1994 to 1995.

## MATERIALS AND METHODS

### Sample size and subjects

A total of 1018 children aged 1-7 years, born in the period of 1987 to 1994 in Longan County and

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\*Supported by the China Medical Board, New York, Inc., Grant No.93-582.

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Received 1998-11-09

having the vaccination record of three doses of 10 $\mu$ g plasma-derived hepatitis B vaccine (produced by the National Institute for Biological Products, Beijing) according to 0, 1 and 6 month schedule, were selected in terms of cluster sampling as a sample for the observation of long-term effectiveness. Among them 144 children were recruited as subjects for the experiment of booster immunization.

#### Method and dosage of booster

The 144 subjects were divided into two groups. One group of 91 subjects were intradermally injected with a dose of 1 $\mu$ g plasma-derived hepatitis B vaccine, another group of 53 subjects were hypodermically immunized with a dose of 2 $\mu$ g vaccine.

#### Reagents

Hepatitis B radioimmunoassay (RIA) reagent kits were purchased from the National Institute of Biological Products, Beijing.

#### Specimens collection and lab test

Peripheral blood of 3ml-5ml was collected in all samples in April 1994, and from all subjects at 1- and 12 month after the booster, respectively. Serum specimens were kept at -20°C for the test. RIA was used for the detection of anti-HBs, anti-HBc and HBsAg. Anti-HBs-S/N ratio  $\geq 10.0$ , anti-HBc inhabitation ratio  $\geq 75\%$  and HBsAg S/N ratio  $\geq 2.1$  were defined as sera positive. Both scales, anti-HBs mIU/ml GMT and anti-HBs positive rate, were used for comparison of the differences of antibody level between before and after booster, and between different doses of booster vaccine. The following formula was used for calculating anti-HBs mIU/ml:

$$\text{mIU/ml} = 130.75 \left[ \text{EXP} \left( 0.66765 \times \frac{\text{CPM of sample} - \text{CPM of negative control}}{\text{CPM of positive control} - \text{CPM of negative control}} \right) - 1 \right]$$

#### Data analysis

Softwares, dBase-III and SAS, were used for the data base and the statistical analysis.

## RESULTS

### Positive rates for anti-HBs, anti-HBc and HBsAg 1-7 years after immunization

The age distribution of positive rates for anti-HBs, anti-HBc and HBsAg of 1018 immunized children aged 1-7 years after infancy hepatitis B vaccination is shown in Table 1.

Table 1 shows that the anti-HBs positive rate significantly declined from 75.0% of age group of 1-2 years to 48.2% of 7-year age group ( $X^2=51.2$ ,  $P<0.01$ ), while anti-HBc and HBsAg positive rates were not found significantly increased with age ( $P>0.05$ ). The results suggested that the hepatitis B vaccine induced-antibody level in infancy immunization was decreasing year by year after vaccination, however, the difference of hepatitis B virus (HBV) infectious rate between age groups was not statistically significance.

### Change of anti-HBs before and after hepatitis B vaccine booster

The results of comparison of anti-HBs level change of 144 subjects before and in the first and 12th month after booster are shown in Table 2.

Anti-HBs GMT of 144 subjects one month after booster was significantly higher (by 18.3 fold) than that before booster ( $t = 17.4$ ,  $P < 0.01$ ). However, in the 12th month after booster, the anti-HBs GMT of 106 subjects dropped significantly, and there was no difference before and after booster ( $t=1.3$ ,  $P>0.05$ ); and the anti-HBs positive rates were 89.6% in the 1st month and 75.5% in the 12th month, significantly higher than (54.9%) before booster ( $P<0.05$ ). The antibody positive rates of both subgroups with low anti-HBs titer (10mIU/ml-99mIU/ml) and the subjects with negative anti-HBs (<10.0mIU/ml) in the first month after booster were significantly lower than before ( $P<0.01$ ), increasingly recovering in the 12th month.

### Relationship of anti-HBs level before and after booster

Anti-HBs level distribution after booster among the subjects with different antibody level before booster is shown in Table 3.

**Table 1 Positive rates for anti-HBs, anti-HBc and HBsAg of 1018 children aged 1-7 years after infancy hepatitis B immunization in Longan County in 1994**

Age group (yr)	No. of subjects	Anti-HBs( $\geq 10S/N$ )		Anti-HBc( $\geq 75\%$ )		HBsAg( $\geq 2.1S/N$ )	
		n	%	n	%	n	%
1-2	220	165	75.0	1	0.5	2	0.9
3-4	341	178	52.2	13	3.8	9	2.6
5-6	320	144	45.0	8	2.5	7	2.2
7	137	66	48.2	8	5.8	1	0.7
Total	1018	553	54.3	30	3.0	19	1.9

**Table 2 Comparison of anti-HBs level before and after hepatitis B booster in 144 subjects immunized with hepatitis B vaccine**

Time point of observation	No. of subjects	Anti-HBs (mIU/ml)								GMT	<i>t</i>	<i>P</i>
		<10		≥10		≥100		≥1000				
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%			
Before booster	144	65	45.1	58	40.3	20	13.9	1	0.7	10.4		
One month after booster	144	15	10.4	16	11.1	100	69.4	13	9.0	190.6	17.4	<0.01
12 months after booster	106	26	24.5	43	40.6	33	31.1	4	3.8	25.3	1.3	>0.05

\*blood specimens only collected from 106 children in the 12th month after booster.

**Table 3 Distribution of anti-HBs levels one and twelve months after booster among immunized children with different anti-HBs levels before booster**

Anti-HBs (mIU/ml) before booster	No.	Anti-HBs levels (mIU/ml) one month after booster								Anti-HBs levels (mIU/ml) 12 months after booster								
		<10		≥10		≥100		≥1000		<10		≥10		≥100		≥1000		
		<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
<10	65	15	23.1	10	15.4	35	53.9	5	7.7	47	22	46.8	14	29.8	9	19.1	2	4.3
≥10	58	0	0.0	5	8.6	49	84.5	4	6.9	45	4	8.9	24	53.4	15	33.3	2	4.4
≥100	20	0	0.0	1	5.0	15	75.0	4	20.0	13	0	0.0	5	38.5	8	61.5	0	0.0
≥1000	1	0	0.0	0	0.0	1	100.0	0	0.0	0	0	0.0	0	0.0	1	100.0	0	0.0

One month after booster 76.9% of 65 subjects with negative anti-HBs before booster, had anti-HBs ≥10mIU/ml; 91.4% of 58 subjects with anti-HBs low titer possessed anti-HBs ≥100mIU/ml; in 20 of 21 subjects with anti-HBs ≥100mIU/ml antibody increased obviously and decreased in one. Fifteen of 65 subjects with anti-HBs <10mIU/ml before booster, were still anti-HBs negative in the first and 12th month after booster, accounting for 23.1%, in 10 children anti-HBs level increased from 10mIU/ml to 100mIU/ml and in the remaining 40 subjects it was 100mIU/ml or over. Thirty-two individuals of the latter two groups were followed up for 12 months, 7 had antibody negative conversion.

Tables 2 and 3 indicate that most of immunized children possessed immune memory to HBsAg, and also in some individuals who had the vaccine-induced anti-HBs negative-conversion, 6 or 7 years after their infancy immunization.

#### Comparison of anti-HBs level between two booster dosages with different immunization routes

Anti-HBs GMTs of 91 subjects of intradermal 1μg group and 53 subjects of hypodermical 2μg group before booster were 9.3 mIU/ml and 11.3mIU/ml, and no statistically significant difference was found between the two groups ( $t = 0.6, P > 0.05$ ). One month after booster, anti-HBs GMTs of both groups increased to 160.8mIU/ml and 247.2 mIU/ml. However, there was no statistical difference between both groups ( $t = 1.3,$

$P > 0.05$ ). The difference of booster-induced antibody levels between the intradermal group and the hypodermical group was not found in this study.

#### DISCUSSION

The observation should be conducted from two aspects to study the persistence of hepatitis B immunization: ① the trend of change of anti-HBs, anti-HBc and HBsAg after vaccination in the given immunized populations; ② to clarify whether the immunized individuals possess immune memory in the certain period after vaccination. The evidence of their immunological anamnestic reaction to HBsAg can be provided through a booster experiment.

The results of our study in Longan County showed that the anti-HBs positive rate of the immunized children was 48.2% in the 7th year, lower than 75.0% during the first two years after infancy hepatitis B immunization, suggesting that the hepatitis B vaccine-induced protective antibody level was gradually decreasing; however, the positive rates for anti-HBc and HBsAg were not significantly increased with the time after vaccination, but obviously lowered than before immunization. The following two explanations might be used for this phenomenon: ① an assumption that the opportunity exposed to HBV was obviously decreased in the immunized population. In recent years the large-scale infant hepatitis B vaccination did significantly decrease the HBsAg carrier rate among the children aged under 5 years<sup>[2-4]</sup>, while, the HBsAg carrier rate in the

older-age population did not decrease, shown in our another study on the population aged 20-30 years without hepatitis B vaccination in Longan County in 1995. ② A part of immunized population were found to have vaccine-induced anti-HBs negative-conversion, but they might still have immune memory to HBsAg. If these children expose to HBV, they will quickly develop enough protective antibody to avoid becoming a HBsAg carrier. The second explanation has been confirmed through a hepatitis B vaccine booster experiment in our study.

The results of the booster experiment indicated that 61.5% (40/65) of 65 subjects with anti-HBs, anti-HBc and HBsAg negative marker, yielded anti-HBs level of  $\geq 100$  mIU/ml one month after a low dose of hepatitis B vaccine booster, and the post-booster antibody increase of these children was referred to immunological anamnestic reaction, according to the standard that the anamnestic reaction was defined as subjects with the anti-HBs-negative yielding anti-HBs level of  $\geq 100$  mIU/ml four weeks after booster<sup>[6]</sup>. Therefore, the reason why the HBsAg positive rate of immunized population always fluctuated at a low level of around 2% was probably attributable to the fact that they still keep immune memory 6-7 years after the initial vaccination. In 50 of 65 subjects with the anti-HBs and HBsAg-negative the antibody increased obviously after booster. It is interesting that 21.9% (7/23) of those children with anamnestic reaction had antibody negative-conversion in the 12th month after booster, and 23.1% (15/65) of subjects were anti-HBs negative at in the first and 12th month after and before booster. The outcome when these two groups of children expose to HBV should be observed in the future. Of 79 subjects with antibody level of  $\geq$

10 mIU/ml, 68 (86.1%) had antibody level increased by 2-fold or more one month after booster, suggesting that a low dose of hepatitis B vaccine booster can induce extremely high titer of antibody in most of these children.

The results of our research are similar to that of a study on booster 4-5 years after infancy hepatitis B vaccination by Chen Hui-Fang<sup>[7]</sup>. Both studies reveal that the majority of children immunized with China-made, plasma-derived hepatitis B vaccine, can quickly produce antibody anamnestic reaction to HBV (titer  $\geq 100$  mIU/ml) 4-7 years after infancy.

These evidences indicate that low dose of China-made, plasma-derived hepatitis B vaccine in infancy may yield a better persistency of immunization and an ideal protective effect in immunized population. It is suggested that no need for revaccination against hepatitis B in the 7th year after the initial immunization, due to no evidences of booster obtained in our study.

#### REFERENCES

- 1 Xu ZY, Liu CB, Yan TJ, Sha QH, Sun YD, Fu TY *et al*. Evaluation of effectiveness of large-scale hepatitis B vaccination in neonates. *Chin J Virol*, 1991;7(Suppl.):48-52
- 2 Chotard J, Inskip HM, Hall AJ, Loik F, Mendy M, Whittle H *et al*. The Gambia Hepatitis B Intervention Study: follow-up of a cohort of children vaccinated against hepatitis B. *J Infectious Dis*, 1992;166(4):764-768
- 3 Tsen YJ, Chang MH, Hsu HY, Lee ChY, Sung JL, Chen DS. Seroprevalence of hepatitis B virus infection in children in Taipei, 1989: five years after a mass hepatitis B vaccination program. *J Med Virol*, 1991;34(2):96-99
- 4 Wainwright RB, McMahon BJ, Bulkow LR, Hall DB, Fitzgerald MA, Harpster AP *et al*. Duration of immunogenicity and efficacy of hepatitis B vaccine in a yupik Eskimo population. *JAMA*, 1989;261(16):2362-2366
- 5 Xu ZY, Xi LF, Liu CB, Cao HL. Strategies for hepatitis B vaccination in neonates: a cost-benefit analysis. *Chin J Virol*, 1991;7(Suppl.): 53-55
- 6 Aoki SK, Finegold D, Kuramoto IK, Douville C, Richards C, Randell R *et al*. Significance of antibody to hepatitis B core antigen in blood donors as determined by their serologic response to hepatitis B vaccine. *Transfusion*, 1993;33(5):362-367
- 7 Chen HF, Guo ZhY, Zhang YJ, W ZhH, Yang Jzh, Tao ZhH *et al*. Long-term efficacy of hepatitis B vaccine in newborn and revaccination study. *Chin J Epidemiol*, 1994;15(2):76-79

# Effect of lipid on proliferation and activation of rat hepatic stellate cells (I)

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**Subject headings** Hepatic stellate cell; triglyceride; very low-density lipoprotein; cell proliferation

## Abstract

**AIM** To study the effect of lipid (triglyceride and very low-density lipoprotein, VLDL) on proliferation and activation of rat hepatic stellate cells (HSC).

**METHODS** HSC were isolated and cultured from liver of Wistar rats by in situ perfusion with pronase and collagenase and density gradient centrifugation with Nycodenz. HSC proliferation was examined with MTT colorimetric assay.

**RESULT** Triglyceride of 12.5 mg/L had a promoting effect on proliferation of HSC ( $P < 0.05$ ), 25, 50, 100 and 200 mg/L had no effects ( $P > 0.05$ ), but 400 mg/L had an inhibiting effect ( $P < 0.01$ ). VLDL of 6.25 and 12.5 mg/L had no effect on proliferation of HSC ( $P > 0.05$ ), but increased concentration of VLDL could promote the HSC proliferation ( $P < 0.05$ ).

**CONCLUSION** Lipid had an effect on proliferation of HSC. Triglyceride and VLDL may promote HSC proliferation and may be associated with fatty liver and hepatic fibrogenesis.

## INTRODUCTION

Despite the early controversy over the primary cellular source of extracellular matrix proteins in liver fibrosis, compelling *in vitro* and *in vivo* experimental evidence is now available to implicate activated hepatic stellate cells (HSC) in the pathogenetic role<sup>[1-3]</sup>. Mechanisms by which collagen-producing cells are activated under pathological conditions remain unknown and continue to be a topic of research interest<sup>[1,2,4,5]</sup>. Although the role of hepatocytes in lipid metabolism and transportation have been discussed intensively, that of HSC was not known. More and more studies showed that HSC took part in lipid metabolism and transportation<sup>[6]</sup>, their abnormality was related to the pathogenesis of fatty liver and liver fibrosis<sup>[7]</sup>. In order to seek a possible explanation for the role of lipid in activation of HSC in liver fibrogenesis, we observed the effects of triglyceride and very low-density lipoprotein (VLDL) on proliferation of rat HSC.

## MATERIALS AND METHODS

### Animals

Male Wistar rats, weighing 400g-450g were fed ad libitum with standard rodent chow.

### Preparation of HSC

HSCs were prepared by the methods of Friedman *et al*<sup>[8]</sup> and Baroni *et al*<sup>[9]</sup> with slight modifications. The rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg). The liver was perfused in situ through the portal vein with 500ml of calcium-free Gey's balanced salt solution for 10min at a flow rate of 40ml/min-50ml/min and then enzymatically digested with perfusate containing 0.05% collagenase (Sigma) and 0.1% pronase E (Merck) for 10min-15min. After being removed, the liver was cut into small pieces and incubated in 50ml fresh GBSS-BSA containing 0.05% collagenase and 0.1% pronase E and stirred at 37°C for 30min. After passing through gauze, cell suspension was centrifuged at 500×g for 7 min, the supernatant discarded at the cells washed twice further with Dulbecco's modified Eagle's medium (DMEM) (Gibco). The cell suspension was mixed with 18% (W/V) of Nycodenz (Sigma) in GBSS without NaCl. The gradient was centrifuged at 1450

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\*Project supported by the National Natural Science Foundation of China, No. 39670339.

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Received 1998-09-07

×g for 17min at 4°C. The white, diffuse, fluffy band in the lower region of the DMEM layer just above the Nycodenz cushion, which contained highly enriched HSC, was gently aspirated, diluted in about 30ml DMEM and centrifuged at 450×g for 7min at 4°C. The cell pellet was suspended in the incubation medium and seeded in the culture flasks with DMEM containing 20% fetal calf serum. Sterile condition was maintained during the entire isolation and purification procedures. Cell viability was assessed by trypan blue exclusion, and cell counting were conducted in a hemocytometer.

#### Culture and determination of HSC<sup>[8,9]</sup>

Purified HSC suspended in DMEM containing HEPES (15mmol), penicillin (100U/ml), streptomycin (100 mg/ml) and fetal calf serum (20% V/V) were seeded at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> in the culture flasks. The medium was changed 20 to 24 hours after plating, and every 3 to 4 days thereafter. Cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. To evaluate the purity of the cultures, HSC at day 2 or 3 and 7 after plating were tested by immunohistochemistry staining for desmin, lysozyme and factor VIII-related antigen, and by ultraviolet excited fluorescence microscopy at the length of 328 nm.

#### Proliferation of HSC

HSC proliferation was studied by colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma) assay<sup>[10]</sup>. HSC ( $1 \times 10^5$  cells/100 μl) were cultured in multiwell tissue culture plates (96 well/plate) (Corning, New York, NY, USA) for 2 days and 48hrs in the presence or absence of triglyceride and VLDL. Twenty μl of MTT solution (5mg/L) was added to all wells of an assay, and plates were incubated at 37°C for 4hrs. Then 100μl dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 20min-30min at room temperature to ensure that all crystals were dissolved, the plates were read on a E-Liza Mat-300 reader, using a test wavelength of 570nm, a reference wavelength of 630nm. Plates were normally read within 1hr of adding the dimethyl sulfoxide. All samples were analyzed in pentuplicate.

#### Statistical analysis

All data were expressed as mean ±SD. Statistical differences were assessed by the standard *t* test and *P* values of <0.05 were judged to be statistically significant.

#### RESULTS

##### The yield, viability and purity of HSC

The yield of HSC ranged from  $5 \times 10^6$  to  $1 \times 10^7$  cells per liver. HSC displayed fading green-blue fluorescence with fluorescence microscopy at a wave length of 328nm. After the first washing at 24hr, the HSC purity in culture exceeded by 95% as assessed by phase contrast microscopy and ultraviolet excited fluorescence microscopy. Viability of HSC assessed by trypan blue exclusion exceeded by 95%. HSC positive for desmin by immunohistochemistry in primary culture was above 90%, and in subculture above 95%. Lysozyme and factor VIII-related antigen were negative for HSC.

##### Effect of triglyceride on proliferation of rat HSC

Triglyceride was directly administered to HSC at concentrations of 12.5, 25, 50, 100, 200 and 400mg/L, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation in the presence of triglyceride is shown in Table 1. Compared with the contrast ( $0.1395 \pm 0.0276$ ), 12.5 mg/L of triglyceride had a promoting effect on proliferation of HSC ( $P < 0.05$ ), 25, 50, 100 and 200mg/L had no effects ( $P > 0.05$ ), but 400mg/L had an inhibiting effect ( $P < 0.01$ ).

**Table 1 The effects of triglyceride on HSC proliferation**

Group	Concentration (mg/L)	Value of OD
Triglyceride	400	$0.0990 \pm 0.0163^b$
	200	$0.1226 \pm 0.0138$
	100	$0.1212 \pm 0.0275$
	50	$0.1450 \pm 0.0264$
	25	$0.1637 \pm 0.0243$
	12.5	$0.1894 \pm 0.0316^a$
Normal control		$0.1395 \pm 0.0276$

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs normal control.

##### Effect of VLDL on proliferation of rat HSC

VLDL was directly administered to HSC at concentrations of 6.25, 12.5, 25, 50 and 100 mg/L of VLDL, respectively. The HSC proliferation was measured with colorimetric MTT assay. HSC proliferation is shown in Table 2. Compared with the contrast ( $0.1395 \pm 0.0276$ ), 6.25 and 12.5mg/L of VLDL had no effect on proliferation of HSC ( $P > 0.05$ ), but 50 and 100mg/L of VLDL could promote HSC proliferation ( $P < 0.05$  or  $P < 0.01$ ).

**Table 2 The effects of VLDL on HSC proliferation**

Group	Concentration (mg/L)	Value of OD
VLDL	100	0.2202±0.0284 <sup>b</sup>
	50	0.1964±0.0287 <sup>b</sup>
	25	0.1834±0.0498 <sup>a</sup>
	12.5	0.1642±0.0269
	6.25	0.1583±0.0314
Normal control		0.1395±0.0276

<sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.01 vs normal control.

## DISCUSSION

There is now overwhelming evidence that HSC, which reside in the space of Disse, are the principal effectors of hepatic fibrogenesis. However, in contrast to most other conditions, it is far from clear whether the characteristic phenotypic transformation, proliferation and fibrogenesis by HSC is always a response to liver cell injury/or inflammation.

HSCs were demonstrated to present lipoprotein receptor in their surface, contained a large amount of fat droplets in their cytoplasm, and synthesized and secreted apolipoproteins. HSCs were in direct contact with plasma in the Disse's space, accessible to both chylomicron and VLDL triglyceride, and do not need to transport the enzyme to the adjacent endothelium. All these suggested that HSC and hepatocytes were necessary for lipid mobilization and transportation<sup>[1,2]</sup>. Their abnormality was related to the disturbance of lipid mobilization and transportation which made triglyceride concentrate in the liver, inducing fatty liver and liver fibrosis<sup>[12]</sup>. Reeves *et al.*'s study<sup>[7]</sup> showed that the mechanism of HSC activation/proliferation and subsequent fibrogenesis related to alcohol may be unrelated to necroinflammation, and that HSC activation occurred in the absence of hepatitis in alcoholic liver disease and correlated with the severity of steatosis. However, isolated fatty liver without hepatitis occurs more frequently than does steatohepatitis. Thus it is not clear whether the accumulation of fat in the liver is responsible for inflammation or whether inflammation evoked by some stimulus caused cell dysfunction that resulted in steatosis<sup>[12]</sup>. Vicente *et al.*<sup>[11]</sup> studied the lipid metabolism during *in vitro* induction of the lipocyte phenotype in HSC. HSC can produce and bind lipoprotein lipase to their own surface, using it potentially for processing exogenous lipids in the acute phase of lipocyte induction, when the accumulation of lipids was accelerated. In the our

study, we found that triglyceride and VLDL promoted HSC proliferation at certain concentrations. Some animal studies also confirmed that steatosis and collagen content in experimental liver cirrhosis are affected by dietary monounsaturated and polyunsaturated fatty acids<sup>[12,13]</sup>. The great amount of lipid in diet produced fatty liver and liver fibrosis more easily. These experimental evidence indicated that the infiltration of lipid increased the production of fibrous tissue in the liver. The earliest event in the development of fibrosis appears to be activation of HSC by such factors as lipid peroxides<sup>[14,15]</sup>. This caused proliferation of HSC and initiation of fibrogenic cascade in the liver. In addition, the investigation showed that high concentration of triglyceride (400mg/L) had an inhibiting effect on HSC proliferation. It was deduced that high concentration of triglyceride might have a toxic effect on HSC. The precise role of triglyceride and VLDL in the HSC proliferation and activation needs to be addressed in further studies.

## REFERENCES

- Alcolado R, Arthur MJ, Iredale JP. Pathogenesis of liver fibrosis. *Clin Sci*, 1997;92(2):103-112
- Pinzani M. Novel insights into the biology and physiology of the Ito cell. *Pharmac Ther*, 1995;66(4):387-412
- Tsukamoto H, Cheng S, Blaner WS. Effects of dietary polyunsaturated fat on ethanol induced Ito cell activation. *Am J Physiol*, 1996;270 (*Gastrointest Liver Physiol* 33):G581-G586
- Friedman SL. Molecular mechanisms of hepatic fibrosis and principles of therapy. *J Gastroenterol*, 1997;32(3):424-430
- Hautkeete ML, Geerts A. The hepatic stellate (Ito) cell: its role in human liver disease. *Virchows Arch*, 1997;430(3):195-207
- Ramadori G, Rider H, Theiss F, Meyer zum Buschenfelde K-H. Fat-storing (Ito) cells of rat liver synthesize and secrete apolipoproteins: comparison with hepatocytes. *Gastroenterology*, 1989;97(1):163-172
- Reeves HL, Burt AD, Wood S, Day CP. Hepatic stellate cell activation occurs in the absence of hepatitis in alcoholic liver disease and correlates with the severity of steatosis. *J Hepatol*, 1996;25(4):677-683
- Friedman SL, Roll FJ. Isolation and culture of hepatic lipocytes, kupffer cells, and sinusoidal endothelial cells by density gradient centrifugation with stractan. *Anal Bioch*, 1987;161(2):207-218
- Baroni GS, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R *et al.* Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology*, 1998;27(3):720-726
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, 1983;65(1):55-63
- Vicente CP, Guaragna RM, Borojevic R. Lipid metabolism during *in vitro* induction of the lipocyte phenotype in hepatic stellate cells. *Mol Cell Biochem*, 1997;168(1):31-39
- Sheth SG, Gordon FD, Chopra S. Nonalcoholic steatohepatitis. *Ann Intern Med*, 1997;126(2):137-145
- Fernandez MI, Torres MI, Gil A, Rios A. Steatosis and collagen content in experimental liver cirrhosis are affected by dietary monounsaturated and polyunsaturated fatty acids. *Scand J Gastroenterol*, 1997;32(3):350-356
- Leonarduzzi G, Scavazza A, Biasi F, Chiarotto E, Comandola S, Vogl S *et al.* The lipid peroxidation end product 4-hydroxy-2, 3-nonenal up-regulates transforming growth factor  $\beta$ 1 expression in the macrophage lineage: a link between oxidative injury and fibrosclerosis. *FASEB J*, 1997;11(11):851-857
- Montosi G, Garuti C, Lannone A, Pietrangelo A. Spatial and temporal dynamics of hepatic stellate cell activation during oxidant-stress-induced fibrogenesis. *Am J Pathol*, 1998;152(5):1319-1326

# ***In situ* hybridization assay of androgen receptor gene in hepatocarcinogenesis \***

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**Subject headings** hepatocarcinoma; androgen receptor; *in situ* hybridization; liver neoplasm, experimental

## **Abstract**

**AIM** To determine the correlation between expression of androgen receptor (AR) gene and hepatocarcinogenesis.

**METHODS** Male SD rats were used as experimental animals and the animal model of experimental hepatocarcinoma was established by means of 3'-me-DAB administration. Androgen receptor mRNA was detected by a non-radioactive *in situ* hybridization assay in neoplastic and non-neoplastic liver tissues.

**RESULTS** The expression of androgen receptor mRNA was observed only in neoplastic cells and some atypical hyperplastic cells. In the liver tissue of control animal and the remaining normal liver cells adjacent to the carcinoma tissue, no positive signal was seen.

**CONCLUSION** Androgen has an important correlation with hepatocarcinogenesis and the expression of androgen receptor gene might be a mark event during hepatocarcinogenesis.

## **INTRODUCTION**

It is well known that sex hormone plays an important role in regulation of cell growth, organ development and carcinogenesis. But the most majority of studies were focused on the tumors of sex hormone-dependent organs (e.g., mammary carcinoma and prostate carcinoma). By now not much attention has been paid to sex hormone effects on hepatocarcinogenesis. However, some data showed that there is a difference in morbidity and mortality of patients with hepatocarcinoma between male and female<sup>[1,2]</sup>. Hepatic adenomas have been also demonstrated to have a clear relationship with oral contraceptive use, and it was presumed that there may be hormone receptors within the adenoma cells that mediate tumor growth in response to hormonal stimulation<sup>[3]</sup>. In addition, some reported that testosterone stimulated tumor growth and it may enhance the progression of chemically-induced hyperplastic nodules to frank malignancy<sup>[4]</sup>. All these suggested that hepatocarcinoma might also be a hormone-dependent tumor. In this study, male SD rats were used as experimental animals and the animal model of experimental hepatocarcinoma was established by means of 3'-me-DAB administration. Androgen receptor mRNA was detected by a non-radioactive *in situ* hybridization assay in neoplastic and non-neoplastic liver tissues in an attempt to detect the inner link between the expression of androgen receptor gene and hepatocarcinogenesis, and explore its exact mechanism.

## **MATERIALS AND METHODS**

### ***Establishment of animal model of experimental hepatocarcinoma***

Male SD rats were divided into two groups: ① control group: the animals were fed with standard food; ② experimental group: the animal were fed with the food containing 0.6% 3'-me-DAB, after 14 weeks the food were replaced with standard one. The experimental rats were sacrificed at 4, 8, 14, 17 and 24 weeks respectively. The liver tissue was fixed in 10% formaldehyde and embedded in paraffin.

### ***Preparations of RNA probe***

A fragment of androgen receptor cDNA was cloned in a transcription vector Bluescript between *EcoRI*

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\*Supported by the National Natural Science Foundation of China, No. 39570348

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Received 1998-08-21

and *Hind* III. By means of transcription *in vitro*, a RNA probe complementary to the rat AR mRNA was labeled with digoxigenin. The probe was stored at  $-20^{\circ}\text{C}$ .

### *In situ* hybridization

The sections, that represent different stages of hepatocarcinogenesis, were selected for *in situ* hybridization assay. After deparaffin and rehydrate, the sections were fixed in 4% paraformaldehyde again. The hybridization was carried out at  $50^{\circ}\text{C}$  and placed overnight. The hybrids were then revealed by an alkaline phosphatase-conjugated anti-digoxigenin antibody and detected with the detection system of Boehringer Mannheim.

