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***Editorials***

# Some recent works on diagnosis and treatment of gastric cancer

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**Subject headings** stomach neoplasms/ diagnosis; stomach neoplasms/therapy; antibody, monoclonal; gene therapy

Aiming at earlier diagnosis and better result of treatment of gastric cancer, we endeavored to do some laboratory and clinical studies in recent years. The following is a brief sketch of these works.

## PREPARATION AND USES OF MONOCLONAL ANTIBODIES

By means of cell fusion technic we established several hybridoma cell lines capable of producing antigastric cancer monoclonal antibodies[1]. These antibodies were named MG series among which MGa-7, MGa-9, MGa-11 an d MGb-2 were used more widely[2]. The corresponding antigens of these antibodies were different from the known cancer related antigens such as CEA, AFP, X-hap ten, Tn antigen, etc. Their chemical nature was proved to be lipoprotein, glycop rotein, or glycolipid. Immunoelectron microscopy demonstrated that these antigens were chiefly distributed on the cell membrances and/ or in the cytoplasm of the gastric cancer cells, being especially abundant in the microvilli, the foss ate surface of the cell membrane, the coarse endoplasmic reticulum and the micro cysts.

### *Diagnostic uses*

**Immunohistological and cytological diagnosis** Owing to the high specificity of these McAbs, they were used to determine the source of some meta static tumors by immunohistological examination when ordinary histological staining gave ambiguous results[3].

Dysplasia of gastric mucosa is quite common in atrophic gastritis. Its outcome is highly variable. It may remain stationary, retrogress or undergo malignant degeneration after a certain period. Hence it is deemed as a precancerous lesion, causing anxiety to the patients and their relatives and leading to repeated gastroscopy with increased economical burden and physical sufferings. So far there has been no reliable method to predict the relative risk of malignant degeneration. We found that immunohistochemical staining of the gastric mucosa with MGa-7 may serve as an important parameter for judging the relative risk of malignant degeneration. In 156 patients with dysplasia of gastric mucosa, 84 gave positive reaction and 72 gave negative reaction. Patients of each group were followed up for 2 - 4 years by repeated gastroscopies. Among the positive reactors, gastric cancer was detected in 17 cases, 7 of which were in the early stage. Among the 72 negative reactors, no one developed gastric cancer.

**Serological diagnosis** Detection of the MG series McAb corresponding antigens (MG-Ags) in blood either by ELISA[4] or by radioimmunoas say gave positive result in 58.7% to 72.8% of gastric cancer patients[5]. A follow-up study in a group of patients undergoing gastrectomy showed that after the excision of the tumor, MG-Ags in serum decreased in titre or became und etectable, while in those with unresectable gastric cancer or metastasis to remo te sites, the serum titre did not change significantly. Hence it could serve as a preliminary test in the diagnosis for gastric cancer and could be used for the surveillance of relapse after excision and for the appraisal of treatment efficacy. Owing to the simplicity and low cost of these serological tests, they were used as a screening measure in the mass survey for gastric cancer, many symptoml ess cases being discovered and properly treated.

More recently a new method for determination of serum MG-Ags was established through the construction of mAb-pXJ19 chimera[6]. The mAb possesses spe cific affinity for gastric cancer related antigen, the pXJ19 is a recombinant te mplate DNA and can be amplified by a pair of designed primers. This method was much more sensitive for detection of gastric cancer related antigen, yielding positive reaction in 85.2% of gastric cancer patients.