## RESULTS

### *Pathological changes in different stages of hepatocarcinogenesis*

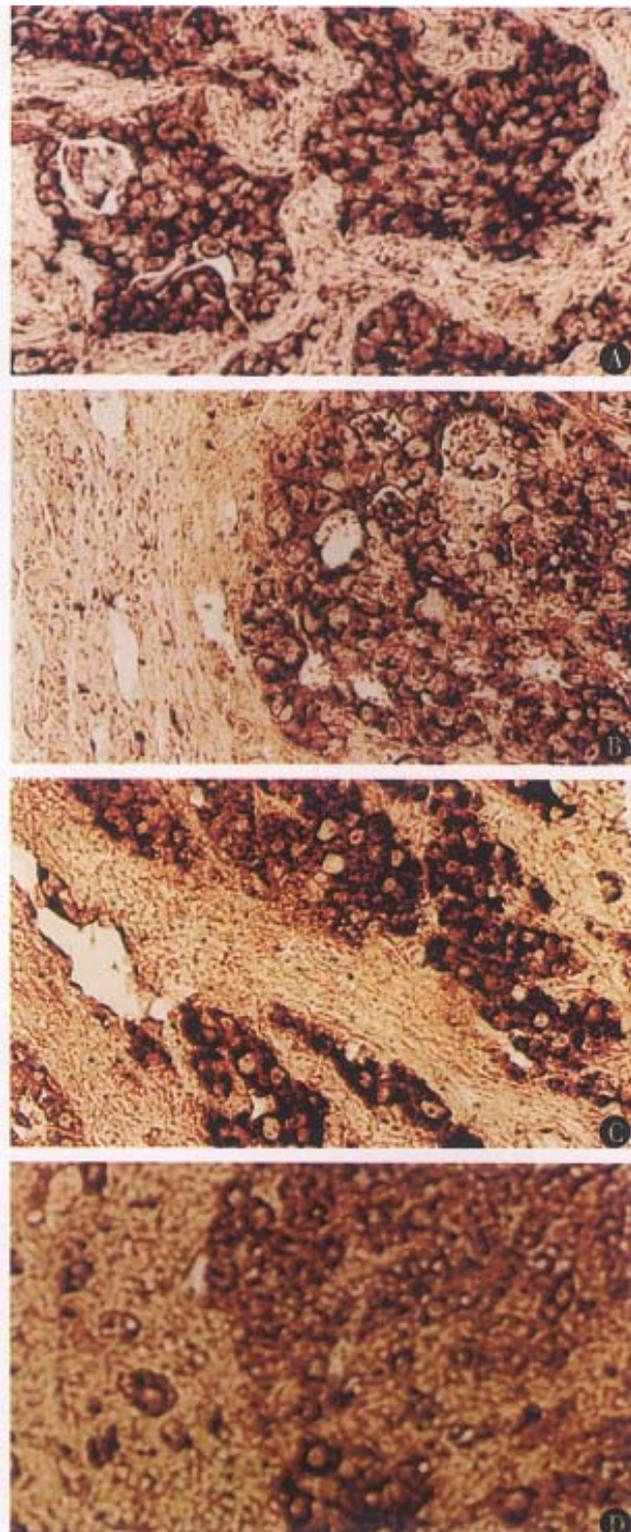
SD rats were divided into 10 groups as shown in Table 1. The specimen from various groups were first used for pathological examination. Results in different stages of experiment has demonstrated a gradual progression of hepatocarcinogenesis. At the 4th and 8th week, hyperplastic foci and nodules appeared, and the carcinoma nodules were seen at the 24th week of experiment.

**Table 1** Groups of experimental animals

	4 weeks	8 weeks	14 weeks	17 weeks	24 weeks
Experimental group	5	5	5	5	5
Control group	3	3	3	3	3

### *Expression of AR gene in different stage of hepatocarcinogenesis*

The selected sections from different groups, which reflect the pathological characteristics of hepatocarcinogenesis in different stages, were used for *in situ* hybridization assay. The results showed that no positive signal of AR expression was seen in the early stage of hepatocarcinogenesis (the 4th and 8th week). The signal of AR expression could be detected until the 14th week of experiment (Table 2), and all the hybridization signal was observed in the carcinoma tissues. In the liver tissues of control animal, and the remaining normal liver cells adjacent to the carcinoma tissue, no positive signal was detected (Figure 1A, B). In the experimental group of the 14th and 17th week, some atypical hyperplastic cells also displayed positive signal in varying degrees (Figure 1C, D). In all of control group, no positive signal was seen.



**Figure 1** Expression of androgen receptor mRNA in hepatocarcinogenesis (*in situ* hybridization).

A: Liver from the 17th week experimental group, shows the expression of AR in the carcinoma tissue.

B: Liver from the 24th week experimental group, shows the expression of AR in the carcinoma tissue and in the remaining normal tissue adjacent to the carcinoma tissue.

C: Liver from the 14th week experimental group, shows the expression of AR in the atypical hyperplastic cells.

D: Liver from the 17th week experimental group, shows the expression of AR in the atypical hyperplastic cells.

**Table 2 Expression of AR in deferent stages of hepatocarcinogenesis**

	4 weeks	8 weeks	14 weeks	17 weeks	24 weeks
Experimental group	-	-	+	+	+
Control group	-	-	-	-	-

## DISCUSSION

It is not clear yet whether androgen plays a role in regulation of hepatocarcinogenesis. But many data showed that there might be a correlation between androgen and hepatocarcinogenesis. The regional data in *Cancer incidence in five continents* demonstrated that the morbidity in men is generally higher than in women, no matter where the incidence is high, moderate or low<sup>[1]</sup>. Besides some men's unhealthy hobby (e.g., excessive drinking) and work surroundings, the potential effects of sex hormone is also a factor which can not be ignored. Another data revealed that the danger of contracting hepatocellular adenoma increased among the women who use oral contraceptives<sup>[5]</sup>. Some also reported that the patients, who use the steroid-hormone stimulating metabolism of androgen over a long period of time for aplastic anaemia therapy, are susceptible to liver cancer. It is also observed that in some patients the survival was prolonged and the tumors were reduced in size after stopping the hormone-therapy<sup>[6]</sup>. In view of these data, it has been presumed that there might be some relationship between androgen and hepatocarcinogenesis. However, this conjecture was only based on the clinical observation and the statistical data, it lacks strong experimental proof yet. Our experimental results showed that the expression of androgen receptor mRNA was observed in the atypical hyperplastic cells and in the cells of hepatocellular carcinoma (the 14th, 17th

and 24th week), yet in the normal liver tissue and the tissue from the early stage of hepatocarcinogenesis (the 4th and 8th week) no positive signal was seen. This result prompted us further that androgen probably is somewhat related to hepatocarcinogenesis. The strong expression of androgen receptor mRNA in hepatocarcinoma cells and no expression or weak expression (the level of expression might be lower than the threshold for detection) in the normal liver tissue and the remaining normal liver cells adjacent to the carcinoma tissue showed that androgen had an important bearing on hepatocarcinogenesis. It should be noted that androgen receptors express also in varying degrees in the atypical hyperplastic cells. Because the atypical hyperplasia is considered as a precancerous stage, the expression of androgen receptor gene in this stage give us much for thought. Is the synchronism of the appearance of precancerous cells and the expression of androgen receptor a mere coincidence or a necessity? This question is well worth further studying. According to these results, we infer further that androgen has an important correlation with hepatocarcinogenesis and the expression of androgen receptor gene might be a mark event during hepatocarcinogenesis.

## REFERENCES

- 1 Okuda K, Ishak KG. Neoplasms of the liver. *Shanghai Science and Technology Publishing House*, 1991:3-9
- 2 Sun HX. Hepatology. Nanjing: *Jiangsu Science and Technology Publishing House*, 1990:676-678
- 3 Masood S, West AB, Barwick KW. Expression of steroid hormone receptors in benign hepatic tumors. *Arch Pathol Lab Med*, 1992, dec;116(12):1355-1359
- 4 d'Arville CN and Johnson PJ. Growth factors, endocrine aspects and hormonal treatment in hepatocellular carcinoma-an overview. *J Steroid Biochem Mol Biol*, 1990, Dec.20;37(6):1007-1012
- 5 Jick H and Herman R. Oral contraceptive-induced benign liver tumors. The magnitude of the problem. *J Am Med Assoc*, 1978;240:828-829
- 6 Guy JT and Smith RE. Androgens and hepatocellular carcinoma. In: Nieburgs HE, eds. Prevention and detection of cancer, part II: Detection, vol.2: Cancer detection in specific sites. *New York:Marcel Dekker*, 1980:217-285

# Sequencing of *p53* mutation in established human hepatocellular carcinoma cell line of HHC4 and HHC15 in nude mice

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**Subject headings** Liver neoplasms; carcinoma, hepatocellular; *p53* gene; mutation; HHC4; HHC15; Tumor cell, cultured

## Abstract

**AIM** To set up cell lines of human hepatocellular carcinoma in nude mice for the research of cell biology and gene therapy.

**METHODS** Xenotransplantation of human hepatoma into nude mice was carried out and the growth rate, histopathology and immunology of the nude mice were studied. The DNA from xenografts were analyzed by HBV gen and PCR amplification of a fragment of *p53* gene exon 7, which were identified by dot blot hybridization, restriction fragments length polymorphism and DNA sequencing.

**RESULTS** HHC4 and hHCC415 cell lines could be successively transplanted in nude mice and the population doubling time was 7 and 5 days respectively. These strains retained the original characteristics of histopathology, secreting AFP and heteroploid karyotypes in human hepatocellular carcinoma. The fragment of HBV gene was detected in the genomic DNA of both hHCC4 and hHCC15, however only hHCC4 secreted HBsAg. The mutation at 250 code (C→A) and 249 code (G→T) were detected respectively in the genomic DNA of HHC4 and HHC15.

**CONCLUSION** The two cell lines are useful material for the studying of cell biology and gene therapy in human hepatocellular carcinoma and provide molecular biological trace of the relationship between high mortality of hepatoma and AFB1 severe pollution of the daily common foods in this district.

## INTRODUCTION

Although the etiopathology of human hepatocellular carcinoma (hHCC) is still unknown, a lot of evidence strongly suggested that infection of HBV and contamination of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) were the inducing factors of the carcinogenesis of hHCC. Xenografts of human tumorous tissue in nude mice usually retain their original morphology, antigen, karyotype and function. These models can be used for several purposes, including assessment of the etiopathology, cell biology, sensitivity of chemotherapy, genetherapy, and so on. hHCC4 and hHCC15 cell lines were established from the patients of HHC in Tong'an district of Xiamen where there was high adjust death rates of hHCC (44.75 per one hundred thousand population from 1987 to 1989) which was the secondary mortality of hHCC in China. In this district, a lot of evidence has been shown that there was severe contamination of AFB1 in daily common foods, such as the oil of peanut (92.9%) and fermented soy beans (44.4%)<sup>[1]</sup>. Also high epidemic infection of HBV was presented in the population in this country (17.6%)<sup>[2]</sup>. Because the relationship between molecular biological changes and etiopathology of HHC in this district is still absent hHCC4 and hHCC15 cell lines in nude mice were studied by cell biology and molecular biology.

## MATERIAL AND METHODS

### Animals

Male and female nude mice, about 4 to 6 weeks old, with BALB/C genetic background were provided by Medical Experimental Animal Laboratory, Cancer Research Center, Xiamen University, where the mice were bred and maintained in vinyl box isolated under specific pathogene free condition. The sterilized food pellet and tap water were given ad libitum.

### Xenotransplantation

Tumor tissues of patients, who underwent partly hepatotomy in the Min-Hai Hospital, Tong'an, were dissected from the primary site in the liver and aseptically minced and placed in cooled culture medium. Several tumor tissue fragments about 2 mm in diameter were transplanted, with trocar, into the subcutaneous tissue of the back of 6 mice within 3

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\*Supported by the Science Foundation of Department of Public Health of Fujian, No.85003-01-11

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Received 1998-11-09

hours after surgical removal of the tumor. Afterwards, a piece of tumor tissue from hHCC15 was orthotopically transplanted into the liver of 21 nude mice for studying the ability of secreting AFP from tumor.

#### **Growth**

Tumors in the subcutaneous tissue or liver were measured in 3 dimensions with calipers for every 7 days. Tumor size was plotted on a graph.

#### **Morphology**

For light microscopy and transition electron microscopy (TEM) the primary tumors of liver and xenografts were fixed and stained by the standard methods.

#### **Assay of AFP, HBsAg and HBcAg**

The Sera from patients and tumor bearing mice were analyzed radioimmunologically with AFP Diagnosis Kit (The Institute of Biochemical Assay and Product, Shanghai). In brief, 100 $\mu$ L of blood was obtained weekly for 6 weeks, by orbital venipuncturing from mice. Two cell lines were inoculated subcutaneously or into liver (for the later only with hHCC15) respectively. HBsAg and HBcAg in the sera of tumor-bearing mice were analyzed with HBsAg and HBcAg Kit (New and Advanced Co. Ltd Xiamen) by ELISA method.

#### **Analysis of chromosome**

According to the standard method of preparing metaphase cell treated with colchicine, chromosome in hHCC4 and hHCC15 cell line was carried out. The number of chromosomes in each spread cell was counted and all of spread cells were done at least for 100.

#### **Amplification of HBV DNA fragment**

The DNA from xenografts were extracted with the standard method of Sambrook and assayed by the HBV Diagnosis Kit (a kind gift from professor Wu Bing-Qun, Dept. of Pathology Beijing Medical University, China). Forward and reverse primer for HBV fragment were designed as follow: F 5'-GGGTGGAGCCCTCAGGCTCAGGGCA-3', R 5'-GAAGATGAGGCATAGCAGCAGGAT-3'. The positive and negative samples were amplified simultaneously as control. The products of PCR were run in 12g/L agarose gel stained with ethidium bromine and photographed.

#### **Amplification of p53 gene fragment**

DNA of two strains was amplified by PCR to produce target of a 110 bp at seventh exon of p53 gene using primers of P1 5'-GTTGGCTCTGACTGTACCAC-3' and P2 5'-CTGGAGTCTTCCAGTGTGAT-3' on DNA Thermal Cycler 480 (Perkin-Elmer/Cetus). The

product of 110bp DNA fragment was identified by 20g/L agarose gel electrophoresis and DNA dot blot hybridization which was performed with hDIG-labeled p53 cDNA probe (2.0kb, cut from reconstructed plasmid ph p53 $\beta$ , a kind gift from Professor Liu Si-Li, Tianjin Medical College). The probe was labeled as the described method of DIG DNA Labeling and Detection Kit (Boehringer Mannheim).

#### **Analysis of restriction fragments length polymorphism (RFLP)**

Five to 10 $\mu$ l of above PCR amplified products were digested with 7 to 10 unit Hae III restriction enzyme at 37 $^{\circ}$ C for 6hr, then precipitated with cooled ethanol. The sediments were analyzed with 150g/L non-denatural polyacrylamide gel electrophoresis, and then stained with ethidium bromide and visualized under UV light.

#### **Sequencing of PCR products**

The 110bp of PCR amplification fragments were purified by standard low-melting point agarose gel electrophoresis method and labeled with fluorescence according to the description of Tag Dye Deoxy<sup>TM</sup> Terminator Cycle Sequencing Kit. The DNA sequence of PCR fragments were analyzed and edited by Applied Biosystems 373A DNA Sequencer.

## **RESULTS**

#### **Transplantation and growth**

The neoplasms of hHCC4 and hHCC15 were presented in the back of 1 in 6 and all 6 mice respectively and showed rapid growth after several generations. A 58% and 100% rate of tumor transplantation were presented in hHCC4 and in hHCC15 respectively. Xenografts were shown a short latency (18.7 days  $\pm$ 4.9 days and 17.5 days  $\pm$ 1.6 days respectively) and almost stable after successive generations. The population doubling time in hHCC4 and hHCC15 cell line was about 7 and 5 days respectively, the growth curves are shown in Figure 7.

#### **Morphology**

Most of the transplanted tumors retained approximately the original morphological characteristics (Figure 1). No metastasis foci was presented in the liver and lung of each tumor-bearing mice within 6 weeks. Ultrastructurally, sinusoid like structure and bile canaliculi were scattered between two cells (Figure 2). Under TEM, fibrillary structure of HBsAg could be seen in the rough endoplasmic reticulum (RER) of carcinoma cells in hHCC4 (Figure 3).

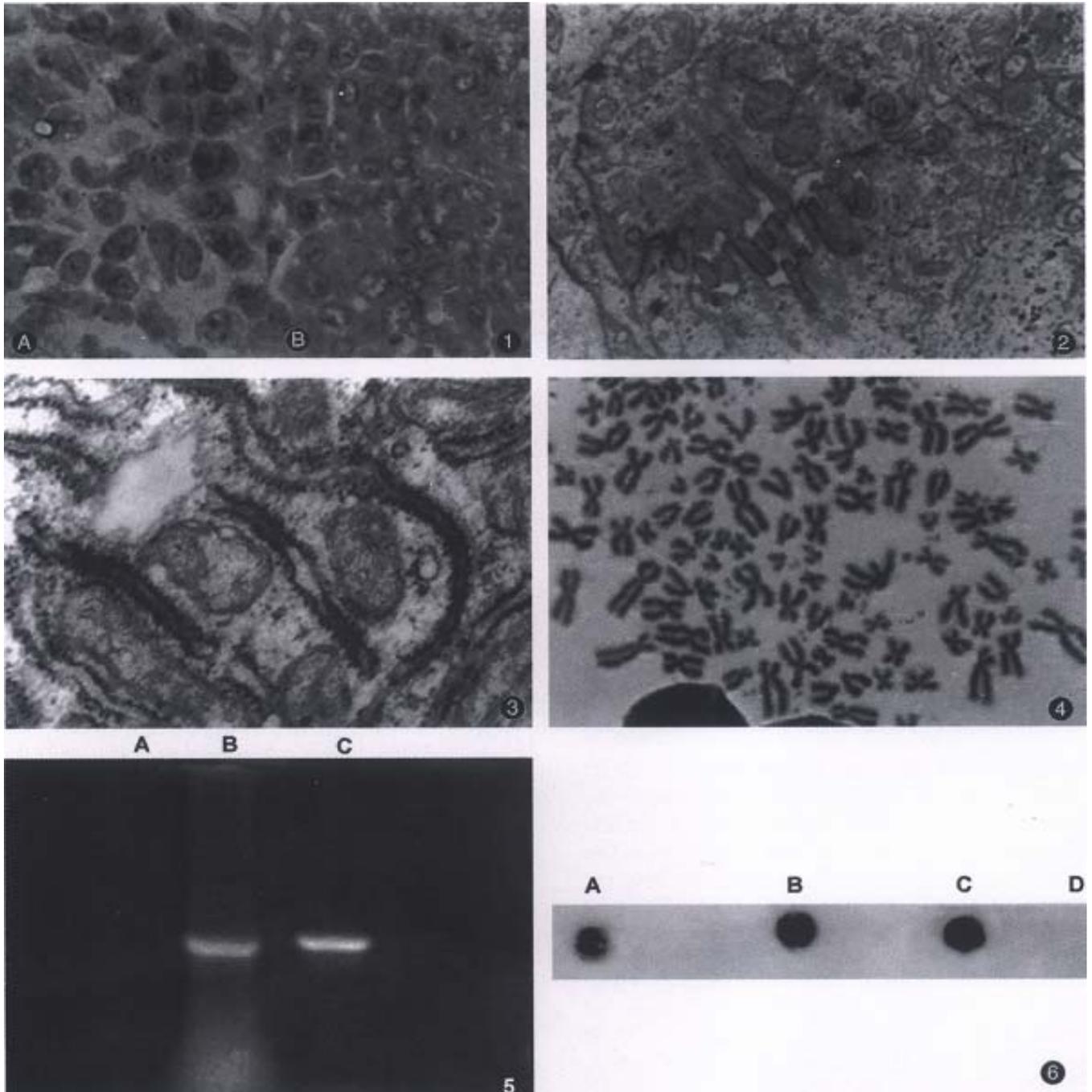
#### **Products of AFP, HBsAg and HBcAg**

Radioimmunoassay disclosed human AFP in sera of both groups mice bearing xenografts of both

strains, (until as large as 100mm<sup>3</sup>), but not for the control group, and their values increased progressively in relation to orthotopic growth of the tumor in hHCC15 (Chart 2). HBsAg could be detected in hHCC4, but not for HBcAg. HBcAg and HBsAg could not be undetected in hHCC15 by ELISA immunoassay.

### Karyology

All evaluable chromosomes of metaphase in hHCC4 and hHCC15 were human chromosomes, and no any mouse chromosomes were seen (Figure 4). A histogram of chromosome counts of 100 cells disclosed the number of chromosomes ranging between 50 to 175, the median number of 106 to 126 in hHCC15 (Figure 9) and 110 to 134 in hHCC4 (data not shown) cell line respectively.



**Figure 1** Light microscope of hHCC4 and hHCC15 showing high nuclear-cytoplasmic ratio. A: HHC4, B: HHC15

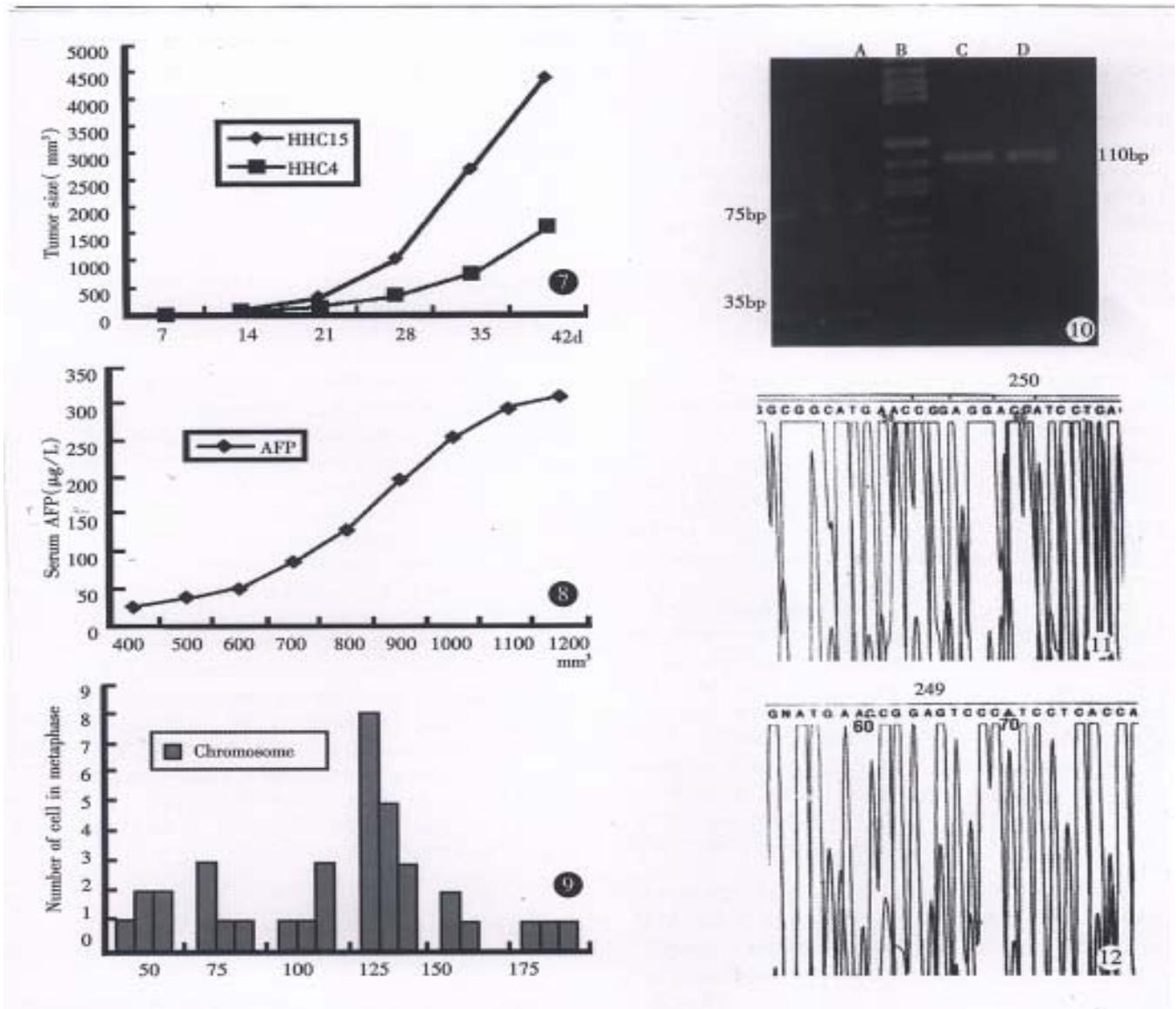
**Figure 2** The bile canaliculi between two carcinoma cells.  $\times 15000$

**Figure 3** The filament of HBsAg in the rough Endoplasmic reticulum of hepatoma cell of hHCC4.  $\times 40000$

**Figure 4** Karyotype of hHCC15.

**Figure 5** Electrophoresis of HBV PCR product from hHCC15. A. negative control, B. HHC15, C. positive control

**Figure 6** Dot blot hybridization of *p53* PCR product. A. hHCC4, B. hHCC15, C. ph *p53* $\beta$  plasmid for positive control, D. pBR322 for negative control



**Figure 7** Growth curves of hHCC4 and hHCC15 in nude mice.

**Figure 8** The relationship between the volume of orthotopical xenograft and the AFP values in the sera of a nude mouse bearing hHCC15.

**Figure 9** The histograms of chromosome numbers in hHCC15 cell passage 21.

**Figure 10** Restriction fragment length polymorphism analysis of PCR products. A. ph p53β plasmid for positive control, B. DNA marker (pBR322/Hae III), C. hHCC4, D. hHCC15

**Figure 11** Dna sequencing results of partial PCR fragment showing C→A mutation at 250 code of p53 gene from hHCC4.

**Figure 12** DNA sequencing results of partial PCR fragment showing G→T mutation at 249 code of p53 gene from hHCC15.

**Amplification of HBV DNA fragment**

PCR products amplified from both hHCC4 (data not shown) and hHCC15 or positive control had similar band running in the same distance in agarose gel, but not any product in negative control sample (Figure 5).

**Identification of PCR products and analysis of RFLP**

DNA extracted from hHCC4 and hHCC15 xenografts and plasmid ph p53β containing wild p53 cDNA were amplified respectively, then the PCR

products were analyzed by DNA dot blot. Figure 6 showed positive hybridization of the PCR products from hHCC4, hHCC15 and ph p53β plasmid with the control negative from plasmid pBR322, which meant the good specificity of the PCR system. With the method of RFLP, it was shown that the product of PCR from ph p53β plasmid, as a contrast of wild p53 cDNA, was digested into two bands of 75bp and 35bp (Line A) and undigested two of 110bp from hHCC4 and hHCC15 (Figure 10). It was suggested that the mutation at seventh exon of p53 gene

could be presented in the xenografts of hHCC4 and hHCC15.

### Sequencing PCR products

The results showed in Figures 11 and 12 were the partial DNA sequence of fragments amplified from hHCC4 and hHCC15 genomic DNA respectively. Figure 11 revealed a CCC→ACC point mutation in 250 code of *p53* gene from xenograft of hHCC4, while Figure 12 showed an AGG→AGT mutation of *p53* gene from xenograft of hHCC15.

### DISCUSSION

Xenografts of hHCC in nude mice usually retain their original morphology, antigen, karyotype and function, such as secreting AFP. It has been reported that both AFP and HBsAg can not be detected simultaneously in nude mouse transplanted with hHCC, but are presented in the cell lines of hHCC *in vitro*<sup>[3]</sup>. The presenting fibrae of HBsAg in the RER of carcinoma cell support the specific function of cells from hHCC4.

A variety of *p53* mutations have been found in a wide spectrum of sporadic tumors, in which the cause of carcinogenesis was still unknown and direct evidence is absent. It has been reported that mutation of *p53* gene was the most common inducing factor in primary advanced hHCC. AFB<sub>1</sub> was the most inducing factor of carcinogenesis in hHCC and specially associated with the mutation of code 249 in *p53* gene, being notable as "hot spots" in hHCC, spreading over South Africa or southeast coast of Asia on the earth<sup>[4,5]</sup>.

Multiple evidence support a closely relationship between AFB<sub>1</sub> and HHC. Rats fed with AFB<sub>1</sub> developed hepatoma in a dose-depend fashion<sup>[6]</sup>. AFB<sub>1</sub> intake might lead the liver to acute necrosis and proliferation of hepatoid-oval cells<sup>[7]</sup>. The more AFB<sub>1</sub> intake daily was, the more necrosis appeared and the oval cells possessing the ability of division continuously divided.

According to the telomere hypothesis of cellular senescence theory, somatic cells, which continuously divided, lead to cell cycle exit and significant telomere erosion and shortage at which the crisis state (M<sub>1</sub>) of cell arrived, which was induced by activating *p53* and *pRb* cascade. It is conceivable that *p53* is proposed to signal a growth checkpoint allowing cell to arrest in G<sub>0</sub> or G<sub>1</sub> state (replicative senescence) by inducing *p21* expression which in turn inactivate cdk/cyclin complex leading to underphosphorylation of the Rb proteins<sup>[8]</sup>. It is reasonable that *p53* gene mutation caused by AFB<sub>1</sub> have the ability to allow cells to overcome M<sub>1</sub>,

leading to an extended lifespan until a second growth checkpoint, M<sub>2</sub> is reached. This rare event, M<sub>2</sub>, is most often associated with the reactivation of telomerase. During the past few years, there has been mounting evidence that the activation of telomerase, a ribonucleoprotein enzyme, is important in maintaining telomere length stability and necessary for the sustained growth of the most cancer<sup>[9]</sup>. It has been reported recently that due to reactivating telomerase, mammary epithelial cells, which were transfected with mutation *p53* gene, could be survive and become immortal *in vitro*<sup>[10]</sup>. The two cell lines provided molecular biological trace of relationship between high mortality of hepatoma and AFB<sub>1</sub> in this district. It shown that carcinoma cells of hHCC4 and hHCC15 possessing telomerase activity (unpublished data) supported the events of *p53* mutation discovered by us.

The carcinogenesis of hHCC is closely related to chronic hepatitis B in which the molecular machanism of hHCC is still a mystery. Futhermore, AFB<sub>1</sub> treatment of trangenic mice integrated with hepatitis B DNA greatly enhanced the development of hepatoma as compared with the mice not treated with AFB<sub>1</sub><sup>[11]</sup>. It is wise to use two cell lines for the disclosing the contribution of carcinogenesis of human hepatoma in coordination of HBV integration and *p53* mutation which were presented in both of the cell lines. Also they are the useful material for cell biology, sensitivity of chemotherapy and gene therapy.

### REFERENCE

- 1 Lin L, Sun CS, Kang TS, Xu WQ, Zhen ZY, Shi MD. Investigation on four common foods polluted by AFTB<sub>1</sub> at Tong'an country. *J Fujian Med Coll*, 1997;26(1):216-218
- 2 Shun CS, Ren JX, Xie CG, Zhen JY, Hong ZN, Zhong WL. Logistic regression analysis of dangerous factors contributed to the carcinogenesis of hepatoma in Tong'an country Fujian province. *J Med Fujian*, 1994;16(1):63-65
- 3 Alexander JJ. Human hepatoma cell lines. In: Okuda K, Ishak KG, eds. Neoplasms of the liver. *Springer Verlag*, 1987:46-56
- 4 Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. *Nature*, 1991;350(4):427-428
- 5 Hollestein M, Sidrausky D, Voylestein B, Harris CC. *p53* mutations in human cancer. *Science*, 1991;253(5):49-53
- 6 Groopman JD, Cain LG, Kensler TW. Aflatoxin exposure in human populations: measurements and relationship to cancer. *Crit Rev Toxicol*, 1988; 19(2):113-145
- 7 Busby WF, Wogan GN. Aflatoxins. In: Searle CE, ed. Chemical carcinogens, 2dn ed. *Washington: American Chemical Society*, 1984:945-1136
- 8 Wright W, Shay J. Time, telomerase and tumours is cellular senescence more than an anticance machanism. *Trends Cell Biol*, 1995;5(4):293-297
- 9 Kim NW, Piatyszek MA, Prowse KR, Hartey 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(23):2011-2015
- 10 Gollahon LS, Shay JW. Immortalization of human mammary epithelial cells transfected with mutant *p53* (273(his)). *Oncogen*, 1996;12(4):715-725
- 11 Sell S, Hunt JM, Dunsford HA, Chisari FV. Synergy between hepatitis B virus expression and chemical hepatocarcinogens in transgenic mice. *Cancer Res*, 1991; 51(4):1278-1285

# Segmental transcatheter arterial embolization for primary hepatocellular carcinoma \*

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**Subject headings** liver neoplasms/therapy; carcinoma, hepatocellular/therapy; embolization, therapeutic; portal veins

## Abstract

**AIM** To evaluate the therapeutic effects of segmental transcatheter arterial embolization for primary hepatocellular carcinoma, and to recognize the manifestation and clinical value of lipiodol overflow into portal veins surrounding the tumors.

**METHODS** A total of 50 cases of nonresectable primary hepatocellular carcinoma underwent segmental transcatheter arterial embolization. Two methods of superselective segmental catheterization were used, one was the method of wire-guiding, and the other the technic of co-axial infusion catheter.

**RESULTS** The 1-, 2-, 3- and 4-year cumulative survival rates of 50 cases with segmental transcatheter arterial embolization for primary hepatocellular carcinoma were 83.8%, 65.4%, 42.9% and 24.5% respectively. The incidence of the lipiodol overflow into portal veins was 64%. The overflow of lipiodol into portal veins, represented as 3-5 grade branches of portal veins visualized by lipiodol, was "star-like" or "tree-like", and there was a relatively large vessel in the center surrounded with radicalized small branches of vessels.

**CONCLUSION** The lipiodol overflow into portal veins was one of the signs of complete embolization for tumors, and may play a partial role in embolizing the portal venous supply for hepatocellular carcinoma.

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\*Supported by "9.5" National Major Project of National Committee of Sciences and Technology, No.96-907-03-02.

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Received 1998-05-10

## INTRODUCTION

Segmental transcatheter arterial embolization (SLp-TAE) has become one of excellent interventional methods for primary hepatocellular carcinoma<sup>[1]</sup>. It was reported that SLp-TAE may play a dual role in embolizing the hepatic arterial and partly portal venous supply for hepatocellular carcinoma. We have performed SLp-TAE since 1990 in our hospital and accumulated some experience and reported it below.

## MATERIALS AND METHODS

### Materials

A total of 50 patients (48 men, 2 women) with nonresectable HCC undergoing SegLp-TAE were studied. They ranged in age from 23 to 71 years (mean, 41.8 years  $\pm$  4.2 years). The clinical symptoms included pain in upper abdomen, abdominal mass and weight loss. AFP was elevated in 44 patients, and cirrhosis occurred in 35 cases. Child's classification showed 15 cases of Child's A, 33 cases of Child's B, and 2 of Child's C. The main tumor measured 1.5 cm - 10 cm with a mean of 6.0cm $\pm$ 1.4cm (7 cases, >10 cm; 28 cases, 5 cm - 10 cm; and 15 cases <5cm). Multiple nodules were found in 10 of the cases.

### Methods

Coaxial infusion catheters of 3.0F with 0.013 inch micro-wire (Target Therapeutic Inc.) were used.

The feeding segmental or sub-segmental artery was detected carefully by celiac arteriography, and variants were excluded by superior mesenteric arteriography and phrenic arteriography. Two methods of superselective segment arterial catheterization were employed: one was wire-guiding catheterization, the other was technic of co-axial infusion catheter.

The volume of 2 ml - 20 ml emulsion (AOE, Adriamycin mixed with lipiodol) were injected, and Gelatin sponge particles were used in some patients. Before embolization, 2 ml - 3 ml of 1% lidocaine was injected to prevent vessel spasm.

## RESULTS

A total of 80 TAE (mean 1.6) were performed by two methods of segmental catheterization (34 by wire-guiding, 16 by co-axial catheter) with 16 cases of sub-segment, and 34 cases of segment in embolization position. Survival period ranged from 3 to 68 months (mean, 1.9 years  $\pm$  1.2 years). The 1-, 2-, 3- and 4-year cumulative survival rates were

83.8%, 65.4%, 42.9% and 24.5% respectively. Complete necrosis was revealed in 3 resected lesions.

## DISCUSSION

It is well known that the blood supply of primary hepatocellular carcinoma is mainly from the hepatic artery, but the portal venous supply is important to its growth, especially, in the edge of tumor. Nakamura<sup>[2]</sup> introduced the segmental transcatheter arterial embolization which may play a dual role in embolizing the hepatic arterial and partly portal venous blood supply for hepatocellular carcinoma, leading to complete necrosis of tumor. The 1- and 2-year survival rates of 50 cases were 83.8% and 62.7% in his reports. In our study, the 1-, 2-, 3- and 4-year cumulative survival rates were 83.8%, 65.4%, 42.9% and 24.5%. The therapeutic results were comparatively good.

Two methods of superselective segmental arterial catheterization were used in our study, one was the wire-guiding catheterization, the other was the technic of co-axial infusion catheter. The successful rate of the former method was 60%-65%. The co-axial catheter was soft, adapted well to the distorted arteries, and could be easily inserted to the segment and sub-segment artery which supplied the tumor. But it is relatively difficult in operation, and need more time of exposure. In our study, we usually inserted the catheter in to proper hepatic artery or lobar artery by the wire-guiding method, before completing the segmental catheterization by the co-axial infusion catheter. The total success rate of segment catheterization was 80%-85%.

In 1988, Nakamura<sup>[1]</sup> discovered in plain abdominal radiograph immediately after injection of oil emulsion, that part iodized oil would overflow into portal veins surrounding the tumors through arteriportal shunt or communications when a relatively large amount of lipiodol was injected into the hepatic artery. In their cases, there was no arteriportal shunt in hepatic angiography. This phenomenon was confirmed by the animal experiment<sup>[3,4]</sup>. The occurrence rate of lipiodol overflow into portal veins was 64% (32/50 cases) in this series. It represented 3-5 grade branches of portal veins visualized by lipiodol which were “star-like” and “tree-like”, or a relatively large vessel in the center surrounded with small radiate branches.

Some authors<sup>[5,6]</sup> found in the resected specimen which demonstrated lipiodol overflow into portal veins, that not only complete necrosis of the tumors was achieved, but also partial necrosis or atrophy occurred in the normal tissues near the tumor. It was suggested that overflow of lipiodol into portal veins was one of the marks of complete embolization for tumors, and may play a partial role in embolizing the portal venous supply for the hepatocellular carcinoma.

There were eighteen cases without lipiodol

overflow into portal veins in our group, which was probably related to different blood supply for the neoplasm. The more affluence of blood supply, the more opportunity of lipiodol overflow. In our group, 71.9% of the cases demonstrated complete deposit of lipiodol in plain radiograph or CT, and only 2 cases with scarce deposit, which suggested that the more complete deposit of lipiodol, the more opportunity of lipiodol overflow through the arteriportal shunt inside the tumor. Additionally, in our practice the treatment was interrupted sometimes by obvious pain resulting from vessel spasm after a bit lipiodol injection, which probably influenced the occurrence of lipiodol overflow into portal veins.

Because primary hepatocellular carcinoma usually has the property of multi-center origin, and micro-tumor metastasis to small branches of portal veins, there were multiple foci beyond the tumor-bearing segment at early stage, which may not be detected by conventional CT or angiography. “Two-steps” method of transcatheter arterial embolization was adopted in some cases in which multiple foci may occur. First, segmental embolization was introduced to the main tumor-bearing segment or sub-segment. Secondly, in patient's tolerance, small dose of lipiodol was injected into the whole liver through the proper hepatic artery, so as not to leave out the micro-foci which escape detection before embolization.

**CONCLUSION** Segmental transcatheter arterial embolization has become one of excellent treatments of primary hepatocellular carcinoma<sup>[8,9]</sup>. The 1-, 2-, 3- and 4-year cumulative survival rates were 83.8%, 65.4%, 42.9% and 24.5% in our group. The long-term prognosis awaits further observations.

## REFERENCES

- 1 Nakamura H, Hashimoto T, Oi H, Sawada S. Iodized oil in the portal vein after arterial embolization. *Radiology*, 1988;167(2):415-417
- 2 Nakamura H, Oi H, Hori S, Takayasa Y, Furui S, Sawada S et al. Outcome of localized hepatocellular carcinoma treated with segmental arteriportal chemoembolization. *Cancer Chemoth Pharmacol*, 1992;31(Suppl 1):S69-S71
- 3 Kan Z, Ivancer K, Hagerstrand I, Chuang VP, Lunderquist A. *In vivo* microscopy of the liver after injection of lipiodol into the hepatic portal vein in the rat. *Acta Radiol*, 1989;30(4):419-425
- 4 Kan Z. Iodized oil injected in the hepatic artery will enter the portal vein [letter; comment]. *J Vasc Interv Radiol*, 1994;5(3):525-526
- 5 Matsuo N, Uchida H, Nishimine K, Soda S, Oshima M, Nakana H et al. Segmental transcatheter hepatic artery chemoembolization with iodized oil from hepatocellular carcinoma: antitumor effect and influence on normal tissue. *J Vasc Interv Radiol*, 1993;4(4):2543-2549
- 6 Khan KN, Nakata K, Kusumoto Y, Shima M, Ishii N, Koji T et al. Evaluation of nontumorous tissue damage by transcatheter arterial embolization for hepatocellular carcinoma. *Cancer Res*, 1991;51(20):5667-5671
- 7 Matsuo N, Uchida H, Sakaguchi H, Nishimine K, Hirohashi S, Ohishi H. Optimal lipiodol volume intracatheter arterial chemoembolization for hepatocellular carcinoma: study base on lipiodol accumulation patterns and histopathologic findings. *Semin Oncol*, 1997;24(Suppl 6):S60-71
- 8 Matsui O, Kadoya M, Yoshikawa J, Gabata T, Takashima T, Demachi H. Segment transcatheter arterial embolization for hepatocellular carcinoma: local therapeutic effect and 5-year survival rate. *Cancer Pharmacol*, 1994;33(Suppl):S84-88
- 9 Nishimine K, Uchida H, Matsuo N, Sakaguchi H, Hirohashi S, Nishimura Y et al. Segmental transarterial chemoembolization with lipiodol mixed with anti-cancer drugs for nonresectable hepatocellular carcinoma: follow-up CT and therapeutic results. *Cancer Chemother Pharmacol*, 1994;33(Suppl):S60-68

# Findings of non-pathologic perfusion defects by CT arterial portography and non pathologic enhancement of CT hepatic arteriography \*

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**Subject headings** liver neoplasms/radiography; carcinoma, hepato cellular/radiography; hepatic arteriography; tomography, X-ray computed

## Abstract

**AIM** To recognize the characteristic findings of non-pathologic perfusion defects with CT arterial portography (CTAP) and nonpathologic enhancement found in CT hepatic arteriography (CTHA).

**METHODS** The manifestations of nonpathologic perfusion defects with CTAP and non-pathologic enhancement found in CTHA were analyzed in 50 patients with primary hepatocellular carcinoma.

**RESULTS** The false-positive rate of perfusion defects detected in CTAP was 15.1%. The shapes of perfusion defects were peripheral wedge, small, round, and patchy. The occurrence rate of non-pathologic enhancement found in CTHA was 22.0%. The shapes of non-pathologic enhancement were small, round, irregular, and wedge.

**CONCLUSION** There was high frequency of non-pathologic perfusion defects detected with CTAP and non-pathologic enhancement found in CTHA. The simultaneous use of both procedures may help decrease the false-positive rate, and increase the veracity of diagnosis for hepatocellular carcinoma.

## INTRODUCTION

CT arterial portography (CTAP) and CT hepatic arteriography (CTHA) are the most sensitive methods of detecting hepatocellular carcinoma<sup>[1,2]</sup>. In recent years, there are more reports on non-pathologic perfusion defects of CTAP and non-pathologic enhancement CTHA. To better recognize and understand the characteristic manifestation of non-pathologic perfusion defects with CTAP and non-pathologic enhancement in CTHA, we analyzed the CT images of 50 cases of hepatocellular carcinoma on CTAP and CTHA in our hospital from January 1995 to January 1998.

## MATERIALS AND METHODS

### Materials

Fifty patients (44 man, 6 women) with hepatocellular carcinoma were examined with CTAP and CTHA in our department. They ranged in age from 21 to 65 years (mean age, 413 years). AFP was elevated in 41 patients. Cirrhosis occurred in 42 cases. Child's classification showed 16 cases of Child's A, 30 cases of Child's B, and 4 cases of Child's C. The tumors measured 0.2cm-5.5cm in size with a mean of 3.6cm. Multiple nodules were found in 42 cases.

### Methods

CTAP examinations were performed with incremental scanning of liver in cranial-to-caudal direction with 8-mm or 10-mm collimation on bi-spiral Elscint Twin Flash scanner (Elscint Corp.). CT images were obtained 25sec-35sec after the initiation of transcatheter (5-F) superior mesenteric artery injection of 30 ml - 40 ml of non-ionic contrast material. Contrast material was injected at a rate of 3.0ml/sec-3.5ml/sec with an automatic power injector (Medrad, Pittsburgh). Conventional angiography was not performed before CTAP. During the catheterization, contrast material administered before CT scanning was limited to 5ml -10ml injected by hand to visualize any aberrant vessels and to facilitate proper catheter placement.

CTHA was done by injecting contrast material into the proper hepatic artery, the common hepatic

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\*Supported by "9.5" National Major Project of National Committee of Sciences and Technology, No. 96-907-03-02

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Received 1998-10-08

artery, or the celiac artery. The volume of 20ml-30ml of contrast material was injected at a rate of 3.0 ml/sec - 3.5 ml/sec. Consecutive scanning of the liver was started 6sec-8sec after the initiation of injection of contrast material. After CTAP and CTHA examination, 3 ml - 15 ml of lipiodol was injected into the hepatic artery, and plain CT scan of liver was performed after two or three weeks (Lipiodol CT, Lp-CT).

## RESULTS

### *Detectability of tumors*

CTAP and CTHA images of 50 cases were interpreted prospectively double-blind by two radiologists, and confirmed pathologically combined with lipiodol deposits in Lp-CT, and 6mo-16mo follow-up (42 tumors of 18 cases proved by operative pathology; 30 lesions of 15 cases by biopsy, and the other 27 cases by Lp-CT and follow-up). A total of 232 tumors were found, including 214 tumors detected by CTAP (the rate of detectability was 92.2%), 209 detected by CTHA (the rate of detectability was 90.1%), and 220 detected by simultaneous use of both procedures (the rate of detectability was 94.8%). Our study did not reveal a statistically significant difference in the sensitivities of CTAP, CTHA and simultaneous use of both procedures.

### *Non-pathologic perfusion defects of CTAP*

A total of 252 perfusion defects were detected with CTAP in the 50 patients, including 38 non-pathologic abnormal perfusion defects in 18 cases with a false positive rate of 15.1%. The shape of perfusion defects was peripheral wedge, small round and patchy. All the non-pathologic perfusion defects were not demonstrated on CTHA images.

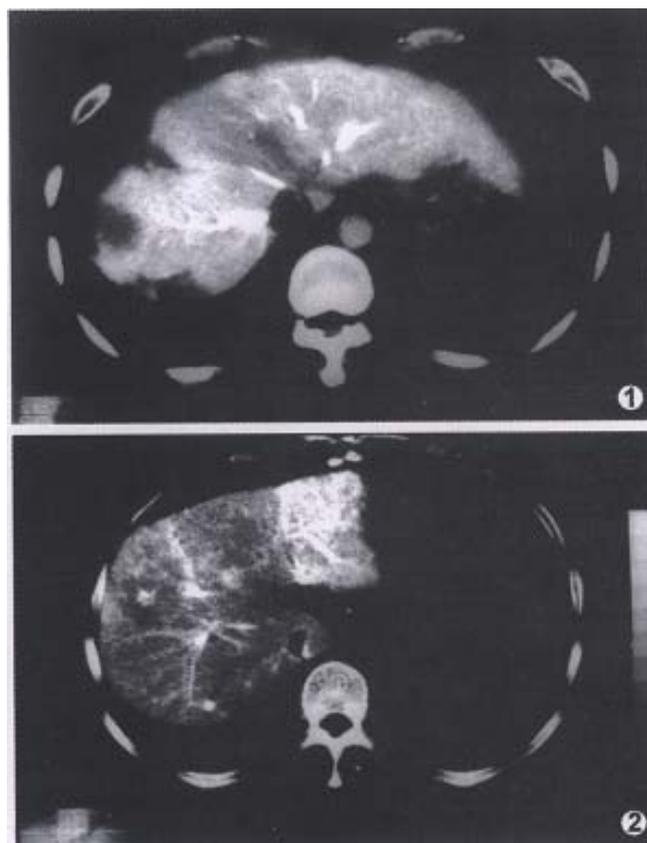
### *Non-pathologic enhancement of CTHA*

A total of 268 enhancement foci were found with CTHA in 50 patients, including 59 non-pathologic enhancement foci in 21 cases with an occurrence rate of 22.0%. The shape of non-pathologic enhancement was small round, irregular and wedge.

## DISCUSSION

In recent years, with the development of spiral CT technique, CTAP and CTHA have become widely used in diagnosis and differential diagnosis of small hepatocellular carcinoma. Compared with non-spiral CT arteriography, spiral CT arteriography has achieved great improvement not only in scanning technique, but also in quality of CT images<sup>[1]</sup>. We scanned the entire liver consecutively, and obtained excellent images of

CTAP and CTHA by the technique of a single breath-hold scanning, with a comparatively small quantity of contrast material (CTAP 30ml-40ml, CTHA 20 ml - 30 ml). Compared with conventionally enhanced CT (contrast material administrated by venous injection), CTAP and CTHA have a high detectability of 80% - 96%<sup>[2]</sup>. In our 50 patients examined with CTAP and CTHA, the detectability rate of CTAP, CTHA and simultaneous use of both procedures were 92.2%, 90.1% and 94.8% respectively.



**Figure 1** CTAP image obtained in a 42-year-old man with recurrence of HCC shows two tumors in right lobe. The peripheral wedge-shaped perfusion defect is non-pathologic perfusion defect, confirmed by biopsy.

**Figure 2** CTHA image obtained in a 45-year-old man shows a small round peripheral enhancement in right lobe which is non-pathologic enhancement. No tumor is found in the operative pathology.

With the widespread use of CTAP and CTHA, there have been more reports on non-pathologic perfusion defects found with CTAP and non-pathologic enhancement with CTHA. The manifestation of pseudolesion in CTAP was noted relatively earlier. Due to aberration of blood supply for some parts of the liver, such as arterio-portal shunt<sup>[3]</sup>, cirrhotic nodule, focal nodular hyperplasia, and focal fatty infiltration, the normal

tissue could show up as perfusion defects in CTAP, especially in the medial segment of the left lobe<sup>[4]</sup>. Irie *et al.* reported that perfusion defects on CTAP are sometimes nonspecific, and suggested that peripheral flat or wedged-shaped perfusion defects indicate benignity. Their study showed that, except for cysts, perfusion defects larger than 1.5cm may indicate malignancy, and combination with CTHA might be helpful in differentiating malignant from benign perfusion defects with CTAP<sup>[5]</sup>. Peterson *et al.* reported that peripheral wedge-shaped perfusion defects on CTAP were nearly uniformly benign, and may be used as a criterion of benignity. They hypothesized that the pathogenesis of these peripheral wedge-shaped perfusion defects may represent variations in the normal portal perfusion of the microvasculature of the peripheral sub-capsular liver parenchyma<sup>[6]</sup>. In our series of 50 cases, the occurrence rate of non-pathologic perfusion defects on CTAP was 15.1%, and peripheral wedge-shaped perfusion defects were the most common (Figure 1).

Since the advent of helical CT technique, CTHA became more widely used in the differential diagnosis for hepatocellular carcinoma<sup>[7]</sup>. Similar to findings of CTAP, pseudo-lesion enhancement of normal liver tissue also occurred in CTHA. Kanematsu *et al.* supposed that local non-pathologic enhancement detected with CTHA might result from the cystic venous drainage or peripheral arterio-portal shunts. They analyzed the frequency, size, location, and shape of local non-pathologic enhancement on CTHA. In a series of 31 patients examined with CTHA, 36 non-pathologic enhancements were found in 14 cases, the occurrence rate being 36.4%. The shapes of non-pathologic enhancement on CTHA appeared round, veriform, irregular, punctate, and wedge-like<sup>[8]</sup>. Although the shape of the enhancement was nonspecific, Irie *et al.* suggested that rim enhancement indicated malignancy in both cirrhotic

and non-cirrhotic liver<sup>[5]</sup>. In our study, the occurrence rate of non-pathologic enhancement on CTHA was 22.0%, and small rounded peripheral enhancement was the most common (Figure 2). In practice, we discovered that the frequency of non-pathologic enhancement was to some extent, related to the depth of catheterization at which CTHA was performed. There was a relatively higher frequency of non-pathologic enhancement in CTHA formed at catheterization of hepatic proper artery, compared with CTHA formed at catheterization of celiac artery trunk. The reason might be the relatively larger volume of contrast material and higher speed of injection in CTHA performed at catheterization of hepatic proper artery. Thus, we did not recommend CTAP or CTHA alone for interpretation of hepatocellular carcinoma. We suggest that the simultaneous use of both procedures help decrease the false positive rate of CTAP and CTHA, and increase the veracity of diagnosis for hepatocellular carcinoma.

## REFERENCES

- 1 Hori M, Murakami T, Oi H, Kin T, Takahashi S, Matsushita M *et al.* Sensitivity in detection of hypervascular hepatocellular carcinoma by helical CT with intra-arterial injection of contrast medium, and by helical CT and MR imaging with intravenous injection of contrast mediums. *Acta Radiol*, 1998;39(2):144-151
- 2 Kanematsu M, Oliver Th-3rd, Carr B, Baron RL. Hepatocellular carcinoma: the role of the helical biphasic contrast-enhanced CT versus CT during arterial portography. *Radiology*, 1997;205(1):75-80
- 3 Tamura S, Kihara Y, Yuki Y, Sugimura H, Shimice T, Adjei O *et al.* Pseudoleion on CTAP secondary to arterio-portal shunts. *Clin Imagin*, 1997; 21(5):359-365
- 4 Matusi O, Kadoya M, Yashikawa J, Gadate T, Kawanori Y. Posterior aspect of hepatic segment IV: Patterns of portal venule branching at helical CT during arterial portography. *Radiology*, 1997;205(1):159-162
- 5 Irie T, Takeshita K, Wada Y, Kisano S, Terahata S, Tamai S *et al.* To evaluation of hepatic tumors: Comparison of CT with Arterial portography, CT with infusion hepatic arteriography, and simultaneous use of both techniques. *AJR*, 1995;164(6):1407-1412
- 6 Peterson MS, Baron RL, Dodd III GD, Zajko AJ, Oliver JH, Miller WJ *et al.* Hepatic parenchymal perfusion defects detected with CTAP: Imaging-pathologic correlation. *Radiology*, 1992;185(1):149-155
- 7 Murakami T, Oi H, Hori K, Kim T, Takahashi S, Tomoda K *et al.* Helical CT during arterial portography and hepatic arteriography for detecting hypervascular hepatocellular carcinoma. *AJR*, 1997;169(1):131-135
- 8 Kanematsu M, Hoshi H, Imado T, Yamawaki Y, Mizuno S. Nonpathological focal enhanced on spiral CT hepatic angiography. *Abdom Imagin*, 1997;22(1):55-59

# Diet and gastric cancer: a case-control study in Fujian Province, China \*

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**Subject headings** stomach neoplasms/etiology; living habits; food habits; risk facto

## Abstract

**AIM** To explore the relationship between consumption of fish sauce, other dietary factors, living habits and the risk of gastric cancer.

**METHODS** From May 1994 to July 1995, a population-based 1:2 case-control study was carried out in high-risk areas of gastric cancer, Changle and Fuqing cities, Fujian Province. Totally 272 cases and 544 age, gender-matched controls were included. Risk state analyses were made by ASRS package.

**RESULTS** Risk state single-factor analysis indicated that gastric cancer risk rose with high intake of fish sauce (OR=2.57), salted vegetables (OR=1.41), salted/fried fish and small shrimps (OR=1.57), low consumption of fresh vegetables (OR=1.95), fresh citrus fruits (OR=1.41), other fresh fruits (OR=1.31), green tea (OR=1.72), exposure to moldy foods (OR=2.32), irregular dinners (OR=5.47) and familial history of malignancy (OR=3.27). No significant relationship was observed between smoking, drinking, salt intake, use of refrigerator and gastric cancer risk. The results of risk state conditional Logistic regression showed that fish sauce, salted dried fish and small shrimps, irregular dinners, familial history of malignancy were included in the best risk set. The summary AOR for the four factors was 75.49%.

**CONCLUSION** High intake of fish sauce, salted foods, moldy foods, irregular dinners and familial history of malignancy were possible risk factors for gastric cancer, whereas fresh vegetables and fruits, and green tea might have protective effects for gastric cancer.

## INTRODUCTION

Changle and Fuqing cities are located in the southeastern part of Fujian Province, China with a high incidence of gastric cancer. However, the causes of gastric cancer still remain unclear. Previous studies indicate that environmental factors may play an important role in the carcinogenesis of gastric cancer, among which, dietary risk factors for gastric cancer were most extensively investigated. Our hypothesis is that the high incidence of gastric cancer may be attributed, to some extent, to some unique dietary habits. Recently a statistically significant relationship between fish sauce consumption, a condiment commonly used by local residents, and the mortality rates from gastric cancer was observed by our ecological study<sup>[1]</sup>. The mutagenicity of fish sauce was also reported by experimental studies<sup>[2]</sup>. N-nitrosamines can also be detected in fish sauce<sup>[3]</sup>. In order to explore further the relationship between consumption of fish sauce and gastric cancer, a population-based 1:2 matched case-control study was carried out from May 1994 to July 1995.

## MATERIALS AND METHODS

### *Selection of cases and controls*

This study was conducted in Changle and Fuqing cities with populations around 600 thousands and 1 million, respectively. All cases histologically confirmed or diagnosed by operation from January 1993 to July 1995 were collected from cancer registry and a quick-reporting system from hospitals. Each case was matched by two randomly selected controls who resided in the same village as index case, with same gender, nationality and age ( $\pm 3$  years). Those who have ever been diagnosed having gastric diseases within the past 3 years were not eligible as controls. Study subjects must have

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\*Supported by "8.5" national major project, No. 95-914-01-10.

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Received 1998-06-06

resided in the two cities for more than 20 years, and can answer questions clearly.

### Investigation

Face to face interviews were made by specially trained interviewers with a structured questionnaire. The items of questionnaire included demographic and socio-economic factors, occupational and medical histories, family occurrence of cancer, use of alcohol and tobacco, and dietary habits. As for dietary habits, we emphasized on the exposure 20 years before. Diet was assessed with a food frequency questionnaire. The subjects were interviewed about the usual frequency of consumption of certain foodstuffs, supplemented with questions about the actual amount consumed per time unit. Then total amount per year was estimated accordingly. The subjects within a matched pair were interviewed by one interviewer.

### Statistical analysis

Data were handled by Epi-info. The statistical analyses, including univariate analysis and conditional logistic analysis were done using ASRS software<sup>[4]</sup>. In univariate analysis, the best cut-points for exposure levels were searched by automatic or forced adjustment, combination, on the criteria of CPDS and AIC. Those factors, which showed association with the risk of gastric cancer in univariate analysis, were further investigated by conditional logistic analysis of risk states to establish a main-effect model.

## RESULTS

Totally 272 pairs were investigated, among which 157 were from Changle City, 115 from Fuqing City; 233 were male and 39 female. The age range for cases was 30-78 years, averaging 58.67 years. No obvious difference in marital status, occupation and education level was observed between cases and controls.