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### *Targeting therapy*

**Immunoconjugates** Since MG series monoclonal antibodies possess specific affinity to gastric cancer tissue, they may carry anticancer agents to the targeted tumor cells. Various immunoconjugates were produced by linking these McAbs ricin, ricin A chain, methotrexate, daunorubicin, adriamycin, epirubicin, mitomycin C, bleomycin, cisplatin and radioactive iodine[7]. After conjugation, both the immunological affinity of the antibodies for the tumor cells and the pharmacological activity of the cytotoxic agents were well preserved. These immunoconjugates exhibited selective cytotoxicity to gastric cancer cells. In vitro studies demonstrated that they could inhibit the growth rate of cancer cells. In vivo experiments showed that they could inhibit the growth of transplan ted gastric cancer in nude mice much more effectively than unconjugated McAb or anticancer agents[8]. In order to demonstrate the process of internalization of these immunoconjugates, we carried out observations by double dynamicel ectron microscopy labeling technic, using strepoavidin-gold and sheep antimouse IgG gold probes[9]. It was found that the main entry fashion of the conjugates was through non-coated microinvagination, followed by coated pits and interiorizion of microvilli. Intracellularly, the endocytosed conjugates were transported from tubovesicular structures to multivesicular bodies and finally to lysosomes, where they were degradated. In the presence of verapamil, they stayed longer in tubovesicular structures, therefore, increased amount of them would remain in the cytosol. Thus cytotoxic effect could be augmented.

**Immunoliposomes** Immunoliposomes are spherical capsules with double-layered phospholipid coats, 40 nm - 120 nm in diameter, incorporating with antigastric cancer monoclonal antibodies. It could entrap a large number (6000 - 10000) of molecules of anticancer agents. By virtue of the specific affinity of the mAb to the gastric cancer related antigen, the immunoliposomes carr ying the anti-tumor agents could be concentrated in the tumor cells manifesting a selective cytotoxic effect on the gastric cancer cells.

**Boron neutron capture therapy** Boron neutron capture therapy (BNCT) is based on the nuclear reaction ($^{10}B_5 + {}^1n_0 - {}^7Li_3 + {}^4He_2$), yielding lithium atom and alpha particles with high LET when $^{10}B$ is irradiated with thermal neutron[10]. The essential factors for a successful BNCT are a large number of $^{10}B$ atoms concentrated in tumor cell and an adequate fluence rate of thermal neutrons. We prepared immunoliposomes, 40nm in diameter, conjugated with mAb MGb-2 and entraping a $^{10}B$ rich compound $(Et\text{-}_4N)_2\text{-}^{10}B_{10}H_{10}$. There were $1.4\times 10^4$ atoms of $^{10}B$ encapsulated and 20 molecules of MGb-2 incorporated per liposome. The immunoliposomes showed specific affinity to the gastric cancer cells and could deliver sufficient amount of $^{10}B$ to them. Thus when irradiated with thermal neutrons, the gastric cancer cells were selectively killed.

## EXPERIMENTAL GENE THERAPY

The development of malignant tumors is believed to be related with the overactiv ation of the oncogenes and the inactivation of the tumor suppressor genes. So it is a logical approach to treat malignant tumors by inhibiting the oncogenes. The oncogenes related with gastric cancer include *c-myc*, *ras*, *c-erbB*-2, *K-sam* , *hst* , n-*myc* , *met* , *p53* (mutant form), etc. and telomerase, cyclin D1, PCNA, etc. are also oncogenic for gastric cancer. In recent years we used experi mental gene therapy in the following ways.

### *Gene transfection of specific ribozyme of c-erb B2*

It was found that the amplification and over expression of c-erb B2 bear a clos e relationship with the occurrence, development and metastasis of malignant tumo rs. A specific ribozyme RZ1 for c-erb B2 mRNA, which can be splitted and inactivated by it, was constructed and transplanted into the gastric cancer cell line SGC 7901. The transfected cell line SGC/RZ1 manifested remarkable changes in growth rate, cell cycle, morphology and tumorigenicity. In comparison with the original SGC-7901 cells, the growth rate was inhibited by 55%. Under flow cytome try, there was a 44% decrease of cells in S phase. Electron microscopy demonstrated vacuole degeneration, karyopyknosis and apoptosis in many cells. In nude mice, the transplanted SGC/RZ1 cells showed a delayed tumor-formation time. The size of the tumor was much smaller than that of the control.