The results of univariate analysis are shown in Table 1. As for dietary habits, consumption of fish sauce, salted vegetables, salted fermented sea products and moldy foods may increase the risk of gastric cancer. However, fresh vegetables, fruits and green tea may have protective effects against gastric cancer. Additionally, irregular dinner and family occurrence of cancer are also risk factors for gastric cancer. No association between the use of alcohol and tobacco, the amount of salt intake, the use of refrigerator and the risk of gastric cancer was observed. To select a possible best subset of risk factors for gastric cancer, conditional logistic analyses of risk states were also made. The results

showed that the best subset of risk factors included fish sauce, irregular dinner, salted fermented sea products and family occurrence of cancer (Table 2). The summary attributable risk for these four risk factors is 75.49%, indicating that these four factors may play an important role in the carcinogenesis of gastric cancer.

**Table 1 Results of univariate analysis on the relationship between fish sauce, other factors and the risk of gastric cancer**

Factors	Exposure	Case	Control	OR	95%CI
Index of smoking <sup>a</sup>	>10	118	241	1	
	≤10	154	303	1.04	0.95-1.13
Hard distilled spirit	≤20kg/y	265	534	1	
	>20kg/y	7	10	1.41	0.63-3.14
Soft distilled spirit	>25kg/y	13	29	1	
	≤25kg/y	259	515	1.12	0.86-1.47
Wine	≤30kg/y	245	494	1	
	>30kg/y	27	50	1.09	0.89-1.33
Beer	>50 bottle/y	42	106	1	
	≤50 bottle/y	230	438	1.33	0.93-1.88
Green tea	>0.75kg/y	47	144	1	
	≤0.75kg/y	225	400	1.72	1.26-2.36 <sup>e</sup>
Fish sauce	<0.4kg/m	198	475	1	
	≥0.4kg/m	74	69	2.57	1.89-3.50 <sup>e</sup>
Salt <sup>b</sup>	≤0.25kg/m	159	347	1	
	>0.25kg/m	113	197	1.25	0.96-1.63
Moldy foods	No	188	456	1	
	Yes	84	88	2.32	1.73-3.09 <sup>e</sup>
Irregular dinners	<3 times/w	114	434	1	
	≥3 times/w	158	110	5.47	4.22-7.09 <sup>e</sup>
Use of refrigerator	Yes	35	78	1	
	No	237	466	1.13	0.85-1.52
Salted vegetables	<2kg/y	157	358	1	
	≥2kg/y	115	186	1.41	1.09-1.83 <sup>d</sup>
Salted fermented sea foods	<1.5kg/y	144	347	1	
	≥1.5kg/y	128	197	1.57	1.21-2.02 <sup>e</sup>
Citrus fruits-c	>2.5kg/y	55	143	1	
	≤2.5kg/y	217	401	1.41	1.03-1.92 <sup>d</sup>
Other fruits	>2.5kg/y	166	366	1	
	≤2.5kg/y	106	178	1.31	1.01-1.71 <sup>d</sup>
Fresh meat, fish, egg, poultry	>25kg/y	105	238	1	
	≤25kg/y	167	306	1.24	0.95-1.61
Fresh vegetables	>25kg/y	212	475	1	
	≤25kg/y	60	69	1.95	1.41-2.70 <sup>e</sup>
Family occurrence of cancer	No	165	454	1	
	Yes	107	90	3.27	2.48-4.31 <sup>e</sup>

<sup>a</sup>smoking index: (amount of smoking<sup>day</sup> × years of smoking)/age of starting smoking; <sup>b</sup>including the salt in fish sauce and soybean sauce; <sup>c</sup>including orange, grapefruit, banana; <sup>d</sup> $P < 0.05$ ; <sup>e</sup> $P < 0.01$ .

**Table 2 The results of conditional logistic analysis of risk states**

Factors	Regression coefficient	Standardized regression coefficient	Adjusted attributable risk <sup>a</sup>
Fish sauce	1.08	3.49	17.81% <sup>c</sup>
Irregular dinners	1.85	8.26	48.93% <sup>c</sup>
Salted sea foods	0.54	2.11	19.69% <sup>b</sup>
Familial history of malignancy	1.19	5.45	27.41% <sup>c</sup>

<sup>a</sup>Comprehensive attributable risk (CAR) = 75.49%; <sup>b</sup> $P < 0.05$ ; <sup>c</sup> $P < 0.01$ .

## DISCUSSION

Fish sauce is one kind of condiments consumed daily by local residents. It is produced by long-term fermentation from several kinds of sea fish. Due to the proteins with amino in the fishes, salted fermented fish products may contain a large amount of important precursors of N-nitroso compounds-amines<sup>[5]</sup>. These precursors may react with nitrite in gastric juice to form N-nitroso compounds internally. Deng et al reported that abstracts of fish sauce from Changle have carcinogenicity and mutagenicity after nitration<sup>[2]</sup>. The amount of N-nitro compounds increased greatly after nitration, and genotoxins can be detected<sup>[3]</sup>. Our ecological study indicated that there was a statistically significant relationship between fish sauce consumption and mortality rates from gastric cancer among 14 counties in Fujian Province<sup>[1]</sup>. The results of this study further supported the point that fish sauce consumption may be an important cause for the high incidence of gastric cancer.

Several case-control studies have indicated that long-term use of refrigerators may decrease the risk of gastric cancer<sup>[6-8]</sup>. However, in our study, refrigerators were not commonly used by local residents. Even among those users, the history of refrigerator use is very short. Therefore, it is impossible to evaluate the role of refrigerator use in the etiology of gastric cancer. However, the deficiency of refrigerator indicated that the consumption of salted foods was very common. Especially in the study area which is located on seaboard, the consumption of salted or fermented sea foodstuffs is very common. In our study, consumption of salted foodstuffs, especially of salted fermented sea products was found to increase the risk of gastric cancer. This is in accordance with the results of Buiatti *et al*<sup>[7]</sup>. Additionally, our study indicated that irregular dinner may be one of etiological factors for gastric cancer. This finding supported the results of our previous ecological study<sup>[1]</sup>. Irregular dinner may

cause injuries of gastric mucosa and promote the effects of carcinogens. A prospective study showed that those with familial history of malignancy, especially gastric cancer, have a higher risk of gastric cancer<sup>[9]</sup>. This is in agreement with our study results. In our study, fresh vegetables showed a protective effect against gastric cancer. This is also true for citrus fruits and other fruits. Their protective effects may be attributed to the vitamin C, which may interrupt the internal formation of N-nitroso compounds. Some studies reported that only consumption of raw vegetables had a protective effect<sup>[6,7]</sup>. However, in our study area, residents do not have such a habit. There were conflicting views on the relationship between the use of alcohol and tobacco and the risk of gastric cancer<sup>[6,7,9,10]</sup>. In our study, no association was observed between the use of alcohol, tobacco and the gastric cancer risk. No interaction between these two factors was found either.

## REFERENCES

- 1 Ye WM, Ti YN, Lin RT, Zhou TS, Cai L, Chen CG *et al*. A study on relationship between fish sauce consumption and mortality from gastric cancer in Fujian province. *J Fujian Med Coll*, 1994;28(4):402-405
- 2 Deng DJ, Zhang RF, Chen Y, Chen CS, Jin S, Zhu SX. Mutagenicity and carcinogenicity of fish sauce from a county with the high risk for gastric cancer in China. *Carcinogenesis, Teratogenesis and Mutagenesis*, 1991;3(1):13-17
- 3 Chen CS, Pignatelli B, Malaveille C, Bouvier G, Shuker D, Hautefeuille A *et al*. Levels of direct-acting mutagens, total N-nitrosocompounds in nitrosated fermented fish products, consumed in a high-risk area for gastric cancer in southern China. *Mutat Res*, 1992;265(2):211-221
- 4 Liu YY. Risk state analysis and its application in biomedicine (I). *Beijing: Science Press*, 1990:83-123
- 5 Zhang RF, Deng DJ, Chen Y, Chen CS, Fan ZF. Analysis on precursors of N-nitrosamines in fish sauce from a high-risk area of gastric cancer. *Chin J Cancer*, 1993;12(5):395-398
- 6 Boeing H, Frentzel B, Berger M, Berndt V, Gores W, Korner M *et al*. Case-control study on stomach cancer in Germany. *Int J Cancer*, 1991;47(6):858-864
- 7 Buiatti E, Palli D, Decarli A, Amadori D, Avellini C, Bianchi S *et al*. A case-control study of gastric cancer and diet in Italy. *Int J Cancer*, 1989;44(4):611-616
- 8 Graham S, Haughey B, Marshall J, Brasure J, Zielezny M, Freudenheim J *et al*. Diet in the epidemiology of gastric cancer. *Nutr Cancer*, 1990;13(1-2):19-34
- 9 Kato I, Tominaga S, Matsumoto K. A prospective study of stomach cancer among a rural Japanese population: a 6-year survey. *Jpn J Cancer Res*, 1992;83(6):568-575
- 10 Hu JF, Zhang SF, Jia EM, Wang QQ, Liu SD, Liu YY *et al*. Diet and cancer of the stomach: A case-control study in China. *Int J Cancer*, 1988;41(3):331-335

# Experimental study on mechanism and protection of stress ulcer produced by explosive noise

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**Subject headings** stomach ulcer/etiology; stomach ulcer/prevention and control; gastric mucosa/pathology; noise/advers effects; stress ulcer

## Abstract

**AIM** To establish an experimental model of stress ulcer produced by explosive noise, and to probe into its mechanism and protection.

**METHODS** The country standard Wistar white rats were randomly divided into control group ( $n=8$ ), which were neither stimulated nor protected, and stimulating group (divided into subgroups A, B and C, including 8 rats each which were decapitated to draw blood for test immediately, 12 hours and 24 hours after stimulation) and prevention group (divided into subgroups A, B and C, having 8 rats each, subgroup A was given cimetidine, B anisodamine and C both drugs). Firing noises of submachine guns were used as inflicting factor. The rats were fasted for 24 hours and stimulated by firing noise for 12 hours. The change of ulcer index, gastric mucosal and related serum hormones were observed.

**RESULTS** Stress ulcer was significant in the stimulating group, and its ulcer index ( $8.6\pm 0.6$ ) was remarkably higher than that in both the control group and prevention group ( $0.3\pm 0.1$ ,  $P<0.01$ ). Its serum gastrin (Gas ng/L,  $294\pm 163$  vs  $63\pm 40$ ,  $P<0.01$ ) and endothelin (ET ng/L,  $181\pm 57$  vs  $135\pm 42$ ,  $P<0.1$ ) were apparently higher than those in the control group, and its serum nitric oxide (NO) level was conspicuously lower than that in the control group (ng/L,  $0.2\pm 0.1$  vs  $0.8\pm 0.5$ ,  $P<0.5$ ), while the serum gastrin level (ng/L,  $556\pm 225$ ) in prevention group was distinctly higher than that in both the control ( $P<0.01$ ) and stimulating group ( $P<0.05$ ). There were no significant differences in the changes of ET and NO between the control

and the stimulating groups.

**CONCLUSION** Stress ulcer model of rats can be successfully established by the stimulation of explosive noise. Gas, ET and NO are related to the formation of stress ulcer, and play an important role in its mechanism. Hepatic function affected by noise is observed in this experiment.

## INTRODUCTION

Explosive noise may produce an enormous adverse effect on human body and mind. It has been known that peptic ulcer occurred much more frequently in war time than in peace time<sup>[1]</sup>. However, there have been no reports about the relationship between explosive sound and stress ulcer in literature. The aim of this study is to establish an animal model of stress ulcer produced by explosive noise and probe into its mechanism, prevention and treatment.

## MATERIALS AND METHODS

### Materials

The healthy country standard Wistar rats were provided by the Animal Center of the Fourth Military Medical University. Submachine guns, a precision pulse counter, and a frequency spectrum analyser were provided by the Chinese PLA 371 Hospital. A PJ-2003/50 G-type  $\gamma$  radioimmunity counter (made in Xi'an), 754 spectrophotometer (made in Hefei), a high-speed and low-temperature centrifuge (made in Tumen), a light microscope and a JEM-2000EX-type transmission electron microscope (made in Japan) were used in this experiment.

### Methods

**Preparation of ulcer model.** Having been fasted for 24 hours, the experimental rats were confined in an isolated room to be tested. Firing noise of submachine guns acted as an inducing factor which had been recorded on a tape and was played to the rats through a loudspeaker at a distance of 20cm-30cm. Examined with the precision pulse counter and the frequency spectrum analyser, the intensity of the firing noise was measured as 110dB(A) and its frequency as 0.25kHz-4.00kHz. After the rats were stimulated successively for 12 hours by gun

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Received 1998-05-18

shot sound, their stomachs were opened and the formation of their stress ulcer was observed.

The rats were randomly divided into three groups: ① Control group, consisting of 8 rats, which were neither stimulated nor protected; ② stimulating group, subdivided into groups A, B and C, including 8 rats each. These subgroups were decapitated to draw blood for test immediately, 12 hours, and 24 hours after stimulation respectively; and ③ prevention group, also subdivided into groups A, B and C, having 8 rats each. Group A was injected 40mg ip with cimetidine 30min before the stimulation; group B given 2 mg ip of anisodamine 30min before and 6 hours after the stimulating; group C administered with above mentioned combined drugs. All the rats used in this study were anesthetized in their abdominal cavities with 1.2mg of 5g/L amobabital sodium after treatment and their abdomens were opened for observation on the changes of their gastric mucosa. After decapitation, two blood samples were taken: one for centrifugation of serum, the other added with EDTA and retardant peptidase for separation of plasma and kept at  $-40^{\circ}\text{C}$  in a refrigerator ready for the detection of hormone and hepatic and renal functions.

Measurements of mucosal damage. According to Guth's method<sup>[2]</sup>, the length of gastric mucosal damage area  $<1\text{ mm}$  was scored 1 point;  $1\text{ mm}-2\text{ mm}$  2 points;  $2\text{ mm}-3\text{ mm}$  3 points;  $3\text{ mm}-4\text{ mm}$  4 points,  $>4\text{ mm}$  scored segmentally. The sum total of the scores of the whole stomach constituted the ulcer index. Tissue and cell structures of all groups were observed and photoed under light microscope and electroscope respectively.

Detection of plasma Gas, ET, NO and hepatic-renal functions. Gas was detected before and after stimulation using the kit produced by the Northern Immunity Agents Research Institute in accordance with the instructions. ET was detected using the kit by the East Asian Immunity Technique Research Institute according to the operational manual. NO was tested by means of the kit provided by the Military Medical Academy according to the manual. Hepatic and renal functions were tested by the laboratory from the serum samples we sent.

Statistics. Both t test and analysis of variance were used for this study.

## RESULTS

### *Observation of gross specimens*

In control group, after the gastric wall was opened, the mucosa was seen intact, tidy and smooth without any ulcer or erosion except for a couple of bleeding spots under the mucosa of 2 rats.

In stimulating group, on the abdominal cavity, different degrees of congestion and edema were

found in serous layers and on the gastric walls, congestion, edema, erosion and ulcer formation came into sight (Figure 1), especially in group B (ulcer index,  $8.4 \pm 0.6$ ), the ulcer index being remarkably different ( $P < 0.01$ ) between group B and the prevention group (ulcer index,  $0.3 \pm 0.1$ ) and control group (ulcer index, 0).

In prevention group, although there were still some congestion, edema, erosion and ulcer formation, these were conspicuously lower than in the stimulating group ( $P < 0.01$ ). The preventive effects proved significantly better in group C' ( $0.3 \pm 0.1$ ) than those in groups A' and B' ( $P < 0.05$ ).

### *Examination under light microscope*

In Control group, mucosal layers were smooth and tidy, glands were arranged in order, and no tendency of inflammatory cell infiltration and submucosal hemorrhage appeared. In stimulating group, in sub group A, B and C, there were interruptions of mucosa, enlarged glandular gaps and damaged parts of gland. Meanwhile, a large amount of RBCs were accumulated among the glands. Within submucous eosinophil, infiltration and capillary thrombosis were detected (Figure 2). In prevention group, all of the 3 subgroups were found intact in mucos, well-structured in glands slightly broadened in gaps with some RBCs sparsely existing beneath mucosa.

### *Examination under electron microscope*

The control group showed intact cell structure, regular secretory granules, and no widened gaps among nuclei. In addition to these, mitochondrion and endoplasmic reticulum were also conspicuous. The structure of microvillus was perfect. On the contrary, the stimulation group exhibited irregular cell structure, with nuclei withered, gaps among nuclei broadened, endoplasmic reticulum expanded, mitochondrion hypertrophic, inflammatory cell infiltration in interstitial, and secretory granules increased (Figure 3). The prevention group in appeared differently. Although there were slight withered nuclei, broadened nuclear gaps, and more or less expanded endoplasmic reticulum, all these were noticeably insignificant as compared with those in the stimulating group (Figure 4).

### *Blood biochemical examination*

Gas was apparently higher in the stimulating and prevention groups than in control group ( $P < 0.05$ , Table 1), being most pronounced in stimulating group B.

ET was obviously higher in the stimulating group than in control group, while there were no significant changes between the prevention and control groups ( $P < 0.05$ ), nor between stimulating

and prevention groups ( $P<0.05$ ).

NO was lower in the stimulating group than in control group ( $P<0.05$ ), especially in group C (Table 1). No substantial difference was seen between the prevention and control groups ( $P<$

$0.05$ ). ALT and BUN showed no remarkable changes among all groups ( $P<0.05$ ), while AST was found higher in the stimulating and prevention groups than in controls ( $P<0.05$ , Table 2).

**Table 1** Serum Gas, ET, NO levels in stress rats ( $n=8$ ,  $\bar{x}\pm s$ , ng/L)

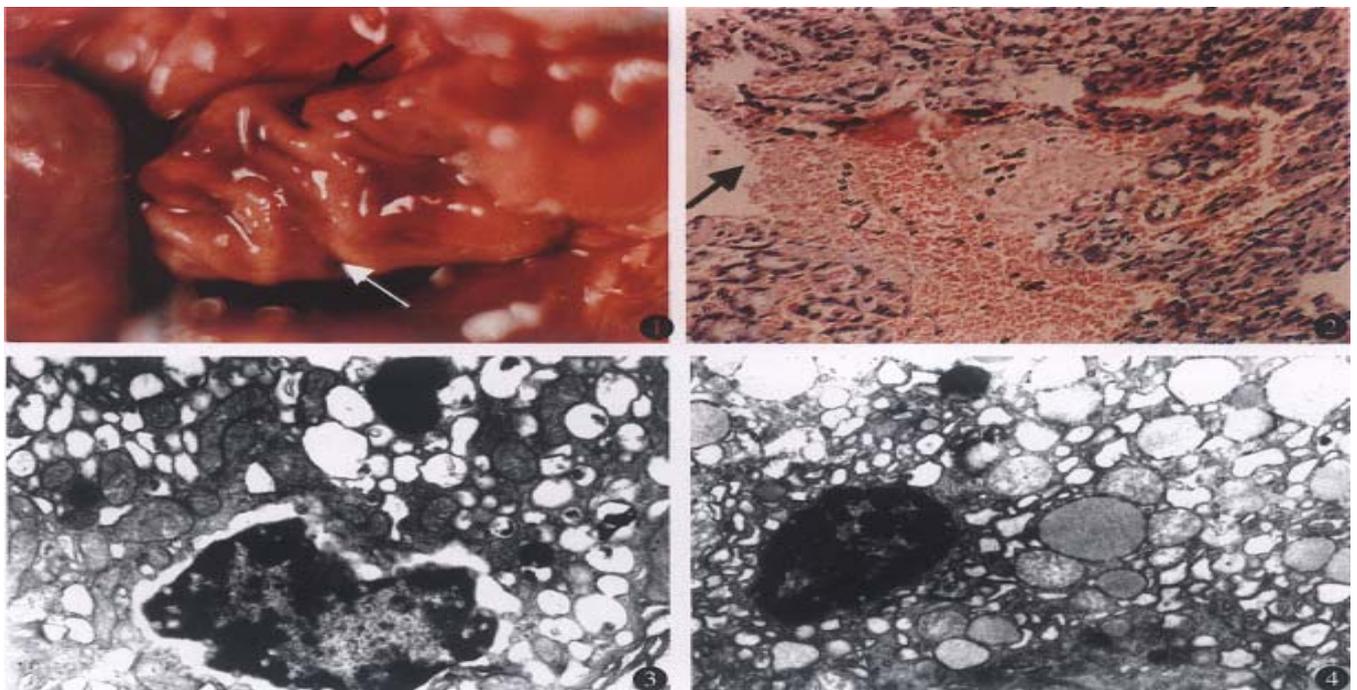
	Controls	Stimulating group			Prevention group		
		A	B	C	A'	B'	C'
Gas	63±40	315±193 <sup>a</sup>	294±163 <sup>a</sup>	70±8	327±93	429±266 <sup>a</sup>	556±225 <sup>b</sup>
ET	135±42	177±87	181±57 <sup>a</sup>	169±26	157±37	158±55	139±43
NO	0.8±0.5	0.5±0.2	0.2±0.1 <sup>a</sup>	0 <sup>b</sup>	0.4±0.3	0.7±0.5	0.7±0.5

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , vs controls.

**Table 2** Serum ALT, AST, BUN level in stress rats ( $n = 8$ ,  $\bar{x}\pm s$ , ng/L)

	Controls	Stimulating group			Prevention group		
		A	B	C	A'	B'	C'
ALT mmol/s	867±67	867±183	997±133	867±117	997±116	804±184	834±100
AST mmol/s	2084±183	2834±583 <sup>a</sup>	2501±450	2450±467	2634±233 <sup>a</sup>	2384±367	2317±283
BUN mmol/L	7.8±0.8	8.4±2.1	7.7±1.5	12.2±1.9	11.5±2.7	8.3±2.1	9.2±1.4

<sup>a</sup> $P<0.05$ , <sup>b</sup> $P<0.01$ , vs control.



**Figure 1** Gross specimens in stimulating group congestion, edema, erosion and ulcer formation.

**Figure 2** Stimulating group: interruptions of mucosa, enlarged glandular gaps, damaged parts and a large amount of RBCs accumulated among glands. HE×10

**Figure 3** Stimulating group exhibited irregular cell structure, with nuclei withered, gaps among nuclei broadened, endoplasmic reticulum expanded, mitochondrion hypertrophic, granules increased. EM×7500

**Figure 4** Prevention group: slight withered nuclei, a little broadened nuclear gaps and more or less expanded endoplasmic reticulum. EM×6000

## DISCUSSION

There are five types of stress ulcers<sup>[3]</sup>, i.e., single bound stress, cold bound stress, socking stress, shock stress and spinal cord injury stress. It has been reported that noise may damage the human body and mind by causing disturbance in the stomach and intestines<sup>[4]</sup> and that ulcer occurred conspicuously more frequently in war time than in peace time<sup>[1]</sup>. Based on these findings and considering that rats are characterized by well-developed adrenalin function, sensitive response to stress and susceptibility to stress ulcer<sup>[5]</sup>, we designed and prepared the stress ulcer model of rats in order to explore its mechanism and prevention and treatment. Our results showed that a typical ulcer model of rats can be successfully established by stimulation with a strong explosive sound continuously for 12 hours. Using Guth's methods<sup>[2]</sup>, the ulcer index was found to be significantly different between stimulating group and both the control group and the prevention group ( $P < 0.01$ ). Such changes as interruptions of mucosa and damage of glands were detected under light microscope (Figure 1). This, therefore, has opened a new path to establish an animal model of stress ulcer.

In an attempt to probe into the underlying causes, we laid special stress on analysing the role of ET, NO and Gas in this disease. To our knowledge, ET serves as a strongest vasoconstrictive substance, while NO functions as a vasodilative one. They are contradictory regulating factors in the blood<sup>[6]</sup>. When balance between the two is lost, the blood flow in gastric mucosa may change and an acute mucosal damage occurs. ET has potent ulcerogenic and vasoconstrictive actions in the stomach where it induces gastric mucosal damage and increases gastric vascular tone<sup>[7]</sup>. In our study, after sound stimulation, serum ET level became conspicuously higher in stimulation group than in control group ( $P < 0.05$ ), indicating ET played an important part in stress ulcer. This was probably because the gun-shot sound overexcited the sympathetic nerves to cause submucosal capillaries to constrict, thus stimulating endodermal cells to produce excessive ET, which, in turn aggravated vasoconstriction and decreased blood flow under mucosa, and, finally, resulted in the erosion and formation of ulcer. NO is produced when arginine guanidine combined with oxygen is converted into nitric oxide under the catalysis of nitric oxide synthetase (NOS), which is the key factor for the production of NO. Kanno *et al*<sup>[8]</sup> reported that an induced NO (iNOS) can be brought about by a variety of cell factors and lipopolysaccharide (LPS) in vascular endothelia and smooth muscles and then produce more and more NO. However, in case of

stress, because of vasoconstriction of gastric mucosa and decrease of blood flow, the production of NO in vascular endothelia can be inhibited, leading to lowered NO level. In addition, erosion and bleeding in mucosa after stress can also cause the synthesized NO to be inactivated by combining with hemoglobin<sup>[9]</sup>, thereby lowering NO level. The decrease of NO level, together with the weakening of mucosal protection and vasodilation, further aggravates erosion of mucosa and formation of ulcer. Our results showed that NO level was noticeably lower in the stimulating group, especially in group C than in control group, which conforms to their findings. After stimulation by explosive noise, with sympathetic nerves overexcited, vagus nerves inhibited<sup>[10]</sup>, cortisol secretion is, of course, increased, resulting in dysfunction of peptidergic nerves in non-adrenergic and non-cholinergic nerves within sympathetic and parasympathetic nerve systems<sup>[11]</sup>, thereby, it causes G-cells to secrete more and more Gas. This may stimulate wall cells to produce excessive gastric acid, pH value in stomach lowered, and  $H^+$  back oozing, leading to inflammation, congestion, edema, erosion in gastric mucosa and even the formation of ulcer. Our results showed that Gas was apparently higher in the stimulating group than in control group ( $P < 0.05$ ). It was discovered under dynamic observation that Gas secretion increased gradually after treatment, reached its peak at the 12th hour, and then returned to normal after 24 hours. All these indicated that Gas not only took part in but played an important role in the process of formation of stress ulcer.

Explosive noise also exerts some harmful effects upon functions of the liver and kidneys. Liu *et al*<sup>[10]</sup> noted that following stimulation by noise, the tension of sympathetic nerves became stronger, and tissue metabolism exuberated, accompanied by vasospasm and tissue ischemia, with the result that the renewal of liver cells hastened and ALT of blood increased. Our results were in agreement with theirs, but insignificant in statistics. This may be due to limited number of samples. AST was remarkably higher in the stimulating group, especially higher group A than in control group ( $P < 0.05$ ). However groups B and C showed a gradual decline. The reason why AST became higher may be that during the stress the sympathetic nerves of rats were so excited that their muscles all over the body kept constricting, and their hearts beat faster for a short time, which caused damages in their skeleton muscle and cardiac muscle cells, and led to the increase of AST release. After removal of stimulation, these actions weakened, cell functions recovered and AST in blood decreased gradually.

The strong noise for a short time, therefore, did little damage to the function of the kidneys.

Our study also proved that among the three prevention groups, group C' for which drugs were given in combination brought best results. Next to this were groups B' and C' treated with anisodamine and cimetidine respectively. Measured by analysis of variance, their ulcer index was significantly different from group A' ( $P < 0.05$ ), corresponding to the previous documents. Following medication, plasma Gas leve rose more remarkably in the prevention group than in the stimulating and control groups. This might be related to the fact that after using H<sub>2</sub> receptor-antagonist and vasodilator agents, the secretion of gastric acid by wall cells was inhibited, and by feedback the secretion of Gas by G-cells at gastric antrium increased. Serum NO and ET did not change conspicuously before and after medication ( $P > 0.05$ ), presumably because H<sub>2</sub> receptor-blocking agents and vasodilator agents reduced the secretion of gastric acid, and abated the stimulation to constriction of submucous blood vessel. On the other hand, it brought about dilatic factors in capillaries, thus the releasing of ET by endodermical cells was weakened. As a result, the producing effect of iNOS was also suppressed so that the formation of NO remained unaffected. Another reason why ET and NO level stayed unchanged was supposedly that the alliviated mucosal erosion and

ulcer reduced the mucosal bleeding and combination of NO with hemoglobin.

From these results, we came to the conclusion that although explosive noise can stimulate the production of stress ulcer, it can be effectively prevented and cured by such medicine as H<sub>2</sub> receptor antagonist and vasodilator agents. Therefore, we believe that use of preventive medicines before war will be of significance for diminution and protection of stress ulcer in wartime.

## REFERENCES

- 1 Zhang XY, Zhang NZ. Modern field of internal medicine (in Chinese). *Beijing: People's Military Medicine Publishing House*, 1997:249-251
- 2 Guth PH, Daures PH, Paulsen G. Topical aspirin plus HCL gastric lesion in the rat. *Gastroenterology*, 1979;76(1):88-93
- 3 Wan JL. Mechanism of formation of stress ulcer (in Chinese). *Chin J Pathophysiol*, 1993;9(5):664-666
- 4 Huang XQ, Wang YZ. Damage of noise to body and mind of patient (in Chinese). *Med Information*, 1995;8(6):279-280
- 5 Shi XY. Medical experimental zoology (in Chinese). *Xi'an: Shaanxi Sci. & Tech Pub House*, 1989:56-62
- 6 Yao Z. Two newfound blood-regulating factors-endothelin and nitric oxide (in Chinese). *Nippon Igaku No Shoukai*, 1994;15(7):527-530
- 7 Battal NM, Hata Y, Ito O, Matsuda H, Yoshida Y, Kawazoe T et al. Reduction of burn induced gastric mucosal injury by an endothelin receptor antagonist in rats. *Burns*, 1997;23(4):295-299
- 8 Kanno K, Hirata Y, Imai T, Marumo F. Induction of Nitric Oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension*, 1993;22(1):34-39
- 9 Lowenstein CJ, Snyder SH. Nitric Oxide: a novel biologic messenger. *Cell*, 1992;70(2):705-711
- 10 Liu ZF, Zhong X, Wu XS, Jiang W, Zhou ZM, Song X. Effects of strong noise on blood viscolity, blood sugar and several enzymes in rats (in Chinese). *Chin J Labor Hyg Occupation Dis*, 1992;10(4):213-216
- 11 Wasnk SA, Pisegra JR, Weerth AD. Brain and gastrointestinal cholecystokinin receptor family. Structure and expression. *Proc Natl Acad Sci USA*, 1992; 89(6):8691-8698

# Effect of devazepide reversed antagonism of CCK-8 against morphine on electrical and mechanical activities of rat duodenum *in vitro* \*

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**Subject headings** duodenum; devazepide; morphine; electrophysiology; cholecystokinin octapeptide

## Abstract

**AIM** To study the antagonism of cholecystokinin octapeptide (CCK-8) against the effect of morphine and its mechanism.

**METHODS** The method and mechanical activities of rat duodenum *in vitro* were recorded simultaneously.

**RESULTS** Acetylcholine (ACh) could increase the amplitude and the number of the spike potential (SPA and SPN) of rat duodenum *in vitro*, followed by the increase of the duodenal contraction amplitudes (CA), showing a positive correlation. Morphine, on the contrary, inhibited the potentiation of ACh, showing a negative correlation. CCK-8 could antagonize the effects of morphine, i.e. the SPA and SPN were increased again, followed by the increase of CA. CCK-A receptor antagonist Devazepide could reverse the antagonism of CCK-8 to the effect of morphine.

**CONCLUSION** CCK-8 could antagonize the effect of morphine which inhibited the potentiation of ACh on the duodenal activities *in vitro*. The antagonistic effect of CCK-8 on morphine was mainly mediated by CCK-A receptor.

## INTRODUCTION

Cholecystokinin octapeptide (CCK-8) is a typical brain-gut peptide. Many data show that CCK-8 has been the strongest endogenous anti-opioid substance up to now. Faris pointed out that CCK-8 could block morphine analgesia in the rat tail flick test<sup>[1]</sup>. Han<sup>[2]</sup> and Xu<sup>[3]</sup> reported respectively that CCK-8 antagonized the analgesic effects of morphine and electroacupuncture (EA), and played an important role in the induction of morphine tolerance and EA tolerance using the behavioral changes and electrophysiological methods. Zetler indicated that morphine and opioid peptides antagonized the hyperfunction of contraction of guinea-pig ileum *in vitro* induced by CCK-8 like peptide<sup>[4]</sup>. Valeri proved that endogenous opioid peptides could antagonize the effects of CCK-8, in the similar experiment<sup>[5]</sup>. But few report about the anti-opioid effect of CCK-8 on the duodenum *in vitro* was found.

In this experiment, the method of simultaneously recording the electrical and mechanical activities of rat duodenum *in vitro* was adopted so as to inquire into the antagonism of CCK-8 to the effect of morphine and its mechanism.

## MATERIALS AND METHODS

### Experimental animals

Twenty-five Wistar rats (Grade II, 195 g - 295 g), Animal Department of Tumour Institute of Heilongjiang Province, were used.

### Experimental animals

Tyrod's solution (made by ourselves); Acetylcholine (ACh, 300nmol/L, Shanghai Third Reagent Factory, China); morphine hydrochloridum (330 nmol/L, Shenyang First Pharmaceutical Factory, China); CCK-8 (0.7nmol/L, Squibb, USA); Devazepide (10nmol/L, Merck Sharp and Dohme Researched Laboratories, USA).

### Experimental method

A rat was given peritoneal anaesthesia with 20%

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\*Supported by the Scientific Technological Foundation of Provincial Education Committee of Heilongjiang in China, No.95-8.

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Received 1998-07-06

urethan (5ml/kg), the peritoneum was opened and one or two segments of 2cm duodenum were cut off under pylorus. The duodenal segments were put into a bathtube containing 50ml Tyrode's solution which was 38°C and saturated with oxygen. Then the bathtube was maintained at 38°C in CS-501 superthermostat. One end of duodenal segment was fixed with resting load of 5g and the other end was connected with a LZ-1 tension transducer according to longitudinal axis of jejunum. Thus contractions of duodenal smooth muscle were recorded. The electrical activities of duodenum were led out by silver adsorptive electrode. Through bioelectrical amplifier, the electrical activities, mechanical contraction and time scale were simultaneously recorded by ST-41 multipurpose polygraph. The parameters were modulated as follows: time constant 0.3 second, high frequency wave filter 30Hz, electrical gain 3, mechanical gain 4, recording paper velocity 5 mm/s<sup>[6]</sup>.

Firstly, normal electrical and mechanical activities of every segment of duodenum was simultaneously recorded. Then 200µl of ACh was injected quickly into the bathtube by a microinjector. Sixty seconds after the injection of ACh, 50µl of morphine hydrochloridum was administered. At 120 seconds and 240 seconds, 40µl CCK-8 and 20µl Devazepide were added respectively.

#### Statistical analysis

Each value was expressed as  $\bar{x} \pm s$ . All data were analyzed with paired *t* test.

## RESULTS

### CCK-8 antagonized the inhibition of morphine to the effect of ACh

Before the injection of ACh, the amplitudes and numbers of the spike potential (SPA and SPN) of 44 duodenal segments and their corresponding contraction amplitudes (CA) respectively averaged

0.70 ± 0.04 mV, 2.41 ± 0.13 and 16.58 mm ± 0.65 mm. At 60 seconds after the injection of ACh, the SPA, SPN and corresponding CA increased to 0.97mV ± 0.05 mV, 2.46 ± 0.11 and 24.50 mm ± 0.99mm, respectively. At this time, morphine was administered. The SPA, SPN and CA decreased to 0.63mV ± 0.04 mV, 2.38 ± 0.08 and 14.73 mm ± 0.69mm respectively at 60 seconds after the injection of morphine. At 120 seconds after adding CCK-8, the SPA, SPN and CA increased to 0.84 mV ± 0.04 mV, 3.29 ± 0.09 and 22.77 mm ± 0.68 mm, respectively. Moreover, all of them showed significant differences (*P*<0.01) when the latter was compared with the corresponding item of the former (Figure 1).

### Devazepide reversed the antagonism of CCK-8 to the effect of morphine

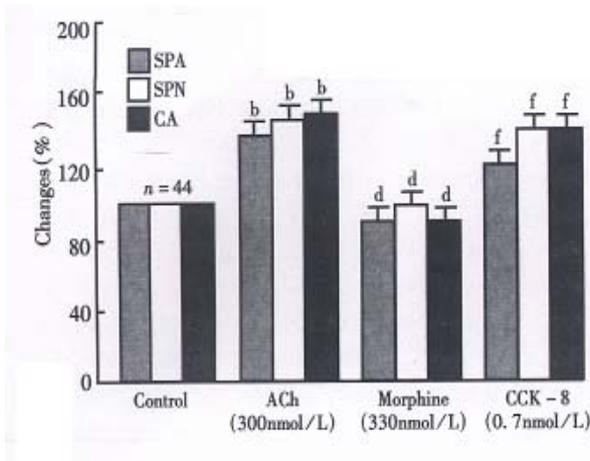
Figure 2 showed the electrical and mechanical change curves of one segment of duodenum simultaneously recorded by multipurpose polygraph. It indicated that the SPA and SPN of the duodenal segment increased, followed by the increase of the CA after the injection of ACh, showing the enhancement of duodenal activities. Whereas the SPA, SPN and corresponding CA were reduced when morphine was administered, showing that morphine inhibited the excitatory effects of ACh. At this moment, the injection of CCK-8 increased the SPA, SPN as well as CA, suggesting an antagonism of morphine effect by CCK-8. On the basis of the above, after CCK-A receptor antagonist Devazepide was administered, the SPA and SPN were decreased again, accompanied by the reduction of CA. It showed that Devazepide reversed the anti-morphine effect of CCK-8. Moreover, every contraction wave occurred after the beginning of spike potential over the slow potential and the ratio between slow potential and contraction wave was 1:1.

The statistical analytical results of 22 duodenal segments are shown in Table 1.

**Table 1** Anti-morphine effect of CCK-8 reversed by Devazepide

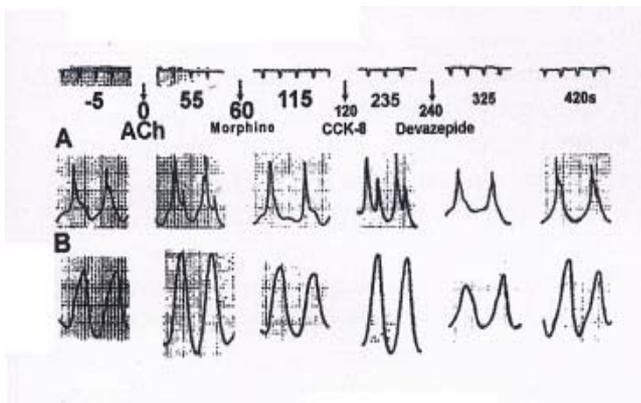
Items	Control <i>n</i> = 22 (0s)	Ach 300nmol/L (60s)	Morphine 330nmol/L (120s)	CCK-8 0.7nmol/L (240s)	Devazepide 10nmol/L (325s)
SPA(mV)	0.70±0.07	0.94±0.09 <sup>b</sup>	0.59±0.06 <sup>d</sup>	0.81±0.07 <sup>f</sup>	0.54±0.05 <sup>h</sup>
SPN	2.71±0.23	3.88±0.15 <sup>b</sup>	2.71±0.09 <sup>d</sup>	3.52±0.13 <sup>f</sup>	2.66±0.18 <sup>h</sup>
CA(mm)	16.40±1.00	24.44±1.63 <sup>b</sup>	13.54±1.04 <sup>d</sup>	22.73±1.00 <sup>f</sup>	13.67±0.66 <sup>h</sup>

<sup>b</sup>*P*<0.01 (ACh vs control); <sup>d</sup>*P*<0.01 (morphine vs ACh); <sup>f</sup>*P*<0.01 (CCK-8 vs morphine); <sup>h</sup>*P*<0.01 (Devazepide vs CCK-8).



**Figure 1** Elimination of inhibited effect of morphine on ACh by CCK-8.

<sup>b</sup> $P < 0.01$  (ACh vs control); <sup>d</sup> $P < 0.01$  (morphine vs ACh); <sup>f</sup> $P < 0.01$  (CCK-8 vs morphine).



**Figure 2** Anti-morphine effect of CCK-8 reversed by Devazepide.

A: electrical activity; B: mechanical contraction; ↓: drug injection; Time scale: 1s.

## DISCUSSION

CCK-8 was the first brain-gut peptide found in human. CCK-8 existed in brain and peripheral tissues of animals and human<sup>[7]</sup>. Previous works indicated that an antagonistic interaction might occur between CCK and opioid peptides. Our experimental results demonstrated that CCK-8 per second did not show any effect, but could selectively

antagonize the effects of morphine which inhibited the potentiation of ACh to rat duodenum *in vitro* with the electrical and mechanical activities. The conclusion was similar to those previous reports<sup>[4,5]</sup>.

Recent receptor binding studies have confirmed the existence of 2 distinct CCK receptor subtypes, i.e. CCK-A and CCK-B receptor were present in both brain and peripheral tissues<sup>[8]</sup>. Devazepide was considerably more potent in inhibiting CCK binding to peripheral-type receptor (CCK-A) than to brain-type receptor (CCK-B)<sup>[9]</sup>. This results showed that Devazepide could reverse the antagonism of CCK-8 to the effect of morphine, therefore it was inferred that CCK-A receptor participates in the anti-morphine effect of CCK-8.

To sum up, our present work firstly demonstrated that CCK-8 could antagonize the elimination of morphine on the potentiations of ACh to duodenal activities, and these effects were mediated by CCK-A receptor. It is suggested that CCK-like peptides and opioid substances together with cholinergic system could regulate the gastrointestinal activities, and provided a new experimental basis for further research in the clinical treatment of the intestinal motility disturbances.

## REFERENCES

- 1 Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science*, 1983;219(4582):310-312
- 2 Han JS, Ding XZ, Fan SG. Is cholecystokinin octapeptide (CCK-8) a candidate for endogenous anti-opioid substrates? *Neuropeptides*, 1985;5(4-6):399-402
- 3 Xu MY, Sun MZ, Zhang LM, Han JS. CCK antagonizes morphine action on activities of pain-related neurons in nucleus parafascicularis. *China J Physiol Sci*, 1992;8(1):6-12
- 4 Zetler G. Antagonism of cholecystokinin-like peptides by opioid peptides, morphine or tetrodotoxin. *Eur J Pharmacol*, 1979;60(1):67-77
- 5 Valeri P, Morrone LA, Pimpinella G, Romanelli L. Some pharmacological characteristics of the guinea pig ileum opioid system activated by cholecystokinin. *Neuropharmacol*, 1990;29(3):231-236
- 6 Yang XP, Xu MY, Ni J, Wang SZ, Bian SL, Gu CR. Electrical change and mechanical contraction of rat jejunum *in vitro* and their relationship. *J Harbin Med Univ*, 1994;28(2):87-90
- 7 Vanderhaeghen JJ, Signeau JC, Gepts W. New peptide in the vertebrate CNS reacting with antigestrin antibodies. *Nature*, 1975;257(5495):604-606
- 8 Hill DR, Campbell NJ, Shaw TM, Woodruff GN. Autoradiographic localization and biochemical characterization of peripheral type CCK receptors in rat CNS using highly selective nonpeptide CCK antagonists. *J Neurosci*, 1987;7(9):2967-2976
- 9 Chang RS, Lotti VJ. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc Natl Acad Sci USA*, 1986;83(13):4923-4926

# Study on the causes of local recurrence of rectal cancer after curative resection: analysis of 213 cases

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Subject headings Rectal neoplasms/surgery; rectal neoplasms/pathology; neoplasm recurrent, local

## Abstract

**AIM** To study the local recurrent rate and the causes of rectal cancer after surgery.

**METHODS** The clinicopathological data of 213 rectal cancer patients and the follow-up information were analyzed. The overall recurrent rate and the recurrent rates from different surgical approaches were calculated. The main causes of recurrence were investigated.

**RESULTS** Among the 213 cases, 73 (34.27%) had local recurrence. The recurrent time ranged from 3 months to 62 months after the first operation. Most of the recurrence (65/73, 89.04%) occurred within 3 years after operation.

**CONCLUSION** Local recurrence had no significant correlation with surgical methods or pathological types, but closely related to Dukes' stages, location of primary tumors and the length of the distal rectum resected. Early resection and a wide tumor free resection margin are key factors to prevent local recurrence.

## INTRODUCTION

For rectal cancer, surgical resection remains the only possible cure. However, long-term survival after surgery is not satisfactory due to local recurrence or distant metastasis. Local recurrence is a major cause of cancer-related morbidity and mortality. To evaluate the rate and find out the causes of local recurrence after radical resection for rectal cancer, we carried out the following study.

## PATIENTS AND METHODS

We studied 213 successive patients (108 males and 105 females) aged 21 to 78 years who underwent curative surgery for rectal cancer between January 1986 to January 1993, in the Institute of Oncology, Hubei Medical University. Primary tumor sites in this series were in the upper segment of their rectum (28), in the middle segment (52), and in the lower segment (133). The pathological types were papillary adenocarcinoma in 33 cases, tubular adenocarcinoma in 121 cases, mucinous adenocarcinoma in 30 cases, villous adenocarcinoma in 10 cases, signet-ring-cell carcinoma in 9 cases, and undifferentiated carcinoma in 10 cases. According to the Dukes' staging system, 50 cases were in stage A, 88 in stage B, and 75 in stage C. The initial operation procedures were Miles operation in 108 cases, Dixon operation in 86 cases and Bacon operation in 19 cases. The recurrence was confirmed by digital rectal examination, ultrasonography, computer tomography (CT) scan, biopsy and pathology, if necessary.

## Statistical analysis

The Chi-square analysis was employed on computer using SAS software to evaluate the difference among different categories, with  $P=0.05$  as the level of significance.

## RESULTS

### Overall rate

Among the 213 cases, 73 (34.27%) had recurrence.

### Time of recurrence

Recurrence within 3 to 24 months after operation happened in 37 cases, within 25 to 36 months in 28

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Received 1998-06-06

cases, and over 37 months in 8 cases. Most of the recurrence (65/73, 89.04%) occurred within 3 years after operation.

#### Site of recurrence

Thirty-five cases recurred in the pelvic cavity, 21 in the anastomosis, 16 in the perineal region and 1 in the abdominal incision.

#### Pathological types and recurrence

The rates of recurrence were higher in mucinous adenocarcinoma and undifferentiated carcinoma than in villous adenocarcinoma, tubular adenocarcinoma, papillary adenocarcinoma and signet-ring-cell carcinoma, although the difference was of no statistical significance ( $P > 0.05$ , Table 1).

**Table 1 Pathological types and local recurrence**

Pathological types	Number	Local recurrence (%)
Tubular adenocarcinoma	121	40 (33.06)
Papillary adenocarcinoma	33	11 (33.33)
Mucinous adenocarcinoma	30	12 (40.00)
Villous adenocarcinoma	10	3 (30.00)
Undifferentiated carcinoma	10	4 (40.00)
Signet-ring-cell carcinoma	9	3 (33.33)
Total	213	73 (34.27)

#### Operational approaches and local recurrence

The rates of local recurrence in Miles, Dixon, and Bacon operation were 37.01%, 31.40% and 31.58%, respectively. The difference was of no statistical significance ( $P > 0.05$ , Table 2).

**Table 2 Operational methods and local recurrence**

Operational methods	Number	Local recurrence (%)
Miles	108	40 (37.01)
Dixon	86	27 (31.40)
Bacon	19	6 (31.58)
Total	213	73 (34.27)

#### The length of distal rectum resected in Dixon operation and anastomotic recurrence

Among the 86 patients who underwent Dixon operation, 27 had local recurrence, 21 of which were anastomotic recurrence. In the 26 cases with a distal resection margin of less than 3 cm, 11 (42.31%) had anastomotic recurrence. However, in the 60 cases with a distal resection margin of greater than 3 cm, only 10 (16.67%) had anastomotic recurrence. The difference was

statistically significant ( $P < 0.05$ , Table 3).

**Table 3 Distal resection margin in Dixon operation and anastomotic recurrence**

Length of distal resection margin	Number	Anastomotic recurrences (%)
<3cm	26	11 (42.31)
≥3cm	60	10 (16.67) <sup>a</sup>
Total	86	21 (24.42)

<sup>a</sup> $P < 0.05$  vs <3cm margin group.

#### Dukes' stages and local recurrence

The rates of local recurrence rose with the increase in Dukes' stages (Table 4).

**Table 4 Dukes' stages and local recurrence**

Dukes' stage	Number	Local recurrence (%)
A	50	6 (12.00)
B	88	30 (34.09) <sup>a</sup>
C	75	37 (49.33) <sup>ab</sup>
Total	213	73 (34.27)

<sup>a</sup> $P < 0.01$ , stage A vs stage B, stage A vs stage C, <sup>b</sup> $P < 0.05$ , stage B vs stage C.

#### Sites of primary tumors and local recurrence

Tumors located in the middle segment of the rectum had a slightly higher rate of local recurrence than those in the upper segment of the rectum ( $P < 0.05$ , Table 5).

**Table 5 Primary tumor sites and local recurrence**

Primary tumor sites	Number	Local recurrence (%)
Upper rectum	28	5 (17.86)
Middle rectum	52	21 (40.38) <sup>a</sup>
Lower rectum	133	47 (37.59)
Total	213	73 (34.27)

<sup>a</sup> $P < 0.05$ , vs middle rectum.

## DISCUSSION

Local recurrence after curative surgery for rectal cancer is a major adverse prognostic indicator.

Although many investigations have been carried out in the prevention, early detection and treatment of this problem, about 7% - 65% of all rectal cancer patients still develop local recurrence<sup>[1-3]</sup>. In our series, the local recurrence rate was 34.27%. We also found that the causes are closely related to Dukes' stages, the length of distal rectum resected and the site of primary tumors, while the

pathological types and operational methods have no significant correlation with the postoperative recurrence.

### Dukes' stages

Dukes' stage is an important factor related to postoperative local recurrence especially the pelvic recurrence. When tumors penetrate the whole rectal wall or metastasize to the regional lymph nodes (stage B and C) the local recurrence rate is 20%-40%. However, when these two negative factors combine together, the local recurrence rate will reach as high as 40%-60%<sup>[4,6]</sup>. In our series of 213 cases, the rate of postoperative local recurrence rose with the advancing stages, which clearly confirms the close correlation between local recurrence and extent of local invasion and regional lymph node involvement. From these observations there is a hope to reduce recurrence if extensive radical resections are routinely performed on patients with Dukes' B or C stage diseases, because these operations will further reduce the unseen residual tumors<sup>[7]</sup>. Other adjuvant treatments such as radiotherapy, chemotherapy or both, may be considered also for these high-risk patients.

### Length of distal resection margin

The nature of transitional mucosa (the mucosa between the normal mucosa and the tumor) has been studied intensively. The transitional mucosa is a highly unstable precancerous lesion which closely links to postoperative recurrence and poor prognosis. The wider this region is, the shorter the post-operative five-year survival will be<sup>[8,9]</sup>.

In clinical practice, when Dixon operation is performed, the length of proximal colon to be resected is seldom limited. However the length of the distal rectum to be resected is limited by several factors, including the preservation of sphincter functions, the available space of pelvic cavity and the operational manipulation. Preservation of sphincters will inevitably limit the length of distal resection margin. Moreover, the lower location of the tumor and the small pelvic cavity set a deep and narrow operation field with very limited exposure, which makes it extremely difficult for the surgeons to achieve a fairly clear distal margin. During the operation, the pulling and tracting will make the distal rectum to be resected seem longer. The intraoperative resection length sometimes is less than the resection length actually required.

The inadequate distal margin means increased chance of residual transitional mucosa or even occult residual cancer cells at the resection margin, which

will eventually result in anastomotic recurrence. In general, a 3cm distal resection is required, while for highly malignant tumors, 5 to 7cm distal margin is necessary<sup>[10,11]</sup>. In our 86 cases of Dixon operation, 21 had anastomotic recurrence. The rates were 42.31% for those with a less than 3cm distal margin and 16.67% for those with a greater than 30cm distal margin.

### Locations of primary tumors

The risk of local recurrence is directly correlated with the location of primary tumors. Cancers at the upper segment of the rectum behave like colon cancer. They are apt to metastasize distantly<sup>[2,3]</sup>. Because of their higher position, better exposure and easier operation manipulation, it is easy to carry out en bloc resection according to the principles of tumor surgery. Therefore, the rate of local recurrence is low. On the other hand, cancers at the middle and lower segments of the rectum are apt to locally recur because of the downward and lateral lymph drainage network and the lack of serosa to ward off local infiltration by the tumor. In these cases even extensive whole pelvic resection often cannot guarantee complete clearance. Therefore, the tendency to local recurrence is relatively high. And there are psychological causes too. For patients with low rectal cancers, Miles operation is the only possible curative treatment. But some of these patients refuse this procedure due to various reasons. Instead they chose Dixon or Bacon operation, which often cannot ensure a true tumor free margin for their conditions. Local recurrence will be unavoidable in some of these patients.

### REFERENCES

- Ballantyne GH, Quin J. Surgical treatment of liver metastases in patients with colorectal cancer. *Cancer*, 1993;71(12):4252-4266
- Vaughn D, Haller DC. Nonsurgical management of recurrent colorectal cancer. *Cancer*, 1993;71(12):4278-4292
- Turk PS, Wanebo HJ. Results of surgical treatment of nonhepatic recurrence of colorectal carcinoma. *Cancer*, 1993;71(12):4267-4277
- Cass AW, Million RR, Pfiarr WW. Patterns of recurrence following surgery alone for adenocarcinoma of the colon and rectum. *Cancer*, 1976;37(6):2861-2865
- Olson RM, Perencevich NP, Malcolm AW, Chaffey JT, Wilson RE. Patterns of recurrence following curative resection of adenocarcinoma of the colon and rectum. *Cancer*, 1980;45(2):2969-2974
- Rich T, Tunderson LL, Lew R, Galdibini JJ, Cohen AM, Donaldson G. Patterns of recurrence of rectal cancer after potentially curative surgery. *Cancer*, 1983;52(7):1317-1329
- Zhang YK, Dong XS, Zhao JH, Liu FS, Zhao TZ, Deng I. The characteristics of lymphatic metastasis in rectal cancer. *J Prac Oncol*, 1989;4(3):134-136
- Colacchio TA, Chabot JA, Zimmerman BW. Differential mucin staining in colorectal neoplasms: Potential clinical application. *Am J Surg*, 1984;147(5):666-669
- Habib N, Salem R, Luck RJ, Blount MA, Rifaat MA, Wood CB. A histochemical methods that predicts local recurrence after curative resection in carcinoma of the colon and rectum. *SGO*, 1984;159(5):436-438
- Goligher JC, Lee PWR, Macfie J, Simpkins KC, Lintott DL. Experience with the Russian model 249 suture gun for anastomosis of the rectum. *SGO*, 79;148(4):517-524
- Yu BM, Jang JT, Shen YX, Wang YC, Zhou XG. Exploration of the operative methods for middle and lower rectal cancers. *J Prac Surg*, 1989;9(11):608-610

# Comparison of long lasting therapeutic effects between succimer and penicillamine on hepatolenticular degeneration \*

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**Subject headings** hepatolenticular degeneration/  
drug therapy; succimer/therapeutic use;  
penicillamine/therapeutic use

## Abstract

**AIM** To compare the long-term effect of succimer (Suc) with that of penicillamine (Pen) in treating hepatolenticular degeneration (HLD).

**METHODS** One hundred and twenty patients with HLD were divided into 2 groups. Group A ( $n=60$ ) received Suc 750mg, po. bid. Group B ( $n=60$ ) received Pen 250mg, po. qid. The period of maintenance treatment varied from 6 months to 3 years, averaging 1.5 years. Symptoms and therapeutic effects were evaluated by modified Goldstein scale.

**RESULTS** The total effectiveness of group A in two different periods of treatment were 80% and 85% respectively, higher than those of group B (58% and 59% respectively) ( $P<0.05$ ). Suc also had obvious curative effects for the patients who failed in the use of Pen. There were fewer side effect in group A than in group B ( $P<0.05$ ). Suc and Pen could increase urinary copper excretion effectively and continually.

**CONCLUSION** Suc is more effective and safer than Pen. Clinically, it can replace Pen as first-choice drug for long-term maintenance therapy of HLD.

## INTRODUCTION

Hepatolenticular degeneration (HLD) is an autosomal recessive disorder that causes changes in the basal ganglia and liver that respectively lead to neuropsychiatric disease, hepatitis and cirrhosis. The patients with HLD have to receive life long decoppering therapy, otherwise the disease will run an invariably fatal course. Penicillamine (Pen) has remained the treatment of first choice for more than forty years because it is readily available and of proven efficacy in some patients. However, the use of this drug is associated with a wide range of toxic reactions and unsatisfactory curative effect for the patients with hepatic type<sup>[1]</sup>. We have used succimer (Suc) to treat HLD since 1990, with satisfactory results. The short-term therapeutic effect of Suc is much better than that of Pen<sup>[2]</sup>. But no study has been carried out to investigate the long-term curative effects of these two copper-binding agents. Our study is to further investigate the long-term therapeutic effects of Suc and Pen.

## MATERIAL AND METHODS

### Subjects

One hundred and twenty patients with HLD were chosen for this study. They were definitely diagnosed after Feb. 1994 and reexamined in our institute from Jan. 1996 to Dec. 1997. They were divided into group A (Suc therapy) and group B (Pen therapy). Based on clinical symptoms, they were classified as neurological type (including Wilson type and pseudosclerosis type) and hepatic type<sup>[3]</sup>. The severity of disease was graded from I to V according to the modified Goldstein method<sup>[4]</sup>. These two groups were comparable for their age, sex, course of disease, period of treatment, clinical classification and severity (Table 1).

### Therapeutic methods

All patients had accomplished synthetical copper-binding therapy with unithiol or EDTA before they started long-term maintenance treatment with Suc or Pen. The period of maintenance treatment varied

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\*Supported by the Natural Science Foundation of Anhui Province, No. 97412001

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from 6 months to 3 years, averaging 1.5 years. The patients of group A were given Suc that was produced by Shanghai Xinya Pharmaceutical Plant. Each pill contains 0.25g mese DMSA. Oral dosage was 20 mg/kg - 30 mg/kg body weight, and administered twice daily. The patients of group B were given Pen produced by Shanghai Xinyi Pharmaceutical Plant. Oral dosage was 20mg/kg-30mg/kg body weight and administered 4 times daily. Considering that both Suc and Pen could chelate other trace and macro-elements in the process of copper-binding, especially zinc element, two hours after taking Suc or Pen, all patients of two groups were given 560mg zinc gluconate. Meanwhile, they were advised to take a copper-poor diet throughout the course of treatment and have their haemogram examined once a week. When patients were reexamined, their clinical conditions were independently judged by two experienced neurologists in our institute. Their haemogram, hepatorenal function, urinary trace and macro-elements were rechecked and compared with previous results. For the patients who did not obtain any effect from treatment, we asked to reexamine 2 months after they inter changed their copper-binding agents between group A and group B.

#### Therapeutic judgement criteria

**Marked effectiveness** Patient conditions were remarkably improved by two grades as compared with those before treatment.