### *Transfection of antisense RNA of PCNA*

Proliferating cell nuclear antigen (PCNA) is a co-factor for DNA polymerase, playing a very important role in the replication of DNA, proliferation of cells and regulation of cell cycle. Inhibition of PCNA expression would bring about changes of malignant behavior of tumor cells. To testify this, we transfected antisense RNA of PCNA into SGC-7901 cells. The transfected cells manifested retardation of growth rate,

degeneration, necrosis and apoptosis. The tumorigenic power in nude mice was inhibited.

### *Tranfection of wild type p53 gene*

Wild type *p53* (w*tp53*) gene is a tumor suppressor gene. When wtp53 cDNA was transplanted into SGC-7901 cells, the transfected cells (SGC7901/ wtp53) manife sted decreased growth rate and prolonged doubling time. Transmission electron microscopy revealed shrinkage of the cells and characteristic morphological features of apoptosis. By flow cytometry, the percentage of cells in G1 phase increa sed while that in S phase decreased in comparison with the parental SGC 7901 cells.

### *Tranfection of herpes simpex virus thymidine kinase (HSV-TK) gene*

HSV-TK can turn the nontoxic ganciclovir (GCV) into phosphorylated GCV, which is a potent inhibitor of DNA synthesis. When HSV-TK-mRNA was transfected into gastric cancer cells and the transfected cells were exposed to GCV in the culture medium, they were killed in a dose-dependent fashion.

### *Transfection of cyclin D1 antisense RNA*

Cycline D1 gene, located on the chromosome 11q13 region, has been found in many cancers, including gastric cancer, and is regarded to be related to carcinogenesis. To testify the effect of inhibiting cyclin D1 in gastric cancer cell, cyclin D1 antisense RNA was first constructed and then was transfected into SGC-7901 cells. The transfected cells displayed a much longer doubling time, an increased percentage of cells in G1-G0 phase, and marked inhibition of tumorigenicity in nude mice.

### *Tranfection of telomerase antisense RNA*

Recent studies indicated that the activation of telomerase is a very important factor for the uncontrolled proliferation of tumor cells. We found that telomerase was detected in 84.2% of 38 cases of gastric cancer, and only 5.2% of normal gastric mucosa. After SGC-7901 cell was transfected with human telomerase anti-sense RNA, its growth rate slowed down in culture. Morphological changes of necrosis and apoptosis occurred, and oncogenic property was reduced.

The above-mentioned preliminary experiments indicate that gene therapy might be a promising approach to the treatment of gastric cancer.

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*Original Articles*

# Relationship between DNA ploidy, expression of ki-67 antigen and gastric cancer metastasis *

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**Subject headings** Ki-67 antigen; neoplasms metastasis; immunoc hemistry; DNA ploidy; stomach neoplasms/pathology

## Abstract

**AIM To evaluate the relationship between the expression of Ki-67 antigen and the pathobiological behaviours of gastric cancers especially their distant metastases.**

**METHODS Fifty-six specimens of gastric cancer routinely fixed in formalin and embedded in paraffin (FFEP) were studied by immunohistochemical method.**

**RESULTS Expression of Ki-67 antigen was significantly related to the distant metastases to liver, ovary and adrenal gland ($P<0.01$), but not related to the histological type, growth pattern, depth of invasion, histological differentiation and the metastases to local lymph nodes ($P>0.05$). Furthermore, the Ki-67 antigen expression was significantly related to the DNA aneuploidy pattern, which is closely related to poor prognosis ($P<0.05$).**

**CONCLUSION Overexpression of Ki-67 can be used as an objective marker of the proliferative activity for predicting prognosis of gastric cancer and metastatic potential to distant organs.**

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## INTRODUCTION

Ki-67 is a mouse monoclonal antibody which recognizes a nuclear antigen expressed in all phases of the cell cycle except Go and early G1[1]. And Ki-67 immunoreactivity can thus be used as biomarker for cell proliferation. Anothermethod to measure cell proliferation is flow cytometry. In our study, we detected 56 gastric cancer tissue specimens immunohistochemically by PcAb-Ki-67 (Dako, A047) and compared with DNA ploidy pattern in order to evaluate the relationship between the proliferative activity of gastric cancer cell and pathobiological behavior of gastric cancer, especially the relationship with the distant organ metastases.