**Improvement** Patient conditions were improved by one grade.

**Ineffectiveness or exacerbation** Patient conditions had no obvious change or became worse.

**Table 1 Characteristics of patients of group A and group B**

Characteristics	Group A (n=60)	Group B (n=60)
Mean age (yr)	20±4.0	18±5.0
Males/Females	34/26	31/29
Mean course of illness (yr)	2.5±2.0	3.0±1.0
Length of treatment		
6mon to 2yrs	40	38
>2yrs	20	22
Clinical classification		
Wilson type	24	26
Pseudosclerosis type	16	15
Hepatic type	10	9
Modified Goldstein Scale		
Grade I	15	18
Grade II	29	27
Grade III	10	11
Grade IV	6	4

## RESULTS

### Clinical effects

We investigated the curative effects of Suc and Pen on HLD in different periods of treatment. The results showed that the total effectiveness of group A was 80% and 85% respectively, higher than those of group B (58% and 59%,  $P<0.05$ ). Among 11 patients of group A who were demanded to replace Suc with Pen because of inefficiency of Suc, 1 (10%) was markedly improved and 2 (18%) improved, with an effectiveness rate of 27%. While in group B, 25 patients who changed to take Suc because of inefficiency of Pen, 5 (20%) were obviously improved, and 12 (48%) improved with an effectiveness rate of 68%. The difference was significant ( $\chi^2=5.1$ ,  $P<0.05$ , Table 2).

**Table 2 Comparison of long-term curative effect of Suc and Pen**

Group	Length of treatment	n	Markedly effective cases (%)	Improved cases (%)	Ineffective cases (%)	Total effective cases (%)
A	6mon to 2yrs	40	10(25)	22(55)	8(20)	32(80) <sup>a</sup>
	>2yrs	20	7(35)	10(50)	3(15)	17(85) <sup>a</sup>
B	6mon to 2yrs	38	5(15)	17(45)	16(42)	22(58)
	>2yrs	22	3(14)	10(45)	9(41)	13(59)

<sup>a</sup> $P<0.05$ , compared with group B.

**Table 3 Urinary trace and macro-elements changes after Suc and Pen therapy ( $\bar{x}\pm s$ )**

Group	Time	n	Copper ( $\mu\text{mol/L}$ )	Zinc ( $\mu\text{mol/L}$ )	Calcium (mmol/L)
A	Before treatment	60	4.4±2.9	4.3±2.5	1.3±0.4
	After treatment	60	19±6.0 <sup>b</sup>	21±7.3 <sup>b</sup>	1.8±0.6
B	Before treatment	60	4.1±2.6	4.7±2.5	1.3±0.4
	After treatment	60	20±6.0 <sup>b</sup>	20±6.9 <sup>b</sup>	1.8±0.5

<sup>b</sup> $P<0.01$ , compared with pre-treatment.

### Side effects

During the period of maintenance treatment, 9 patients (15%) in group A suffered from gingival suffusion and nosebleeding, rash and mild abdominal distension; 22 patients (37%) in group B mainly manifested with high temperature, rash and cytopenia. In both group A and B, there were 5 (5/9) and 16(16/22) patients who had the above side effects from 6 months to 2 years. These side effects could be abated or stopped after the patients were given haemostatic, leukocyte-increasing drugs and antiallergics. No patient in both groups had to stop his treatment because of side effects. The incidence of side-effects in group A was notably lower than in group B, the difference being statistically significant ( $\chi^2=7.36$ ,  $P<0.01$ ).

### Laboratory studies

Long-term copper-binding treatment, remarkably increased the urinary copper and zinc excretion ( $P < 0.01$ ), indicating that both Suc and Pen could effectively facilitate urinary copper and zinc excretion (Table 3). In group B, the white blood counts of patients were decreased, but there was no statistical significance compared with the results of pre-treatment, and no significant changes in hepatorenal function either.

### DISCUSSION

HLD is an autosomal recessive inheritant disease, abnormalities in serum ceruloplasmin, urinary copper excretion and copper accumulation in the liver have for many years suggested a primary defect in copper metabolism as the cause. By now, the gene responsible for HLD was located in chromosome 13q14.3, encoding a P-type ATPase. At least 70 different mutations have since been described in this copper-ATPase gene in individual with HLD<sup>[5]</sup>. Long-term anticopper therapy is necessary for patients to maintain their normal life. All neurologists considered Pen as a drug of first choice before Suc was clinically adopted. Suc is a new broad spectrum heavy metal antidote with low toxicity and high water solubility. It is easily discharged through urine after taken orally. We began to Suc to cure HLD in 1990 and found that it could improve effectively neurologic symptoms and facilitate evidently biliary copper excretion besides increasing clearly urinary copper excretion<sup>[6,7]</sup>.

Comparing the long-term therapeutic effects between Suc and Pen on HLD, we found that Suc was Superior to Pen ( $P < 0.05$ ) because the former caused clinical symptoms to exacerbate less frequently than the latter did. Suc also had obvious curative effects for the patients who failed in Pen treatment. Side effects incidence of Suc was obviously lower than that of Pen ( $P < 0.01$ ). Laboratory results showed that long-term use of Suc or Pen could increase effectively urinary copper and zinc excretion ( $P < 0.01$ ). As Suc has better short- and long-term therapeutic effects, less side effects and permanent urinary copper excretion function, we recommend that Suc should be used as a drug of first choice in long-term maintenance therapy of HLD.

### REFERENCES

- 1 Yang RM, Bao YC, Jiang TZ, Ren MS. Side effects of penicillamine in treating 128 patients with hepatolenticular degeneration. *New Drugs Clin Remed*, 1986;5(2):91-93
- 2 Ren MS, Yang RM, Zhang B, Xu SH. Comparison of therapeutic effects between unithiod, succimer and penicillamine on hepatolenticular degeneration. *Chin J New Drugs Clin Remed*, 1998;17(1):23-25
- 3 Walshe JM. Wilson's disease. In: Vinken PJ, Bruyn GW, Klawans HL, eds. *Extrapyramidal disorder: handbook of clinical neurology*. Vol. 5 (49). Amsterdam: Elsevier, 1986:223-228
- 4 Yang RM. Hepatolenticular degeneration. Ed 1. Hefei: Anhui Science and Technology Publishing House, 1995:204-205
- 5 Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW. The wilson disease gene: spectrum of mutations and their consequences. *Nature Genet*, 1995;9(2):210-217
- 6 Ren MS, Yang RM. Clinical curative effect of DMSA and impact on biliary trace elements of HLD. *Chin J Nervous Mental Dis*, 1990;16(6):32-35
- 7 Ren MS, Zhang B, Yang RM, Han YZ, Wang X. Clinical study of integrated traditional and western medicine in hepatolenticular degeneration. *WCJD*, 1998; 6(1):7-9

# An experimental study in etiologic effect of pancreas divisum on chronic pancreatitis and its pathogenesis \*

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**Subject headings** pancreatitis/etiology; pancreatitis/physiopathology; pancreas divisum/physiopathology; pancreas divisum/etiology; chronic diseases

## Abstract

**AIM** To investigate the etiologic association of pancreas divisum (PD) with chronic pancreatitis and to clarify its pathogenesis.

**METHODS** A PD canine model was established in 32 dogs. The dogs were randomly divided into 4 groups ( $n=8$ ). Group I: The communicating branch between the dorsal and ventral pancreatic ducts was partly ligated Group IIa: The communicating branch was amputated and completely ligated Group IIb: The dorsal duct was amputated and ligated at 2mm distance to the minor papilla. Group III: A sham operation without any amputation or ligation was performed. Before and after operation, the activities of serum phospholipase A2 (PLA2) and amylase (Ams) were assayed and the basal pressures of the ducts were measured when secretin was injected. Pancreatic ductography and the pathologic examination were made.

**RESULTS** The activities of serum PLA2 and ams in Group I, IIa, and IIb were significantly increased 5 - 80 days after operation. At sacrifice, the basal pressures of the ventral duct were significantly higher 30min-60min after provocation in Group I, IIa and IIb. The pressures of the dorsal duct were significantly increased in Group IIb but no difference in Group I and IIa. Under light microscopy the fibrosis of interlobus and periducts, the destruction of acini and infiltration of inflammatory cell in dorsal and ventral pancreas were found in Group IIb. But in Group I and

IIa, this findings were present only in ventral pancreas. The electron microscopy showed that in ventral pancreas of Group I and IIa and the dorsal and ventral pancreas of Group IIb, the rough endoplasmic reticulum of the acinar cells showed granules-scaling, fusion and dilatation. The zymogen granules decreased and the mitochondria was swollen.

**CONCLUSION** PD is one of etiologic factors in chronic pancreatitis. The pathogenesis is the functional obstruction of the minor papilla at the peak stage of secretion.

## INTRODUCTION

The patients with pancreas divisum (PD) were considered to have a higher risk for chronic recurrent pancreatitis. But the ant the etiology and pathogenesis are still unclear. We established a canine model of PD in 32 dogs for investigating the etiologic association and clarifying the pathogenesis.

## MATERIALS AND METHODS

### *Animal model*

Thirty-two healthy adult dogs of both sexes weighing about 10kg were used. Prior to the experiments, they were fasted for 24 hours. Under the sodium pentobarbital anesthesia, an abdominal midline incision was made and the head of pancreas was exposed. The partial ligation or amputation of the pancreatic duct was randomly made in 4 groups. Group I: the communicating branch between the dorsal and ventral pancreatic ducts was partly ligated at the middle segment ( $n=8$ ). Group IIa: the communicating branch was amputated and the remaining stump was ligated ( $n=8$ ) Group IIb: the dorsal pancreatic duct was amputated and ligated at a 2-mm distance from the minor papilla ( $n=8$ ).

### *Pancreatic enzymes assay*

The activities of serum phospholipase A2 (PLA2) and amylase (Ams) were assayed before ligation

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Received 1998-06-10

and 5, 10, 15, 30, 50, 80, 120, 150 and 180 days after operation by the method of radioimmunoassay<sup>[1]</sup> and blue starch respectively.

### **Manometry of pancreatic duct**

The pressure of the dorsal and ventral pancreatic duct was measured before ligation and at sacrifice at 0, 15, 30, 60 and 90 min after intravenous injection of secretin (Kabi Vitrum, Stockholm, Sweden) at a dose of 11 U/kg. A polyvinyl catheter with a 0.8mm inner diameter and 1.0mm outer diameter was used. The probe contained an end orifice measuring 0.8mm in diameter and was filled with sterile saline. The perfusion took place at a constant rate of 0.25ml/min. The pressure was recorded on a thermal pen recorder (Nippon Sanei 360, Tokyo, Japan).

### **Pancreatic ductography**

Pancreatography was performed by retrograde perfusion<sup>[2]</sup> via the major and minor papilla cannulation with 180mgI/ml Omnipaque (Nycomed Co, Norway) before operation and at sacrifice for investigating the pancreatic ductal changes.

### **Tissues preparation for light and electron microscopy**

The specimens for light microscopy were fixed with 10% formalin solution and embedded with paraffin wax. The sections were HE stained. Blocks of tissues (1.0mm<sup>3</sup>) cut from pancreas and duodenal papilla for electron microscopy were fixed with 2.5% glutaraldehyde, postfixed with 1.0% OsO<sub>4</sub> dehydrated with ethanol, and embedded with Epon 812. The sections stained with uranyl acetate and lead citrate were examined under the Hitachi H-600 electron microscopy.

### **Statistical analysis**

Results were expressed as mean±SD. Comparisons among groups were made using Scheffe's multiple test or Spearman's rank correlation analysis.

## **RESULTS**

### **Biochemical assay**

In Group IIb, the activities of the serum PLA<sub>2</sub> and Ams were significantly increased after operation, especially during the postoperative 15-80 days. The activities in Groups I and Group IIa were slightly increased during the postoperative 5-15 days, but there was no statistically difference as compared with preoperation. There were no changes in the activities in Group III before and after operation.

### **Manometries of pancreatic duct**

Before operation, the basal pressures of the dorsal and ventral duct were 0.78 kPa±0.21 kPa and 0.69 kPa±0.24 kPa, respectively. When the secretin was administered, they were slightly increased at 30 and 60 min, but with no statistical difference (all  $P>0.05$ ). During 60min-90min, the pressures returned to the preoperative levels.

### **At sacrifice**

In ventral duct, the basal pressures in Groups I, IIa, IIb and III were 0.80 kPa±0.3 kPa, 0.79 kPa±0.28 kPa, 0.64 kPa±0.20 kPa and 0.83 kPa±0.24 kPa, respectively. There was no significant difference as against preoperation. After secretin injection, the pressure at 30 min was significantly higher than that before operation in Groups I, IIa and IIb. At 60 min, the pressures in Groups I and IIa returned to the preoperational level at 90 min. In dorsal duct, the pressures were significantly increased in Group IIb ( $P>0.001$ ). In Groups I, IIa and III, there were no significant differences before and after secretin injection.

### **Pathological changes**

Light microscopy. In ventral pancreas, interlobular or/and periductal fibrosis, the destruction of acini and infiltration of inflammatory cells were found in Group IIb, slight periductal fibrosis in Groups I and IIa, and no abnormal histological changes in dorsal pancreas in Groups I and IIa, but chronic pancreatitis was present in Group IIb. The duodenal papilla was histologically normal.

Electron microscopy. The rough endoplasmic reticulum of the dorsal and ventral pancreatic acinar cells in Groups I, IIa and IIb showed granulescaling, fusion and vacuolar dilatation. The zymogen granules decreased and their electron density reduced. The mitochondria were swollen and the space around the nucleus was increased.

## **DISCUSSION**

PD had been recognized by the 17th century and it was found in approximately 7% of western autopsies. A higher incidence of chronic pancreatitis was discovered among the patients with PD, but their etiologic association has not been established, because so far there has been no animal models concerning PD. We established the animal model in 32 dogs through partial ligation or amputation of the communicating branch between the dorsal and ventral pancreatic duct. Not only the anatomic shapes but also the pathophysiological bases were similar to that in human. PLA<sub>2</sub> presents in the pancreas as an inactive zymogen, and inappropriate

intrapaneatic activation of PLA2 is thought to play an important role in the development of pancreatitis. In this study, the PLA2 activities in Groups IIa and IIb, especially in Group IIb were significantly increased, indicating the possibilities of pancreatitis. It was reported that PLA2 activities could be used to evaluate the severity of acute pancreatitis<sup>[3]</sup>. In Group IIb, due to all the pancreatic secretions occurring through the major papilla, not as the other groups, it was not surprising that more severe pancreatitis was induced.

The previous studies concerning the drainage of the pancreatic secretions were to measure the diameter of the duct by means of CT or ultrasound during secretin provocation<sup>[4,5]</sup>. It was not accurate because dilatation occurred in the pancreatic ducts when the periductal fibrosis was obvious. In the present study, the pressures of the ventral duct in Group IIb at 30 and 60min were significantly higher than that before provocation. It implied the existence of physiologic obstruction. Chronic ventral and dorsal pancreatitis was found in Group IIb. But the similar changes were not seen in Groups I and III, and slight in Group IIa, and the

duodenal papilla was histologically normal, confirming the association of pancreatitis with relative obstruction of secretion drainage. In Groups I and IIa, the communicating branch between the ventral and dorsal ducts were partly ligated or amputated. The juice secreted from the dorsal and ventral pancreas drained from the minor and major papilla, respectively. So, it is not likely to block the drainage. In Group IIb, all of the secretions drained via the major papilla. To block the drainage at the peak stage of the secretion was not avoided. Therefore, we have come to the conclusion. That there was clear etiologic association between PD and chronic pancreatitis.

## REFERENCES

- 1 Warsaw AL, Simeone JF, Schapiro RH, Warsaw BF. Evaluation and treatment of the dominant dorsal duct syndrome (pancreas divisum redefined). *Am J Surg*. 1990;159(1):59-66
- 2 Lu WF, XU P, Zhu CR, Wu BH. The ERCP diagnosis and its significance of pancreas divisum. *Chin J Radiol*. 1996;30(3):175-178
- 3 Kitagawa M, Hayakawa T, Kondo T, Shibata T, Sakai Y, Sobajima H et al. The diagnosis value of serum pancreatic phospholipase A2 in pancreatic diseases. *Gastroenterol Japonica*. 1991;26(1):62-64
- 4 Lindstrom E, Ihse I. Dynamic CT scanning of pancreatic duct after secretin provocation in pancreas divisum. *Dig Dis Sci*. 1990;35(10):1371-1378
- 5 Warsaw AL, Simeone J, Schapiro RH, Hedberg SE, Muller PE, Ferrucci JT. Objective evaluation of ampullary stenosis with ultrasonography and pancreatic stimulation. *Am J Surg*. 1985;149(1):65-72

# Preliminary study on the production of transgenic mice harboring hepatitis B virus X gene \*

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**Subject headings** hepatitis B virus; gene, viral; transgenic animals; liver neoplasms; diseases models, animal

## Abstract

**AIM** To establish transgenic mice lineage harboring hepatitis B virus X gene and to provide an efficient animal model for studying the exact role of the HBx gene in the process of hepatocarcinogenesis.

**METHODS** The HBx transgenic mice were produced by microinjecting the construct with X gene of HBV (subtype adr) DNA fragment into fertilized eggs derived from inbred C57BL/6 strain; transgenic mice were identified by using Nested PCR; expression and phenotype of HBx gene were analyzed in liver from transgenic mice at the age of 8 weeks by RT-PCR, pathologic examination and periodic acid-schiff staining (PAS), respectively.

**RESULTS** Five hundred and fourteen fertilized eggs of C57 BL/6 mice were microinjected with recombinant retroviral DNA fragment, and 368 survival eggs injected were transferred to the oviducts of 18 pseudopregnant recipient mice, 8 of them became pregnant and gave birth to 20 F1 offspring. Of 20 offsprings, four males and two females carried the hybrid gene (HBx gene). Four male mice were determined as founder, named X1, X5, X9 and X15. These founders were back crossed to set up F1 generations with other inbred C57BL/6 mice or transgenic littermates, respectively. Transmission of HBx gene in F1 offspring of X1, X5 and X9 except in X15

followed Mendelian rules. The expression of HBx mRNA was detected in liver of F1 offspring from the founder mice (X1 and X9), which showed vacuolation lesion and glycogen positive foci. **CONCLUSION** Transgenic mice harboring HBx gene were preliminarily established.

## INTRODUCTION

Despite overwhelming epidemiological evidence linking persistent Hepatitis B Virus (HBV) infection and the development of hepatocellular carcinoma (HCC)<sup>[1]</sup>, the pathogenetic mechanisms by which the virus contributes to liver cell transformation remain elusive. The case is mainly the limited host range and the lack of *in vitro* culture systems to propagate it. With the development of embryo microinjection technology, leading to the production of transgenic animals, it provided a powerful tool for studies of gene expression and creation of animal models of human diseases.

The HBx gene is one of the four genes in the HBV genome. It encodes a viral transactivator, the HBx antigen (HBxAg). This protein has been shown to convert immortalized mouse fetal hepatocytes into a fully malignant phenotype. Moreover, NIH 3T3 cells stably transfected with a HBx expression plasmid are carcinogenic in nude mice. Interestingly, recently it has been reported that the P53 and HBx proteins can be co-immunoprecipitated from HBV-related HCCs. Complexing of P53 protein by HBx protein should inhibit its DNA consensus binding and transcriptional transactivator function and provide a basis for the ways in which this interaction might contribute to malignant transformation<sup>[2]</sup>.

These observations suggest that HBx may play a part in the molecular pathogenesis of HCC in humans. In support of this hypothesis, it has been shown that high level expression of the HBx gene can lead to HCC in transgenic mice<sup>[3-5]</sup>, although others have not observed the induction of HCC in independently derived X gene transgenic mouse strains<sup>[6-8]</sup>. Noticeably, the mice that develop HCC

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\*Supported by the National the 9th 5-year Program of China, No. 969060107 and the Army Natural Science Foundation of China, No. 96MO92

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Received 1998-06-10

were produced and maintained on a CD1 background that displays a high spontaneous rate of HCC. This might suggest that the HBx protein functions are a cofactor in the process of hepatocarcinogenesis, and that it may not be sufficient to induce HCC by itself<sup>[9]</sup>.

To investigate the exact role of the HBx gene in the process of hepatocarcinogenesis. The HBx transgenic mice were produced by microinjecting the HBx gene of HBV (subtype adr) DNA fragment into fertilized eggs derived from inbred C57BL/6 strain mice that displays a lower spontaneous rate of HCC, and its phenotype were preliminarily analyzed by pathologic examination and PAS.

## MATERIALS AND METHODS

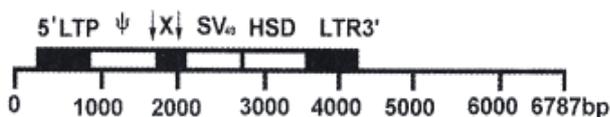
### Materials

Various restriction enzymes and other reagents. Modifying enzymes ECOR I, Hind III, BamHI, T4 DNA ligase, dNTP, Taq DNA polymerase, MMLV reverse transcriptase, etc, purchased from Promega, Sangon and Sigma Company, respectively.

Mice. C57BL/6 mice (clean animal) were purchased from Animal Center. Shanghai.

### Methods

**Construction of recombinant plasmid.** PSHDX42, a 6787bp recombinant retroviral vector plasmid containing the X gene of HBV (subtype adr) was constructed by DNA recombinant technique (Figure 1) (Plasmid constructs is to be published elsewhere).



**Figure 1** Structure of recombinant plasmid PSHDX42  
X: HBx gene; ↓: BamHI

**Production of HBx transgenic mice.** Plasmid PSHDX42 was linearized with Ecor I and purified with QIAGEN. It was microinjected into the male pronucleus of the fertilized eggs of C57BL/6 mice. The injected eggs were then transplanted into the oviduct of pseudopregnancy mice. Operated mice got pregnant and given birth.

**Detection of transgenic integration.** Genomic DNA was extracted from transgenic mice tail (about 1cm -1.5cm) at age of 2-3 weeks. Total DNA was extracted by the method described by Sambrook<sup>[10]</sup>.

**Nested-PCR.** The primers were designed on human HBX (subtype adr) X gene sequence (synthesized by Cybersyn Biotechnology Company). The sequence of primer for the PCR were as follows:

Outer primer 1: 5'-CATGGCTGCTCGGGTGTGCT-3'  
2: 5'-ATTAGGCAGACGTGAAAAG-3'  
Inter primer 3: 5'-CTTTGTCTACGTCCCGTCGGCGCTGAATC-3'  
4: 5'-CAGTCTTTGAAGTATGCCTCAAGGTCGGT-3'

The PCR reaction I contained 5μl of 10×PCR Buffer, 3μl of 25mM Mg<sup>2+</sup>, 3μl of 15mM dNTP, 1.5μl of 20pmol each of outer primer, 2U of Taq DNA polymerase, 1μg sample DNA. A 50μl total reaction volume was obtained by adding sterile water, the reaction procedure of PCR I was: beginning 5min at 95°C, then 30 cycles of 94°C denature for 50 sec, annealing at 55°C for 1min and extension at 72°C for 1min. The PCR reaction II contained 5μl of 10×PCR buffer, 3μl of 25mM Mg<sup>2+</sup>, 3μl of 15mM dNTP, 3μl of 10pmol each of inter primer, 2.5U of Taq DNA polymerase, 1μl-2μl PCR I products. The reaction procedure of PCR II was as follows: beginning 5 min at 94°C, 30 cycles of 94°C denature for 50 sec, annealing at 60°C for 50 sec and extension at 72°C for 1 min, and final extension at 72°C for 10min. A 1.8% agarose gel was prepared and loaded with 10μl PCR II products, then electrophoresed at 45vol for 70min, and the result of electrophoresis was observed.

**Detection of transgenic expression.** Tissue samples were removed from F1 transgenic mice liver at the age of 8 weeks and frozen in liquid nitrogen. Total RNA was extracted by the method described by Sambrook<sup>[10]</sup>.

**RT-PCR.** The sequence of primers for RT-PCR were previously described as outer primer 1,2. First stranded cDNA was synthesized as follows: The RT reaction contained 1μg RNA, 5μl of 10×PCR buffer, 3μl of 15 mM dNTP, 1μl of RNasin (80U/μl), 2μl 10pmol outer primer 1 or 2, 1μl MMLV (200U). A 50μl total reaction volume was obtained by adding sterile DEPC treated water and RT was followed at 37°C for DNA polymerase, 3μl of 10pmol outer primer 1,2 respectively. The reaction procedure of PCR was described as PCR I.

**Histological procedures and cytohistochemical staining.**

Liver tissues from F1 transgenic mice at the age of 8 weeks were divided into two portions: one portion fixed in 10% buffered formalin for Hematoxylin and Eosin as well as PAS stainin, the other quickly frozen in liquid nitrogen and used for RNA analysis.

## RESULTS

### *Establishment of transgenic mice*

Recombinant retroviral DNA fragment was microinjected into the male pronucleus of the 514 fertilized gees of C57-B1/6 mice, and 368 survived eggs were transferred to the oviducts of 18 pseudopregnant recipient mice, 8 of them became pregnant and gave birth to 20 offspring mice. The zygotes survival rate and birth rate were 71% (368/514) and 5.4% (20/368). DNA extracted from tail of 20 offspring mice were screened by PCR amplification (Figure 2), and the results showed that four males and two females carried the hybrid gene (HBx gene). Four male mice were determined as founders, named X1, X5, X9 and X15. Total integration rate and efficiency of transgene was 30% and 1.1%, respectively.

### *Establishment of HBx transgenic mice lineage*

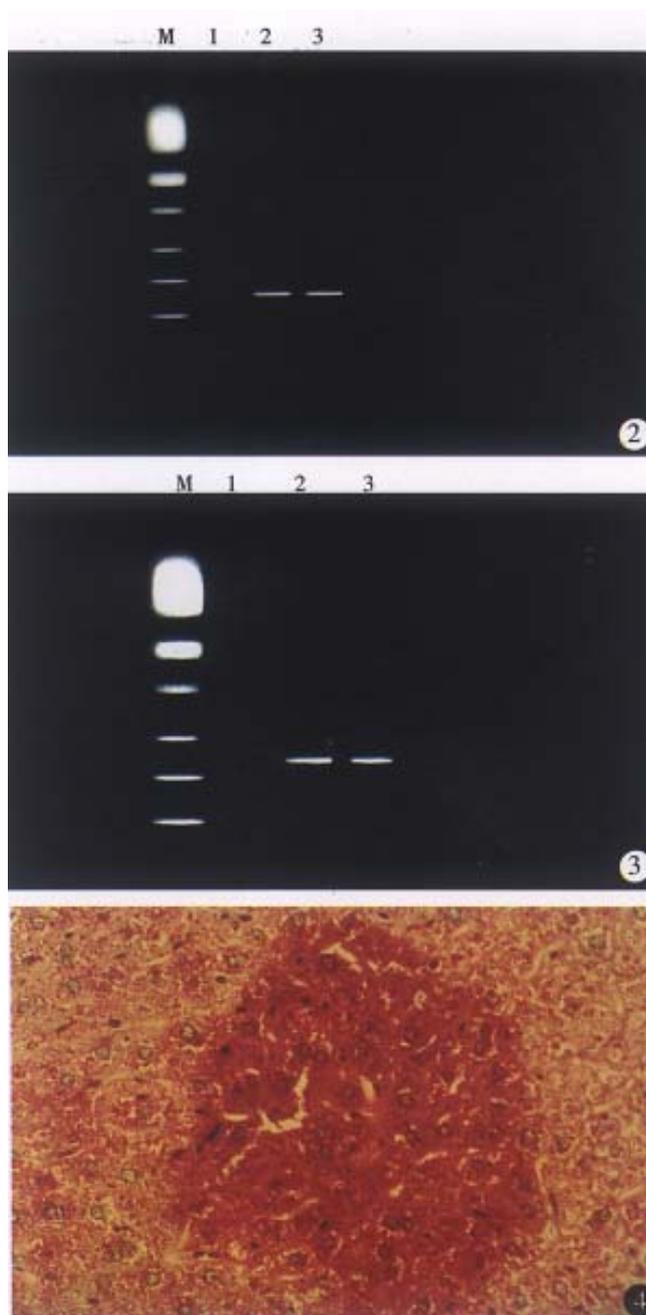
The founders (X1, X5, X9 and X15) were back crossed to set up F1 offspring with other inbred C57BL/6 mice or transgenic littermates. Of 20 F1 mice from X1, 9 F1 mice carried the hybrid gene; of 18 F1 mice from X5, 8 F1 mice carried the hybrid gene; of 17 F1 mice from X9, 8 F1 mice carried the hybrid gene. Surprisingly, no mice integrated from F1 generation of X15 were detected by PCR.

### *Expression of HBx gene in F1 mice*

We performed RT<sup>2</sup>PCR to examine whether HBx transgenes express in liver tissues of F1 mice of transgenic mouse lineages (X1, X5 and X9). The PCR products (439bp) were detected in the liver of F1 mice of transgenic mouse lineage (X1, X9) (Figure 3). NO Band was detected in the liver of F1 offspring of transgenic mouse lineage X5.

### *Phenotypic characteristics of transgenic F1 mouse*

The liver from the F1 offspring of lineage (X1, X9) showed the presence of areas of altered hepatocytes which were made up of cells with poorly stained cytoplasm and PAS positive foci (Figure 4), but not in their normal littermates.



**Figure 2** Electrophoresis analysis of Nested PCR products amplified from DNA obtained from tail tissue. lane 1, normal mouse (negative control); lane 2 and 3 (transgenic mice) showed positive bands (280bp). M, PCR marker (SABC).

**Figure 3** Electrophoresis analysis of RT-PCR products. Lane 1, normal mouse liver tissue (negative control); lane 2 and 3 transgenic mice liver tissue showed positive bands (439bp). M, PCR standard marker (SABC).

**Figure 4** Two-month old transgenic mouse liver showed glycogen positive foci PAS×200

## DISCUSSION

Despite the increased frequency in the generation of transgenic animals, the methodologies for introducing exogenous DNA into mammalian early embryos have remained essentially unchanged since

the pioneering work of Gordon in the early 1980s<sup>[11]</sup>. To date, the most widely used method is microinjection of foreign DNA into the pronuclei of fertilized eggs. The method has enabled the production of transgenic animals from various mammalian species, but the success rate of generating a transgenic animal is extremely low. In this study, the zygotes survival rate and birth rate were 71% and 5.4% and efficiency of transgene was 1.1%. The low efficiency of transgene may be influenced by many factors such as DNA structure, purity and concentration, gene vector and fertilized eggs as well as mouse strains, etc<sup>[12]</sup>. The result of F1 generation mice integration demonstrated that foreign gene can stably transmit to next generation in a normal Mendelian fashion. As for F1 generation from founder X5 low integration efficiency, it was thought that the founder X5 was likely to mosaics. The mechanism of DNA integration into mouse chromosomes following microinjection is unknown. One possibility is that spontaneous breaks occur in chromosome, possibly exacerbated by the microinjection technique and DNA repair endonuclease, and these breaks are sites for integration of linear DNA<sup>[12]</sup>

When the transgene results from random integration of a DNA fragment injected into fertilized eggs the pronucleus, its level of expression in the resulting transgenic animal varies and depends upon factors, such as the site, transgene copy number, genetic background of mouse, particularly promoter/enhancer of fusion gene. In this study, HBx gene under the control of retroviral promoter/enhancer LTR and SV40 promoter, the expression of HBx gene was detected by RT-PCR in liver from partial transgenic mice lineage (X1, X9). The results demonstrated that the retroviral promoter/enhancer LTR and SV40 promoter were probably

functional, and HBX gene expression was influenced by different integrated site.

Above all initial histological examination of liver in HBX gene transgenic mice revealed the presence of pathological change (vacuolation lesions) and glycogen positive foci. The results of phenotype observation were similar to KIM's<sup>[3]</sup>, and were consistent with their preneoplastic lesions, and further identified HBx transgenic mice model established. To sum up, we have produced transgenic mice harboring HBx gene. This work has laid a solid foundation for further study on the function of HBx gene in hepatocarcinogenesis, successively analysis on these transgenic mice are being undertaking.

## REFERENCES

- 1 Beasley RP, Hwang LY. Epidemiology of hepatocellular carcinoma, viral hepatitis and liver disease. In: GN Vyas, JL Dienstag, JH Hoofnagle. eds. *New York, Grune and Stratton, Inc.* 1994:109-224
- 2 Feitelson MA, Duan LX. Hepatitis B virus X antigen in the Pathogenesis of chronic infections and the development of hepatocellular carcinoma. *Am J Pathology*. 1997;150(4):1141-1157
- 3 Kim CM, Koike K, Saito I, Miyamura T, Jay G. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature*, 1991;351(23):317-320
- 4 Koike K, Moriya K, Iino S, Yotsuyanagi H, Endo Y, Miyamura T et al. High level expression of hepatitis B virus HBx gene and hepatocarcinogenesis in transgenic mice. *Hepatology*, 1994;19(4):810-819
- 5 Yoo YD, Ueda H, Park K, Flanders KC, Lee Y LK, Jay G et al. Regulation of transactivator. *J Clin Invest*, 1996;97(2):388-395
- 6 Lee TH, Finegold MJ, Shen RF, Demayo JL, Woo SL, Butel JS et al. Hepatitis B virus transactivator X protein is not tumorigenic in transgenic mice. *J Virol*, 1990;64(12):5939-5947
- 7 Perfumo S, Amicone L, Colloca S, Giorgio M, Pozzi L, Tripodi M et al. Recognition efficiency of the hepatitis B virus polyadenylation signal is tissue specific in transgenic mice. *J Virol*, 1992;66(11):6819-6823
- 8 Billet O, Grimber G, Levrero M, Seye KA, Briand P, Joulin V et al. In vivo activity of the HBV core promoter: tissue specificity and temporal regulation. *J Virol*, 1995;69(9):5912-5916
- 9 Chisari FV. Hepatitis B Virus transgenic mice: insights into the virus and the disease. *Hepatology*, 1995;22(4):1314-1325
- 10 Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual* 1. 2nd ed. Cold Spring Harbor Laboratory Press, 1989:343-361
- 11 Gordon JW, Scangos GA, Plotkin DG, Barbosa JA, Ruddle Fu. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci USA*. 1980;77(14):7380-7384
- 12 Eringer RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc Natl Acad Sci USA*, 1985;9(82):4438-4442

# Presence of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells \*

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**Subject headings** liver neoplasms; BEL-7404; Fas gene; Bcl-2 proteins; gene expression; apoptosis

## Abstract

**AIM** To study the expression of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells in order to analyze the possible relationship between cell growth regulation by alpha-fetoprotein(AFP) and Fas/Bcl-2 proteins.

**METHODS** BEL-7404 human hepatoma cells were maintained in RPMI 1640 medium supplemented with 10% new-born calf serum. Cells adhered to coverslips were used to detect Fas and Bcl-2 protein expression by the avidin-biotin complex (ABC) immunocytochemical assay.

**RESULTS** Immunocytochemical study showed that essentially all the BEL-7404 human hepatoma cells could express Fas and Bcl-2 proteins, although in various amount. No positive staining for Fas and Bcl-2 proteins was observed when cells were incubated with non-relevant sera, to establish the specificity.

**CONCLUSION** Fas apoptosis signals and Bcl-2 rescue/survival signals from apoptosis are expressed in BEL-7404 human hepatoma cells. The finding strongly implies that AFP-mediated cell apoptosis and growth enhancement are potentially associated with Fas and Bcl-2 proteins present in those cells.

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\*Supported by National Postdoctor Science Foundation of China, No. 199711.

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Received 1998-07-15

## INTRODUCTION

Alpha-fetoprotein (AFP) is a major serum protein present in the early stages of development in mammals and other vertebrates, which virtually disappears in adult life. This protein is mainly synthesized by the fetal liver and the yolk sac. But, AFP may reappear in some tumors such as human primary hepatocellular carcinoma. It has also been found that AFP, like other serum proteins, can bind to specific membrane receptors on some cells such as macrophages, T lymphocytes, hepatoma cells, breast carcinoma cells and so on. AFP has immunoregulatory functions in a variety of experimental systems and some experimentally induced autoimmune diseases in animals can also be inhibited by the administration of AFP<sup>[1]</sup>. Our recent studies demonstrate that human AFP stimulates the proliferation of hepatoma cells *in vitro*<sup>[2]</sup>. Further investigation found that high concentrations of AFP resulted in hepatoma cell growth arrest<sup>[3]</sup>, which suggests that AFP may be a biphasic cell growth regulator. However, the mechanisms of the growth-regulatory properties exhibited by AFP are largely unstudied.

Apoptosis routinely occurs during embryogenesis, histogenesis, metamorphosis, endocrine-dependent tissue atrophy and normal adult tissue turnover. Moreover, tumor regression is also often mediated through apoptosis as a result of X-irradiation and chemotherapeutic exposure in cancer cells<sup>[4,5]</sup>. The detection of apoptotic cells and apoptotic bodies is based on several well-established morphological features. These features include cell shrinkage, disconnection with neighboring cells, nuclear chromatin condensation, maintenance of cytoplasmic membrane integrity, strong eosinophilic cytoplasm and lack of an inflammatory reaction. Fragmentation of the cells leads to the appearance of membrane-bound apoptotic bodies. Apoptotic bodies are defined as small, roughly spherical or ovoid cytoplasmic fragments, some of which contain nuclear fragments. A previous report demonstrated that AFP and AFP-receptor antibody blocked the induction of apoptosis in HL-60 leukemia cells in culture<sup>[6]</sup>. But, high concentrations of AFP was found to induce apoptosis in human hepatoma cells<sup>[7]</sup>. These results have strong implications that growth-regulatory activity of AFP is, at least in

part, related to affecting apoptosis process.

It has now been ascertained that cell apoptosis signals and rescue (survival) signals from apoptosis are mediated by a cell-surface transmembrane protein termed Fas and a cytoplasmic protein termed Bcl-2<sup>[8-10]</sup>. The human Fas protein (also designated APO-1) is a 48kDa cell surface glycoprotein that belongs to a family of receptors that includes CD 40, nerve growth factor receptors and tumor necrosis factor receptors. A series of studies indicate that apoptosis is mediated by the intercellular interactions of Fas with its ligand (Fas-L) or effectors. On the other hand, bcl-2 has been identified as an apoptosis inhibitor. The Bcl-2 protein (molecular mass 25kDa) is encoded by a gene involved in the *t*(14,18) chromosomal translocation and plays a central role in the inhibition of apoptosis. On the basis of Genbank identification, an amino acid sequence resembling a Fas-like peptide stretch has been detected in human AFP (39% identity, 23 amino acids in length). In a similar fashion, a sequence identifying with a Bcl-2-like amino acid stretch can also be discerned in human AFP (41% identity, 17 amino acids in length)<sup>[11]</sup>. Therefore, we speculate that Fas and Bcl-2 proteins in human hepatoma cells may contribute to the influence of AFP on apoptosis and growth regulation. We hereby investigated the expression of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells with avidin-biotin complex (ABC) immunocytochemical method.

## MATERIALS AND METHODS

### *Cell lines and culture conditions*

A human hepatoma cell line, BEL-7404, was maintained in RPMI 1640 medium (Gibco) supplemented with 10% new-born calf serum, at 37°C, 5% CO<sub>2</sub> and 100% humidity. The RPMI 1640 medium was replaced with fresh medium every two to three days. For ABC assay, cells at a density of 5 × 10<sup>3</sup> cells/mL were grown on coverslips, and adhesive cells were directly used. Cell viability was determined by mixing the cell suspension with 0.5% trypan blue (1:1).

### *Immunocytochemical assay*

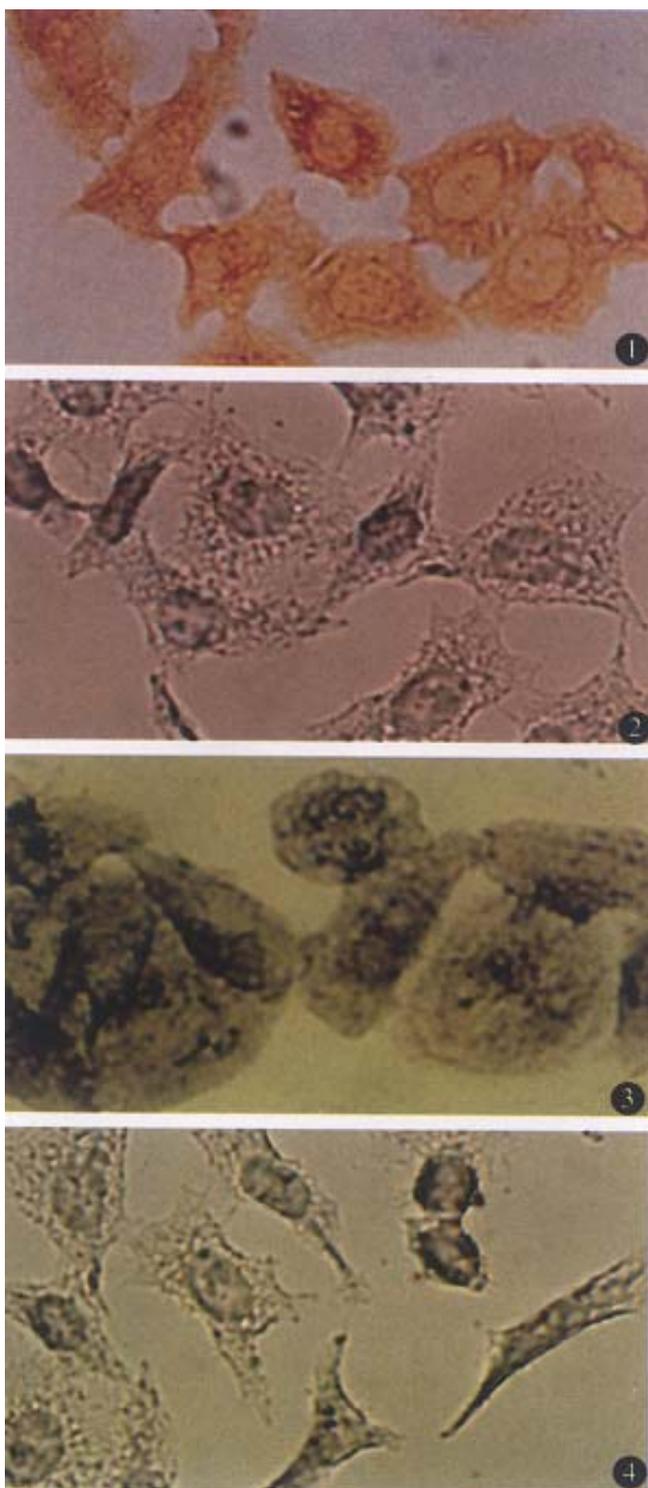
Antibodies used included a rabbit polyclonal antibody to Fas protein (Santa Cruz), and a mouse monoclonal antibody to Bcl-2 protein (Dako). As described by the manufacturers, the antibody against Fas protein is an affinity-purified antibody raised against a peptide corresponding to amino acids 260-279 mapping at the carboxy terminus of Fas protein of human origin. The specificity of the antiserum for Fas was confirmed by Western blotting and immunohistochemistry. The antibody

against Bcl-2 protein belonged to mouse IgG1 subclass. The antibody reacted specifically with Bcl-2 protein as demonstrated by immunoblotting and immunoprecipitation.

The monoclonal and polyclonal antibody reactivities were visualized using Vectastain ABC kit (Vector) following the vendor's instructions with a few modifications<sup>[12]</sup>. Briefly, adhesive cells were fixed in 4% paraformaldehyde for 5min-8min at room temperature, then treated with 0.05M Tris-buffered saline (TBS, pH 7.2) containing 0.4% Triton X-100 for 15min at room temperature. The coverslips were then incubated with 1% bovine serum albumin in TBS containing 0.4% Triton X-100 for 30min at 37°C. For the detection of Fas protein, prior to incubation with antibodies, endogenous peroxidase activity was irreversibly inhibited by treatment with 5% hydrogen peroxide in methanol solution at room temperature for 30min. After washing in TBS, primary antibodies to Fas were applied for 1h at 37°C. After washing 3 times, coverslips were incubated with biotinylated sheep anti-rabbit secondary antibodies at 37°C for 45min followed by avidin biotin peroxidase complex at 37°C for 45min. For color development, a peroxidase substrate solution, 0.05% 3,3'-diaminobenzidine (Sigma) in TBS containing 0.01% hydrogen peroxide, was applied for 5min-10min at 37°C. For the assay of Bcl-2 protein, primary antibodies for Bcl-2 were reacted with the cells for 1h at 37°C. After incubation with the biotinylated secondary antibodies (horse anti-mouse antibodies) for 45min at 37°C, the coverslips were treated with alkaline-phosphatase-conjugated avidin-biotin complex for 1h at 37°C. Development reagents contained 33μl of nitroblue tetrazolium salt (75mg/mL in 70% dimethylformamide) and 25μl of 5-bromo-4-chloro-3-indocyl phosphate (50mg/mL in dimethylformamide) in 7.5mL TBS. For color development, cells were incubated in the color solution for up to 4h at 37°C in the dark. The coverslips were finally rinsed in water. The primary antibodies were replaced by control non-relevant sera to monitor specificity of ABC immunocytochemical staining.

## RESULTS

On the basis of preliminary experiments, rabbit anti-human Fas antibody was used at a dilution of 1:80, sheep anti-rabbit IgG at 1:50, and rabbit ABC at 1:100, respectively. The dilution of mouse anti-human antibodies against Bcl-2 was 1:100. Horse anti-mouse IgG and mouse ABC were used at 1:80 and 1:100, respectively. The use of appropriately diluted reagents minimized troublesome nonspecific background staining.



**Figure** Detection of Fas and Bcl-2 proteins in BEL-7404 human hepatoma cells. (1) Fas antigen was stained by the avidin-biotin-peroxidase complex. Reaction products were visible in almost all the cells. (2) Same as in 1, except that rabbit anti-human polyclonal antibody to Fas was replaced by normal rabbit serum. (3) BEL-7404 cells were incubated with mouse anti-human monoclonal antibody to Bcl-2. Positive grains detected by alkaline-phosphatase-conjugated avidin-biotin complex were also visible in almost all the cells. (4) Same as in 3, except that the primary antibody to Bcl-2 was replaced by normal mouse serum. Original magnification  $\times 200$ .

Figure 1 shows ABC detection of Fas antigen in BEL-7404 cells. When primary antibodies to Fas were incubated with BEL-7404 cells, large numbers of brown grains were detected on the membrane of essentially all the cells, although the grain distribution varied. Treatment of the cells with normal rabbit serum resulted in a marked reduction in the number of specific grains (Figure 2). When the primary antibodies against Bcl-2 were used, purple grains were present diffusely throughout the cytoplasm of BEL-7404 cells with sparing of the nuclei. The number of grains in the cytoplasm varied, but essentially all the cells, including those undergoing mitotic division, were considered to contain Bcl-2 protein (Figure 3). However, when cells were incubated with normal mouse serum under the same experimental condition, no accumulation of grains in the cells was observed (Figure 4).

Overdevelopment of the color reaction can produce a high level of non-specific staining, but this was significantly reduced by careful monitoring of the reaction after the addition of color reagents.

#### DISCUSSION

For study of gene expression, immunocytochemical staining can be used to detect final products of gene expression and their location in cell, though it cannot be used to detect directly genes. In the present study, Fas and Bcl-2 proteins have been detected by ABC immunocytochemical method in cultured BEL-7404 human hepatoma cells. We consider that ABC assay is rapid, reliable, sensitive and economical to use, and there are no known health hazards associated with the reagents used, which provides a very useful tool for investigating the gene expression<sup>[12]</sup>.

Some studies demonstrate that cell apoptosis is mediated by the intercellular interactions of Fas with its ligand or effectors, and Bcl-2 protein can inhibit apoptosis<sup>[8-10]</sup>. As mentioned in the "Introduction", amino acid sequences of Fas and Bcl-2 proteins can be detected on some domains of human AFP<sup>[11]</sup>. Moreover, Bcl-2 site is localized adjacent to a proposed hinge region of human AFP molecule, which allows rotational flexibility. A conformational change in the tertiary structure of AFP, possibly induced by excessive ligand binding, can expose such a Bcl-2 site, which is normally hidden in a molecular crevice. According to the present result, taken together with previous published data, we speculate that dimerization or binding of low concentrations of AFP with its normal molecular configuration to Fas protein could blunt the apoptosis signals, resulting in enhancement of cell growth. On the other side,

dimerization or binding of high concentrations of AFP with possible conformational change to the exposed Bcl-2 signal site could block the rescue/survival signals, causing the induction of apoptosis. In this fashion, AFP might function in both the up and down-regulation of cell growth by employing a binding or dimerizing mechanism to apoptotic mediators.

The oncoproteins are products of the proto-oncogenes, which now include *myc*, *myb*, *fos*, *jun*, *ski*, *ets*, *cbl*, *erb A* and possibly many others. Most oncoproteins have turned out to be transcription factors, which function as molecular switches that sense incoming signals and modulate the transcription of specific genes. Many oncoproteins have functional partners with which they heterodimerize to bind DNA, such as *fos* and *jun*, *myc* and *max*, etc. Protein-protein and protein-DNA interactions of the oncoproteins are often mediated through helix-loop-helix and leucine zipper motifs. Tumor suppressor proteins (*p53* and retinoblastoma protein) can function as growth suppressors. It has been found that AFP gene promoter can be regulated by some transcription factors such as AP-1<sup>[13]</sup>, although the activation of the oncogenes *c-fos*, *c-jun* and *c-myc* might not directly *in vivo* the basal level of AFP gene expression in hepatoma cell lines<sup>[14]</sup>. The mutation of *p53* gene occurred in the early stage of hepatocarcinogenesis may be correlated with the initiation of hepatocarcinogenesis, and mutant P53 protein probably related to the reactivation of AFP gene<sup>[15]</sup>. On the other hand, Genbank computer-generated sequence identities detected on human AFP domains demonstrated that amino acid identities for the oncogenes *c-erb A*, *c-myc*, *rel*,

*myb*, *ras* ranged from 29% to 54% over lengths from 13 to 24 amino acid, largely in human AFP domains 1 and 2. In comparison, the tumor-suppressor (retinoblastoma, Rb) protein identities appear to reside only on domain 1 of human AFP<sup>[11]</sup>. Thus, it is possible that the growth-regulatory activity of AFP may also be mediated by some oncoproteins and/or tumor suppressor proteins. The exact relationship between AFP growth regulation and oncoproteins as well as tumor suppressor proteins still needs to be further investigated in the future.

## REFERENCES

- 1 Wang XW, Xie H. Significance of alpha-fetoprotein in the development of novel therapeutic agents. *Drugs Fut*, 1998;23(7):in press
- 2 Wang XW, Xu B. Stimulation of tumor-cell growth by alpha-fetoprotein. *Int J Cancer*, 1998;75(4):596-599
- 3 Dudich E, Semenkova L, Gorbatova E, Dudich I, Khromykh L, Tatulov E *et al.* Growth regulative activity of human alpha-fetoprotein for different types of tumor and normal cells. *Tumor Biol*, 1998;19(1):30-40
- 4 Kerr JFR, Winterford CM, Harmon BV. Apoptosis: its significance in cancer and cancer therapy. *Cancer*, 1994;73(8):2013-2026
- 5 Marx J. Cell death studies yield cancer clues. *Science*, 1993;259(5):760-761
- 6 Laderoute MP, Pilarski LM. The inhibition of apoptosis by AFP and the role of AFP receptors in anti-cellular senescence. *Anticancer Res*, 1994;14(6):2429-2438
- 7 Semenkova LN, Dudich EI, Dudich IV. Induction of apoptosis in human hepatoma cells by alpha-fetoprotein. *Tumor Biol*, 1997;18(5):261-273
- 8 Goyns MH. Apoptosis-the key to future anticancer therapy? *Drugs Fut*, 1995; 20(6):601-608
- 9 Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood*, 1992;80(5):879-886
- 10 Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol*, 1994; 124(1):1-6
- 11 Mizejewski GJ. Alpha-fetoprotein as a biologic response modifier: relevance to domain and subdomain structure. *Proc Soc Exp Biol Med*, 1997;215(4): 333-362
- 12 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled anti-body (PAP) procedures. *J Histochem Cytochem*, 1981;29(4):577-580
- 13 Bois-Joyeux B, Thomassin H, Richard F, Ikononova R, Denissenko M, Danan JL. Several transcription factors participate in the functioning of the alpha-fetoprotein gene promoter. *Bull Cancer*, 1995;82(7):541-550
- 14 Tournier-Thurneyssen I, Feldmann G, Bernuau D. Nuclear oncogenes and alpha-fetoprotein gene expression in hepatoma cell lines. *Tumor Biol*, 1993;14 (4):201-212
- 15 Zou HQ, Tang ZY, Ye SL. Mutation of *p53* gene and expression of alpha-fetoprotein during hepatocarcinogenesis. *Chin J Med*, 1994;74(8):474-475

# A comparison between Zhishi Xiaopiwan and cisapride in treatment of functional dyspepsia \*

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**Subject headings** Dyspepsia/therapy; Zhishi xiaopi; cisapride; gastric emptying

## Abstract

**AIM** To compare the therapeutic effect of the herbal medicine Zhishi Xiaopi with that of Cisapride in the treatment of functional dyspepsia (FD).

**METHODS** Fifty-one FD patients were randomized into Herbal group ( $n = 27$ ) and Cisapride group ( $n = 24$ ). Two two groups were given a four-week treatment of *Zhishi Xiaopiwan* 100ml, tid, a.c. and Cisapride 5mg, tid, a.c. respectively. Patients' symptoms were assessed and 39 patients' (22 of Herbal group and 17 of Cisapride group) gastric liquid emptying times were measured with ultrasonography before and after the treatment.

**RESULTS** The therapeutic effective rates of Herbal group and Cisapride group were 81.49% and 87.50% ( $P > 0.05$ ). The half gastric emptying time (GET<sub>50</sub>) and gastric emptying time (GET) of healthy controls and FD patients were 36.12min±10.22min vs 52.95min±13.49min and 87.07min±21.11min vs 120.74min±23.08min ( $P < 0.001$ ). The GET<sub>50</sub> and GET of Herbal group before and after the treatment were 51.63min±13.15min vs 45.62min±10.82min and 117.34min±23.29min vs 103.26min±22.19min ( $P < 0.01$ ). The results of Cisapride group were 54.66min±14.14min vs 40.95min±11.29min and 125.12min±24.47min vs 95.49min±22.31min ( $P < 0.01$ ). The differences in values (median) of GET<sub>50</sub> and GET for Herbal group and Cisapride group before

and after treatment were 5.75min vs 17.18min and 13.22min vs 33.54min ( $P < 0.05$ ).

**CONCLUSION** Delayed gastric emptying is one of the pathogenesis of FD. Both *Zhishi Xiaopi* pills and *Cisapride* can effectively alleviate the symptoms of FD and accelerate gastric liquid emptying. The effect of *Zhishi Xiaopiwan* on enhancing gastric motility is comparable with but less than that of *Cisapride*.

## INTRODUCTION

Dyspepsia is a common syndrome, Outpatients in gastrointestinal clinics complaining of dyspeptic symptoms amount to about 30%-40% of the total visits. Among them, more than half have no organic lesions after examination. So it is called functional dyspepsia (FD). Some of the patients with FD present symptoms suggestive of delayed gastric emptying. So gastrointestinal hypomotility is considered one of the pathogenesis. In this study, we compared the therapeutic effect of *Zhishi Xiaopiwan* made of (herbal medicine) with *Cisapride* in the treatment of FD by assessing symptomatic improvement and measurement of gastric liquid emptying time.

## MATERIALS AND METHODS

### *Diagnosis of functional Dyspepsia*

Epigastric pain, discomfort or bloating, postprandial fullness, eructation, nausea and other upper abdominal symptoms lasting at least for 4 weeks; esophagitis, peptic ulcer, upper gastrointestinal erosion and neoplasm were excluded by endoscopy; diseases of liver, gallbladder, pancreas and lower gastrointestinal tract by laboratory, ultrasound and X-ray examinations; and diabetes, hyperthyroidism, connective tissue diseases and history of abdominal surgery were excluded.

### *Patients and healthy controls*

Fifty-one patients meeting the above diagnostic criteria were randomly divided into two groups:

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Received 1998-11-09

Herbal group, 27 patients (average age, 45.1±9.67 year; range, 25-63 years; a ratio of men/women, 10±17) and Cisapride group, 24 patients (average age, 48.5±12.9 years; range, 26-72 year; a ratio of men/women, 13:11). Healthy control group consisted of 10 volunteers without gastrointestinal symptoms (average age, 41.4±11.75 years; range, 25-58 year; a ratio of men/women, 7:3).

**Symptom assessment**

Five symptoms (epigastric pain or discomfort, bloating, postprandial fullness, eructation and nausea) were assessed for each patient once a week or two weeks. Symptoms were assessed according to Stanghellini criteria<sup>[1]</sup>: 0 = absent, 1 = occasionally present and not affecting patients' daily activities; 2 = present moderately often, slightly affecting their activities; 3 = present moderately or more often, affecting considerably patients' activities. The symptom complex index (SCI) was calculated by dividing the summation of all presenting symptoms' scores with the number of symptoms.

**Therapeutic efficacy assessment criteria**

Noticeable efficiency (NE): SCI was less than pre-treatment value by 2 or more; efficiency: SCI was less than pre-treatment value by 1 or more but less than 2; and inefficiency: SCI was less than pre-treatment value by less than 1.

**Measurement of gastric liquid emptying**

Gastric liquid emptying was measured in one vertical section of antrum with real time ultrasonography as used by Marizo<sup>[2]</sup>. All medication was stopped for 3 days and the patients were fasted for 12 hours before the test. Milk of 250ml with a total caloric value of 597KJ, prewarmed to 37°C, was used as a testing meal.

**Treatment**

Herbal group was treated with Zhishi Xiaopiwan in the form of decoction (100ml three times a day before meal) for 4 weeks. Zhishi Xiaopiwan consisted for the following ingredients: *Citrus aurantium* L.15g, *Officinal Magnolia Bark* 12g, *Tangshen Asiabell Root* 10g, *Largehead Atractylodes Rhizome* 10g, *Poria* 12g, *Ternate Pinellia Tuber* 10g, *Golden Thread Rhizome* 3g, *Nardostachys Rhizome and Root* 6g, etc. If pain was severe, *Paniclate Swallowwort Root* 10g and *Corydalis yanhusuo* W.T.Wang 15g were added. If bloating was severe, *Finger Citron* 10g, *Akebi Fruit* 10g and *Costus Root* 10g were supplemented. If eructation and nausea were severe, *Inula Flower* 10g and *Bamboo Shavings* 5g were added. If there

was regurgitation, *Ark Shell* 30g and *Cuttle Bone* 30g were used. Cisapride group was treated with Cisapride (5mg three times a day before meals) for 4 weeks.

**Statistical analysis**

Student's *t* test,  $\chi^2$  test, rank sum test and linear regression were used and *P* values less than 0.05 were considered significant.

**RESULTS**

**Therapeutic efficiency of symptoms**

Both Zhishi Xiaopiwan and Cisapride could effectively alleviate patients' symptoms (Tables 1-3). There were no significant difference between the efficiencies of the two medications on the symptoms of the FD, and in improvement SCI between two groups after treatment. The total efficacy rates (NE +E) of Herbal group and Cisapride group were 81.49% and 87.5% respectively (*P*>0.05). During treatment, 3 patients of Cisapride group had fewer gastrointestinal symptoms (1 with lower abdominal pain, 2 with loose stool). Patient in Herbal group had no side reactions, but with no statistical significance ( $\chi^2 = 3.59, P > 0.05$ ).

**Table 1 Comparison of the therapeutic efficiency for symptoms relief**

Symptoms	Group	Cases	NE <sup>a</sup> (n)	E <sup>a</sup> (n)	IE <sup>a</sup> (n)	EF <sup>a</sup> (%)
Epigastric pain	Herbal	16	1	10	5	68.75 <sup>b</sup>
	Cisapride	15	4	7	4	73.33
Bloating	Herbal	22	10	8	4	81.89 <sup>b</sup>
	Cisapride	18	13	3	2	88.89
Postprandial fullness	Herbal	19	9	8	2	89.47 <sup>b</sup>
	Cisapride	16	12	3	1	93.00
Eructation	Herbal	14	5	7	2	85.71 <sup>b</sup>
	Cisapride	17	10	4	3	82.35
Nausea	Herbal	6	3	3	0	100.00 <sup>b</sup>
	Cisapride	8	2	4	2	75.00

<sup>a</sup>NE: noticeable efficiency, E: efficiency, IE: inefficiency, ER: efficiency rate; <sup>b</sup>*P*>0.05 v Cisapride group.