## MATERIALS AND METHODS

### *Materials*

Fifty-six specimens of gastric cancer were collected from Cancer Institute of China Medical University. Among these 56 cases, no metastasis was found in 7 cases, 12 were accompanied with liver, 4 with ovarian, 1 with adrenal and 47 with lymph node metastasis. Tissue blocks from primary and metastatic tumours were chosen from each case.

### *Methods*

PcAB to human Ki-67 antigen (A047) was used in this study to identify the proliferative activity of gastic cancer cell. The dilution for Ki-67 was 1:100. Sections were immunostained using the avidin-biotin-peroxidase complex method and pressure cooking was used to unmask Ki-67 antigen[2].

### *Evaluation of immunostaining*

Four semi-quanti quantitative classes were used for grading: negative(-), no positive cells; weak positive (+), positive cells < 10%; moder ately positive (+ +), the positive cells between 10%-50%; strong positive (+ + +), the positive cells >50%.

DNA ploidy was measured by flow cytometry, the detailed procedures and the stand ard of evaluation followed the method reported previously[3].

## RESULTS

Expression of the Ki-67 antigen was not related to WHO's classification and Lauren's classification ($P<0.05$). It was not related to the depth of local invasion of gastric cancer ($P>0.05$), growth pattern

($P > 0.05$) and local lymph nodes metastasis ($P > 0.05$). But the expression of Ki-67 was significantly related to the distant organ metastases ($P < 0.005$, Table 1) and also related to DNA aneuploidy pattern(Table 2).

**Table 1 Relationship between expression of Ki-67 antigen and metast asis of gastric cancer**

| Metastasis (Mets) | *n* | Expression of Ki-67 antigen | |
|---|---|---|---|
| | | +—+ +(%) | + + +(%) |
| Non-Mets | 7 | 3(42.9) | 4(57.1) |
| LN Mets | 32 | 23(71.9) | 9(28.1) |
| Distant organ Mets | 17 | 3(17.6) | 14(82.4)[b] |
| Total | 56 | 29(51.8) | 27(48.2) |

[b]$P<0.01$, *vs* LN (lymph node).

**Table 2 Relationship between expression of Ki-67 antigen and DNA ploidy**

| DNA ploidy | *n* | Expression of Ki-67 antigen | |
|---|---|---|---|
| | | +—+ +(%) | + + +(%) |
| Di(Tetra)ploid | 35 | 22(62.9) | 13(37.1) |
| Aneuploid | 21 | 7(33.3) | 14(66.7)[a] |

[a]$P<0.05$.

## DISCUSSION

Proliferative activity of cancer cells was closely related to the biological behavior of carcinoma, especially the invasion, metastasis and prognosis. In this study, the results showed that Ki-67 could be used as a marker to measure the proliferative activity of gastric cancer cells and predict the potential of metastasis to distant organs of gastric cancer. The method was simple and quick. The detection of Ki-67 antigen could be used as a useful marker to foretell the high risk of the metastases to distant organs and predict the prognosis of gastric cancer.

DNA aneuploidy was one of the markers of malignant tumour cells. Xin, *et al* had reported that aneuploidy DNA pattern may be related to the development of distant organ metastases, especially through the blood vascular system[4]. The results of this study showed that DNA aneuploidy was related to the expression of Ki-67, the latter was also closely related to the distant met astases ($P < 0.01$). These suggested that the expression of Ki-67 and aneuploidy DNA pattern are two objective markers which may be valuable in predicting high potential of metastases to the distant organs, and the combined detecti on of these two markers could be a more useful method for predicting metastases to the distant organs and prognosis.