**Table 2 Comparison of symptoms complex index**

Group	Cases	Before treatment	After treatment	Differential values
Herbal	27	2.28±0.32	1.02±0.60 <sup>a</sup>	1.26±0.56 <sup>b</sup>
Cispride	24	2.19±0.40	0.71±0.55 <sup>a</sup>	1.49±0.56

<sup>a</sup>*P*<0.001 v before treatment, <sup>b</sup>*P*>0.05 v Cisapride group.

**Table 3 Comparison of the clinical therapeutic efficiency rate**

Group	Cases	NE <sup>a</sup> rate %(n)	Ea rate %(n)	IEa rate %(n)
Herbal	27	25.93 (7) <sup>b</sup>	55.56 (15) <sup>b</sup>	18.51 (5) <sup>b</sup>
Cisapride	24	37.50 (9)	50.00 (12)	12.50 (3)

<sup>a</sup>NE: noticeable efficiency, E: efficiency, IE: inefficiency; <sup>b</sup>*P*>0.05 v Cisapride group.

### Gastric liquid emptying

Gastric liquid emptying tests were done in 10 healthy volunteers and 39 FD patients. Half gastric emptying time (GET<sub>-50</sub>) and total gastric emptying time (GET) of health controls were 36.12 min ± 10.22min and 87.07 min ± 21.11 min. Those of FD patients were 52.95 min ± 13.49 min and 120.74 min ± 23.80min, both of which were longer than those of healthy controls ( $P < 0.001$ ). If the normal GET<sub>50</sub> range was set as from 16.09 to 56.15 (mean ± 1.96 SD), there were 14 (35.90%) patients with delay of gastric emptying.

Both Zhishi Xiaopiwan and Cisapride could shorten the gastric liquid emptying time (Table 4). After treatment, there was still significant difference between the gastric emptying time of Herbal group and control group ( $P < 0.05$ ), but there was no significant difference between those of Cisapride group and control group ( $P > 0.05$ ). The median difference of GET<sub>50</sub> and GET of Herbal group before and after treatment were 5.75 min and 13.22min respectively, and those of Cisapride group were 17.18 min and 33.54 min ( $0.25 < P < 0.05$ ). So the effect of Zhishi Xiaopiwan in enhancing gastric emptying is less than that of Cisapride.

**Table 4 Comparison of gastric emptying time between before and after treatment**

Group	Cases	GET <sub>50</sub> (min)	GET (min)
Control	10	36.12±10.22	87.07±21.11
Herbal	Before treatment	51.63±13.15 <sup>a</sup>	117.34±23.29 <sup>a</sup>
	After treatment	45.62±10.82 <sup>b,c</sup>	103.26±22.19 <sup>b,c</sup>
Cisapride	Before treatment	54.66±14.14 <sup>a</sup>	125.01±24.47 <sup>a</sup>
	After treatment	40.95±11.29 <sup>b,d</sup>	95.49±22.31 <sup>b,d</sup>

<sup>a</sup> $P < 0.01$  v control group, <sup>b</sup> $P < 0.01$  v before treatment, <sup>c</sup> $P < 0.05$  v control group, <sup>d</sup> $P > 0.05$  vs Control group.

### DISCUSSION

In this study, we found 35.9% of FD patients had delayed gastric liquid emptying, which is similar to the results of 23%-55% reported previously<sup>[3,4]</sup>. This indicates that quite a few FD patients have gastric hypomotility. Gastrointestinal manometric techniques<sup>[1,5]</sup> showed that during fasting, the cycles of Migrating Motor Complex (MMC) of FD patients are less than those of healthy subjects. No matter during fasting or digestive period, the amplitude and frequency of the contractions of antrum and duodenum of FD patients are all less than those of healthy subjects. During digestive period, the numbers of duodenal propulsive peristalsis and the coordinating contractions between antrum and duodenum are less than those of healthy subjects.

Ultrasonographic techniques<sup>[6]</sup> also showed that the postprandial antrum contractions of FD patients are incomplete with small waves of irregular rhythm, as compared to the complete, even and rhythmical peristalsis of healthy subjects. The gastrointestinal hypomotility and the incoordination between antrum and duodenum may induce delay of gastric emptying. As an agonist of 5-HT<sub>4</sub> receptor, Cisapride can act on the receptors of the intermediate and terminal neurons of myenteric nerve plexus in gastrointestinal smooth muscle and improve the gastrointestinal motility by promoting cholinergic nerves to release acetylcholine. It can increase the amplitude and frequency of the gastric contractions and the numbers of coordinating contractions between antrum and duodenum to accelerate gastric emptying.

Symptomatology of FD is very similar to that of "Piman Zheng", a name of disease in Traditional Chinese medicine with bloating as the chief complaint. Zhishi Xiaopiwan is the commonly used Chinese medicine for "Piman Zheng". So we chose this medicine to treat FD and compared it with Cisapride. The results showed that Zhishi Xiaopiwan could effectively ameliorate the symptoms of FD, and its total efficiency rate (81.49%) was not significantly different from that of Cisapride (87.5%). Gastric emptying examinations indicated that Zhishi Xiaopiwan also could accelerate the gastric liquid emptying, although its effect was less than that of Cisapride. Its effect on enhancing gastrointestinal motility was proven in previous animal experiments. The main ingredient of Zhishi Xiaopiwan-*Citrus aurantium L.* could prolong the canine intestinal active duration of MMC by reducing the duration of the phase I and prolonging the duration of the phase II<sup>[7]</sup>. Other ingredients, such as *Tangshen Asiabel Root*, *Rhizome Atractylodes macrocephalae*, *Ternate Pinellia Tuber* and *Golden Thread Rhizome*, could increase the frequency of rat gastric electric spikes and amplitude of gastric contractions and regulate the disturbance of gastric electric rhythm. *Rhizome Atractylodes macrocephalae* might act on cholinergic receptors to activate the gastric movement<sup>[8,9]</sup>.

Not all FD patients had delayed gastric emptying. This indicates that gastrointestinal hypomotility is not the sole pathogenesis of FD, other factors such as hypersensitiveness of gastric mucosa, disturbance of gastric accommodation and failure of gallbladder contraction might also be involved in the pathogenesis<sup>[10]</sup>. The inconsistency between the symptomatic relief and improvement of gastric emptying by Zhishi Xiaopiwan also suggests

that the action of the herbal medicine to improve symptoms might be through mechanisms other than promoting gastric motility, which should be further studied in the future.

In conclusion, we found that delay of gastric emptying was one of the pathogenetic factors of FD, and both Zhishi Xiaopiwan and Cisapride could ameliorate the symptoms of FD and accelerate the gastric liquid emptying in certain percentage of patients. In this aspect, Cisapride is better than the herbal medicine we used.

## REFERENCES

- 1 Stanghellini V, Ghidini C, Maccarini MR, Paparo GF, Roccrinaldesi, Barbara L. Fasting and postprandial gastrointestinal motility in ulcer and non-ulcer dyspepsia. *Gut*, 1992;33(2):184-190
- 2 Marzio L, Giacobbe A, Conoscitore P, Facciorusso D, Frusciante V, Modoni S. Evaluation of the use of ultrasonography in the study of liquid gastric emptying. *Am J Gastroenterol*, 1989;84(5):496-500
- 3 Marizo L, Difelice F, Grazia M, Imbimbo B, Lapenna D, Cuccurullo F. Gallbladder hypokinesia and normal gastric emptying of liquids in patients with dyspeptic symptoms. A double-blind placebo-controlled clinical trial with cisapride. *Dig Dis Sci*, 1992;37(2):262-267
- 4 Wang BE, Liang XM, Jia BQ, Chen BW, Ke MY, Lin SL *et al.* A controlled multicentre clinical trial on Cisapride in treatment of functional dyspepsia. *Chin J Internal Med*, 1995;34(3):180-184
- 5 Zhang SL, Wang ZH. Effect of Helicobacter pylori on the motility of antrum and duodenum in FD patients. *Chin J Digest*, 1995;15(Suppl):76-77
- 6 Jin ZD, Zhou DW, Xu GM. Clinical study on measuring gastric motility with real time ultrasonography. *Chin J Digest*, 1997;17(1):59
- 7 Bi QH, Yang DZ, Yin CZ, Ding AL. Effect of Zhishi on the small intestinal myoelectric activity. *Acta Medica Sinica*, 1991;6(1):39-42
- 8 Chen Z, Li SR, Han Y, Tian GQ, Yuan GC, Chen XY. Clinical and experimental study on non-ulcerative dyspepsia treated with Shenxia Heweining. *Chin J Intergrated Tradit Western Med*, 1994;14(2):83-85
- 9 Ma XS, Fan XP, Chen Z, Li CW, Xin YS. Effect and mechanism of Rhizome Atractylodes Macrocephalae on gastrointestinal motility in animals. *Chin J Digestion*, 1996;16(5):261-264
- 10 Lin J. Advance in the pathogenesis of FD. *Chin J Gastroenterol*, 1997;2(1):51-53

# Effects of Fuzhenghuayu decoction on collagen synthesis of cultured hepatic stellate cells, hepatocytes and fibroblasts in rats \*

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**Subject headings** Fuzhenghuayu decoction; collagen synthesis; hepatic stellate cells; hepatocytes; fibroblasts

## Abstract

**AIM** To study the mechanism of Fuzhenghuayu (FZHY) decoction on anti-liver fibrosis.

**METHODS** FZHY 10% decoction sera was incubated with rat normal subcultured hepatic stellate cells (HSC) and fibrotic primarily cultured HSC, normal and fibrotic hepatocytes and subcultured skin fibroblasts separately. Cell intracellular and extracellular collagen synthesis rates were measured by the method of [<sup>3</sup>H] Proline impulse and collagenase digestion.

**RESULTS** For primarily cultured HSC and hepatocytes, both of intracellular and extracellular collagen synthesis rates decreased in the drug sera group. For the normal subcultured HSC and primarily cultured hepatocytes, the extracellular collagen secretion was decreased obviously by the drug sera, and intracellular collagen synthesis rates were inhibited to some extents. For fibroblasts, both intracellular and extracellular collagen synthesis rates were inhibited somewhat, but no significant differences were found.

**CONCLUSION** The mechanism of FZHY decoction on anti-liver fibrosis may be associated with inhibition of liver collagen production.

## INTRODUCTION

Liver fibrosis is the common pathological feature of chronic liver diseases, and is closely associated with changes of liver cell functions. In order to investigate the mechanisms of Fuzhenghuayu decoction action on liver fibrosis, the drug serum was collected and incubated with cultured rat hepatic stellate cells (HSC), hepatocytes and fibroblasts, and then cellular functions were observed.

## MATERIALS AND METHODS

### Animals

Wistar male rats were purchased from Shanghai Animal Center, Chinese Academy of Sciences, among them, rats weighing 180g-200g were used for isolations of hepatocytes, 350g-450g rats were used for isolation of HSC. SD rats, pregnant for 12-14 days were the gifts of Shanghai Institute of Family Planning and used for skin fibroblast isolation and culture. All rats were maintained with food and water available *ad libitum*.

### Reagents

Minimum essential medium Eagle (MEM), 199 Medium (M199) and Dubocal modified Eagle's Medium (DMEM) were purchased from GIBCO, USA. Pronase E, type IV collagenase, Metrizimide, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) from Sigma Co., USA. And L-[5-<sup>3</sup>H] proline ([<sup>3</sup>H]Pro) from Amersham Co., England.

### Drug

Fuzhenghuayu (FZHY) decoction consists of *Cerdecaps*, *Semen Persiciae*, *Radix Salviae Miltiorrhizae*, etc. Shanghai Zhonghua Pharmaceutical Factory made the decoction into a kind of fluid extract. Each gram of the fluid extract contained 2703g of the above raw herbs.

**Cell isolation and culture** HSC isolation and culture were performed according to the modified Friedman method<sup>[1]</sup>, and hepatocyte isolation according to the modified method<sup>[2]</sup>. Fibroblast followed E Zheng's method<sup>[3]</sup>.

**Model establishment**<sup>[4]</sup> Male Wistar rats received 0.5% DMN dissolved in 0.15mol/L NaCl, at a dose of 10μl of DMN/100kg i.p., for 3 consecutive days each week for 3 weeks. The pair fed controls

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\*Project supported by the National Natural Science Foundation of China, No. 39570889

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Received 1998-07-16

received an equivalent amount of saline.

**Drug sera were prepared** Using our own method<sup>[5]</sup>.

**Grouping and drug sear incubation** The cultured cells were divided into control and drug serum groups. The control was incubated with 199 medium containing 10% normal rat serum, and the drug group was incubated with 199 medium containing 10% drug serum, for 72h in HSC and fibroblast cells, for 48h in hepatocytes and 24h for fibrotic HSC.

**Assay of collagen synthesis rates** Greets method was used<sup>[7]</sup>.

**Statistics** Two tails student t test was used for statistical analysis.

## RESULTS

### Effects on HSC collagen synthesis

The drug sera markedly inhibited the intracellular collagen synthesis in normal subcultured HSC, and both intracellular and extracellular collagen productions in fibrotic HSC.

**Table 1** Effects of drug sera on HSC collagen synthesis rate ( $n = 4$ , %  $\bar{x} \pm s$ )

Group	Normal subcultured HSC		Fibrotic HSC	
	Intracellular	Extracellular	Intracellular	Extracellular
Drug sera	0.31±0.21	2.70±0.10 <sup>a</sup>	0.12±0.09 <sup>a</sup>	0.80±0.34 <sup>b</sup>
Control	0.57±0.37	4.15±0.95	0.33±0.10	1.72±0.53

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs control.

### Effects on hepatocyte collagen synthesis

The drug sera could inhibit extracellular collagen synthesis more obviously in fibrotic hepatocytes, than in the normal cells. The drug sera could also inhibit intracellular collagen synthesis of fibrotic hepatocytes (Table 2).

**Table 2** Effects of drug sera on hepatocyte collagen synthesis rate ( $n = 4$ , %  $\bar{x} \pm s$ )

Group	Normal subcultured HSC		Fibrotic HSC	
	Intracellular	Extracellular	Intracellular	Extracellular
Drug sera	0.34±0.05	0.23±0.04 <sup>a</sup>	0.84±0.16 <sup>a</sup>	0.43±0.12 <sup>b</sup>
Control	0.31±0.09	0.30±0.02	1.24±0.50	0.60±0.14

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs Control.

### Effects on NIH/3T3 fibroblast collagen synthesis

The drug sera could inhibit both intracellular and extracellular collagen synthesis to some extent, but no significant difference was found (Table 3).

**Table 3** Effects of drug sera on NIH/3T3 fibroblast collagen synthesis rate ( $n = 4$ , %  $\bar{x} \pm s$ )

Group	Intracellular	Extracellular
Drug sera	1.53±0.50 (6)	8.89±3.66 (6)
Control	2.03±0.75 (5)	12.62±1.03 (4)

In bracket was the case numbers.

## DISCUSSION

Collagens are the main components of extracellular matrix, which play an important role in keeping liver structure and functions. If collagen production increased or decomposition decreased, its metabolism would break and lead to liver fibrosis. It was found that HSC could transform to myofibroblasts under stimulation of cytokines induced by hepatocyte injury, and was the key cell for synthesis and secretion of collagen in liver. In the paper, both normal subcultured HSC and fibrotic HSC showed significance in collagen production. Although hepatocyte has the function of collagen production, which is low in normal hepatocytes, and increased obviously in fibrotic hepatocytes. This was also observed in our study. Fibroblasts had many subtypes, all of which could produce extracellular matrix, and were used for cell models in investigating liver fibrosis instead of HSC<sup>[8,9]</sup>. In this study, rat skin fibroblast showed ability of collagen production.

Besides anti-etiology therapy, regulation of collagen metabolism, including inhibition of collagen synthesis and increase of collagen decomposition, could protect or delay the formation of liver fibrosis, while inhibition of collagen production in liver is one of key steps for anti-liver fibrosis. In our previous clinical and animal studies, Fuzhenghuayu decoction showed good effects on liver fibrosis<sup>[10-13]</sup>. In the present study, serum was collected from rats fed on Fuzhenghuayu decoction by seropharmacological method and incubated with 3 kinds of cells. The results showed that the drug sera could decrease normal and intracellular collagen synthesis of fibrotic hepatocyte, decrease extracellular collagen production in subcultured HSC and normal hepatocyte, inhabit fibroblast collagen production and intracellular collagen production in HSC to some extent, but no action on intracellular collagen synthesis in normal hepatocytes. It is suggested that one of important mechanisms of Fuzhenghuayu decoction action on liver fibrosis may be the inhibition of liver collagen production.

## REFERENCES

- Xu LM, Liu C, Liu P, Liu CH, Gu TH, Li FH *et al.* Efficient and steady Ito cells isolation method. *J Cell Biol*, 1995;17(3):143-145
- Nagambula Bieqi. Primary hepatocyte culture. *Tokyo Academic Press*, 1986:3-10
- E Zheng. Cell culture technology. *Beijing: People's Health Press*, 1993:10-17
- Ala-Kpkkp L, Pihlajaniemi T, Myers JC, Kivirikko KI, Savolainen E. Gene expression of type I, III and IV collagen in hepatic fibrosis induced by dimethylnitroamine in the rats. *Biochem J*, 1987;244:75-79
- Liu C, Liu CH, Liu P, Xu LM, Hu YY. Inhibitory effect of Fuzhenghuayu decoction on Ito cell proliferation in rats. *Chin J Trad Med Tech*, 1997;4(2):97-99
- Greets A, Vrijnsen R, Rauterberg J, Burt A, Schellinck P, Wisse E. *In vitro* differentiation of fat-storing cells parallels marked increase of collagen synthesis and secretion. *J Hepatol*, 1989;9(1):59-68
- Chojkier M, Lyche KD, Filip M. Increased production of collagen in vivo by hepatocytes and nonparenchymal cells in rats with carbon tetrachloride induced hepatic fibrosis. *Hepatology*, 1998;8(4):808-814
- Zhang SX, Li J. Study of ultramicro-structure in fibroblast. *Acta Anat*, 1986;16(3):301-303
- Peferson TC, Isbrucker RA. Fibroproliferation in liver disease: role of monocyte factors. *Hepatology*, 1992;15(2):191-197
- Liu C, Liu P, Hu YY, Xu LM, Zhu JL, Xie HM. Effects of Fuzhenghuayu decoction on liver fibrosis. *J TCM*, 1994;35(10):602-604
- Liu C, Liu P, Chen GC, Lu P, Hu YY, Xu LM. Effects of Fuzhenghuayu decoction on serum HA and LN in chronic active hepatitis and liver cirrhosis. *Chin J Trad Med Tech*, 1995;2(Suppl):67-72
- Liu C, Liu P, Chen GC. Clinic observation of effects of Fuzhenghuayu decoction on chronic type B hepatitis. *Chin J Trad Med Tech*, 1996;3(5):13-15
- Liu P, Hu YY, Liu CH. Effects Fuzhenghuayu decoction on liver fibrosis and Ito cells in liver fibrotic rat induced by DMN. *Chin J Trad Med Tch*, 1997;4(2):99-101

# Three-dimensional structure of lymphatics in rabbit stomach \*

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**Subject headings** stomach; corrosion casts; lymphatics; three-dimensional structure

## INTRODUCTION

Recently, the stomach lymphatics have been studied, but there are different opinions on the lymphatic distribution of the stomach layers<sup>[1-6]</sup>. There has been no reported in China. Describing the three-dimensional organization of the stomach lymphatics and revealing the correlation of the three-dimensional and the two-dimensional and organization.

In our study in the rabbit with the lymphatic corrosion cast with Mercox and semithin section methods we investigated the relationship of the three-dimensional organization with the drainage of the stomach lymphatics, which may provide the evidences of lymphology, pathology and the clinical medicine.

## MATERIALS AND METHOD

Twelve rabbits of both sexes were used, two of them, undergone the procession of the semithin section of electron microscopy, were observed under light microscopy. The other ten were used for the lymphatic corrosion casts.

The Mercox (CL-2B-5, Japan Velene Hospital, Tokyo,) diluted to 25%-30% (V/V) with methyl methacrylate monomer was injected in and around the mucosal submucosal, layers of the stomach. Shortly before the injection, a curing agent (MA, Japan Vilene Hospital Tokyo) was added to the injection medium to give a concentration of 1% (W/V). The injected parts of the stomach were

removed and placed in a hot water bath (60°C) for 3hrs. They were put in concentrated NaOH (15%-20%) at about 60°C until tissue elements were completely corroded away. The lymphatic corrosion casts were cut into blocks and observed under a SEM (S-520) (with an accelerating voltage of 10-15kv).

## RESULTS AND DISCUSSION

### *The lymphatic of mucosal and submucosal layers*

The samples filled with resin which were in the mucosal and the submucosal layers clearly showed the three-dimensional organization of the lymphatic capillaries and the lymphatics. There was a layer of the lymphatic capillary network in the deep layer of the tunica mucosa between the bottoms of gastric gland and the muscularis mucosa. The networks extended short tube with blind ends into gastric glands. The tubers were called intergland circular cones. which were 20µm-30µm in diameters. The cones were round, hook, V and finger in shap. In the cardia and the fundus of the stomach the cones were sparse and connected to the lymphatic capillary networks of the tunica mucosa. In the body of the stomach 2 or 3 circular cones were connected in one group. The roots of the circular cones were connected to the sinus (50µm-60µm in diameters), then drained to the lymphatic capillary networks of the tunica mucosa (Figure 1).

Donini has observed the lymphatic capillary networks of the subepithelium were in the stomach pylorus. We observed the lymphatic capillaries between the bottoms of the tunica mucosa gland and the muscularis mucosa, but did not observe the lymphatic capillaries were in the subepithelium. Our observations were similar to Han's studies<sup>[5]</sup>. We found the lymphatic capillaries were in the tunica mucoa, but no thick lymphatics. A large number of lymphatic capillaries and lymphatics were found in the tela submucosa. The lymphatic capillaries formed a coarse network. The lymphatics (vessels) also formed a coarse plexus. The corrosion casts of the lymphatic clearly showed the three-dimensional organization of the lymphatics. The diameter of the lymphatic capillary was 10 µm - 30 µm, but the lymphatic vessel's diameter was 30 µm - 100 µm.

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\*Project supported by the National Natural Science Foundation of China, No. 39070462

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Received 1998-04-20

The size of the meshes of the lymphatic vessels were varied and interconnected, triangular, oval and polygon in shape. The semithin sections also showed rich lymphatic capillaries and lymphatic vessels in the stomach tela submucosa. On the surface of the lymphatic casts, we found marked constrictions characteristic of bicuspid valves (Figure 2).

#### *The lymphatics of the tunica muscularis*

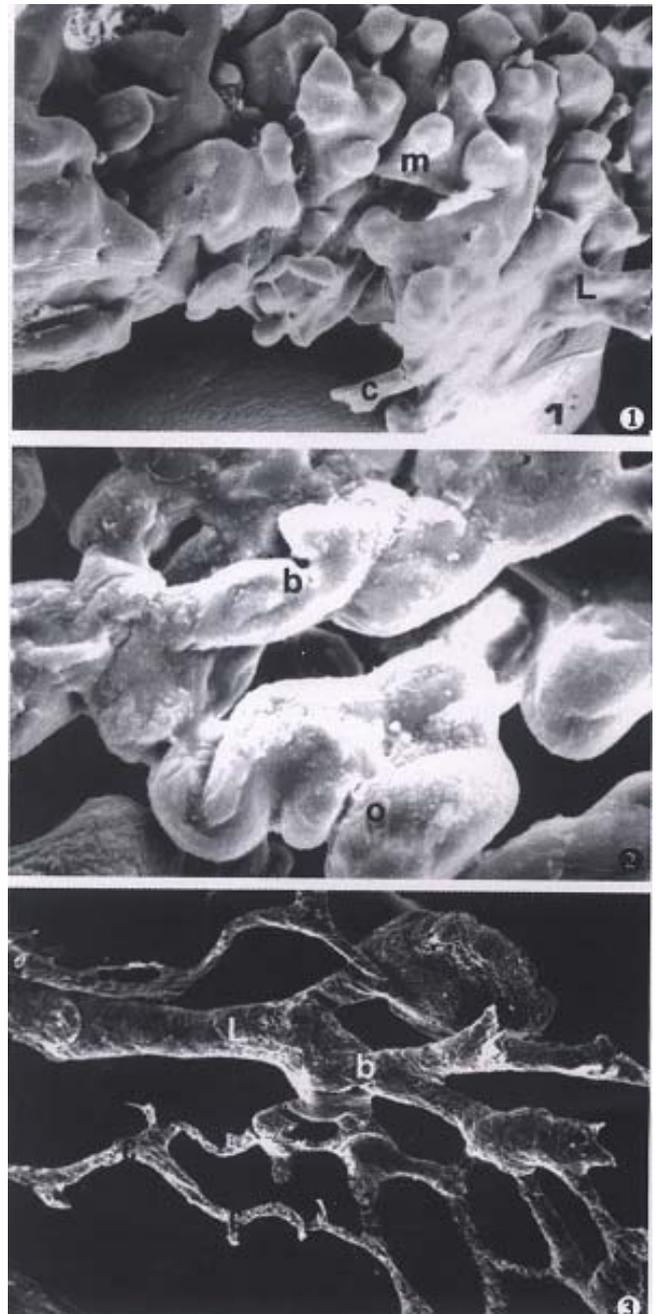
A rich lymphatic capillaries and lymphatics were found in the stomach tunic muscularis. Some the lymphatic capillaries of 7 $\mu$ m-30 $\mu$ m in diameter extended the short branches with blind ends. The diameter of the lymphatic was 30  $\mu$ m - 80  $\mu$ m. Between three muscular layers there were lymphatic capillaries and the lymphatic vessels. The lymphatics were string of beads in shape and interconnected to plexus. There were break ends of the anastomotic channels to the superficial and the deep part from the lymphatics of the tunic muscularis. It suggested that the lymphatics of the tunica muscularis were interconnected with both the lymphatics of the tela submucosa and the lymphatics of the tunica serosa. The surface of the lymphatic casts in the tunica muscularis there were folds which run parallel to the lymphatic major axis. The imprints of endothelial nuclei were denser than other layers. On the surface, we could see the transverse imprints which were induced by the smooth muscle contraction. Between the lymphatic capillaries and lymphatic vessels of the tunica muscularis there were anastomotic channels which existed in each layer of perimysium. The lymphatics and the lymphatic capillaries of the tunica muscularis were seen in the histological sections.

O<sub>ctrovekhev</sub> thought that there was not any lymphatic capillary. But Nariadchikova pointed out that the lymphatic vessels and the lymphatic capillaries of the tunica muscularis only existed among the three layer's muscularis but not in each muscular layer. Our experiment proved that in the connective tissue there were both lymphatic capillaries and lymphatic vessels in each smooth muscularis layer. We also observed the lymphatic capillaries among perifascicular parts of each muscular layer.

#### *The lymphatics of the tunica serosa*

There were both the lymphatic capillaries and the lymphatic vessels in a deep part of the tunica serosa (Figure 3). The meshes of the lymphatic capillary network and the lymphatic plexus in the layer were larger than those of the tela submucosa and the tunic muscularis. The meshes presented in willow leaf, oval or triangular shape. The lymphatic

capillaries and the lymphatic vessels were also observed in the semithin sections of the layers under the light microscopy.



**Figure 1** The interglandular circular cones of tunica mucosa (m) the lymphatic capillaries (c) and the lymphatics (L) of the tela submucosa. SEM $\times$ 150

**Figure 2** The lymphatic networks of the tela submucosa. The constriction (b) of the cast surface presenting the bicuspid valves; the ovoid or fusiform indentations (o) presenting the endothelial nuclei of the lymphatic. SEM $\times$ 550

**Figure 3** The lymphatic capillaries (c) and the lymphatic vessels (L) of the tunica serosa. The constriction (b) presenting the impression of the bicuspid valves. SEM $\times$ 200

Donin reported that only in the curvatura ventriculi minor and major did the lymphatic

The lymphatic, capillaries  
of the tunica mucosa



The lymphatic capillaries of  
the tela submucosa

The lymphatic vessels of  
the tela submucosa



The lymphatic capillaries  
of the tunica muscularis



The lymphatic vessels  
of the tunica muscularis



The lymphatic capillaries  
of the tunica serosa



The lymphatic vessels  
of the tunica serosa

The aggregate lymphatics vessels



The part lymph nodes

The drainage correlation of the lymphatics of all the layers.

capillaries of the tunica serosa exist. Rakhan thought the lymphatic capillaries of the tunica serosa only existed in the parts of pylorus. Ohtani<sup>[3]</sup> pointed out that only the lymphatic vessels existed in the longitudinal muscle layer. In our lymphatic casts and semithin sections we observed both the lymphatic capillaries and the lymphatic vessels existed in the tunica serosa.

- 1 Wassieev W. A scanning electron microscopy study of peritoneal stomata in different peritoneal regions. *Anat Anz*, 1998;180(2):137-143
- 2 Ji RC. Enzyme-histochemical study on postnatal development of rat stomach lymphatic vessels. *Microvasc Res*, 1997;54(1):1-12
- 3 Ohtani O. Microvasculature as studied by the microvascular cirrosion casting scanning electron system. *Acta Histol Jpn*, 1983;46(1):1-4
- 4 Kato S. Histochemical localization of 5'-nucleotidase in the lymphatic endothelium. *Acta Histochem Cytochem*, 1990;23(5):613-617
- 5 Han MD, Wang YX, Zhao LH. Ultrastructure of intramural lymph capillaries of the stomach in the rat. *Acta Anatomy Sinica*, 1990;23(2):129-133
- 6 Zhao LH, Wang YX, Wang GX. Fine distribution of the lymph vessels in the stomach as studied by enzyme-histochemical method. *Acta Anatomy Sinica*, 1993;24(1):97-100

REFERENCES