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# *In situ* detection of tumor infiltrating lymphocytes expressing perforin and fas ligand genes in human HCC *

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**Subject headings** carcinoma, hepatocellular; lymphocytes; fas ligand genes; perforin; *in situ* hybridization; liver neoplasms

## Abstract

AIM **To investigate the expression of perforin and fas-ligand (fas-L) of tumor infiltrating lymphocytes (TILs) in human hepatocellular carcinoma (HCC).**

METHODS **By *in situ* hybridization and immunohistochemistry, the perforin and fas-L gene expression of TILs was studied in 20 HCC cases.**

RESULTS **Positive expression of perforin and fas-L genes was detected in 16 HCC cases. One patient had expression of perforin and fas-L genes in the majority of TILs and survived 1.5 years after tumor resection without HCC relapse. This seems that the presence of a large number of activated T cells might be beneficial for the antitumor immunity. In other cases, less than 10% o f TILs were able to express perforin and fas-L genes.**

CONCLUSION **Although there were a number of T cells in HCC, only few of them were immunoactive and able to kill tumor cells. It seems important to promote further proliferation of these activated T cells *in vitro* or *in vivo*.**

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## INTRODUCTION

Cytotoxic T lymphocytes (CTLs) play a major role in killing tumor cells. Two pat hways have been described by which a cytotoxic cell may induce lysis of its target[1,2]. The first pathway is called perforin pathway. T cell receptors (TCRs) of CTLs binding with MHC antigens on the tumor cells induce the release of granules filled with perforins and granzymes, and perforins then attack the target cell surface, followed by granzymes entering into the cell and killing it. The second is fas-L pathway. Fas-L from activated T cell binds with Fas antigen on the tumor cell surface, directly causing cell apoptosis. Although we applied TILs to treat tumor several years ago[3], this is the first domestic report studying whether activated T cells around the tumor express the two killing genes, perforin gene and fas-L gene. We studied the expression of perforin and fas-L genes of TILs in the HCC specimens using *in situ* hybridization and immunohistochemistry to find out whether there are T cells with killing activities in HCC tissues.

## MATERIALS AND METHODS

### *Materials*

Specimens were obtained from 20 HCC patients (16 men and 4 women; ranging in age from 25 to 64 years with a mean of 47 years) who underwent tumor resection from January to June, 1994 in our hospital. Among these patients, 17 were associated with liver cirrhosis. The control specimens were from the normal liver tissues of 3 patients with hepatic angioma. The normal liver tissues and HCC tissues were quickly frozen in liquid nitrogen within half an hour after removed from the bodies of the patients, and stored at -70℃. The specimens cut from the marg in between tumor and paratumor areas were embedded with O. C. T, fixed with 40mL/L-paraformaldehyde, gradiently dehydrated with ethanol, and then stored at -70℃. Human fas-L cDNA was kindly presented as a gift by Dr. Nagata of J apanese Bioscience Institute, and human perforin cDNA by Dr. Kevin Y.T. Thia of Australia Austin Institute. Rabbit-anti-human fas-L polyclonal antibodies were purchased from Santa Cruz Biotech Company of USA. Rat-anti-human

perforin monoclonal antibody was donated by Dr. Eckhard. R. Padack of the Medical Academy of Miami University, USA. Digoxin labeling and detection kit was purchased from Boehringer Mannheim Company of Germany. The immunohistochemistry kit of streptavi din-biotin amplification system was a-product of WAK Company of Ger many.

### *Methods*

**HE staining**

***In situ hybridization*** Probe labeling proceeded according to the method of random primer labeling presented by the digoxin labeling kit of Boehringe r Mannheim Company. In situ- hybridization was performed following a previously published protocol[3]. The concentration of the probe was $1\times10^3$ μg/L and cells with blue granules under microscope were regarded as positive.

**Immunohistochemistry** The specimens were processed according to the ABC method, and visualized with DAB. Cells with brown granules under microscope were regarded as positive.

## RESULTS

### *HE staining and immunohistochemistry*

A few lymphocytes with negative perforin and fas-L expression were seen in the 3 normal liver tissues, while in the 19 HCC specimens, there was a various number of TILs, most in the mesenchyma of the tumor and a few in the parenchyma. Furt hermore, in another HCC case (No.14) which had not-experienced relapse for 1.5 years after tumor resection, there were a large number of TILs with positive expression of perforin and fas-L not only in the mesenchyma of the tumor, but also extensively in the parenchyma of the liver (Figures 1-3). TILs of 15 patients had positive perforin and fas-L expression with a positive rate below 10%. In the other four cases, although there was a various number of TILs infiltrating in the tumor mesenchyma, no TILs expressed perforin and fas-L. There was no relationship between the number of TILs in the liver tissue and the positive rate of perforin and fas-L expression.

### *In situ hybridization*

There were no perforin and fas-L positive-hybridization signals in the 3 normal liver specimens. In HCC specimens, perforin and fas-L expression of TILs showed strong positivity in one case (No.14), mild positivity in 15 cases and negativity in 4 cases, indicating that perforin and fas-L expression in transcriptive level was parallel to that in protein level in HCC.

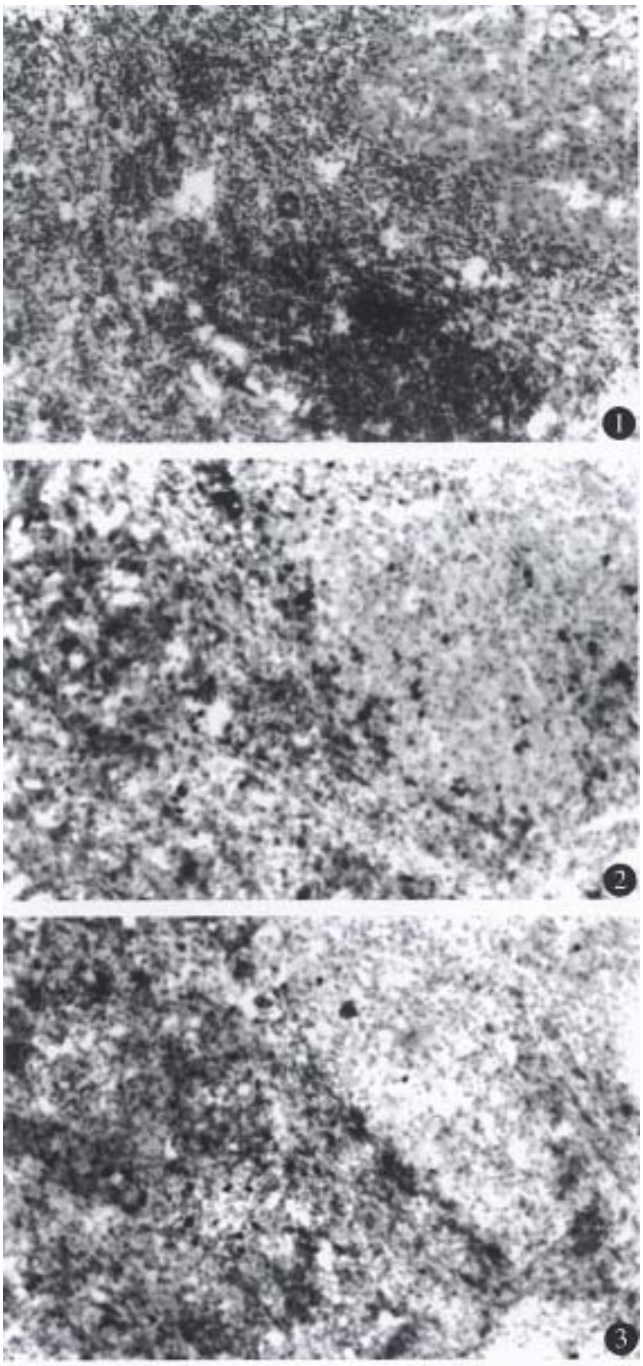

**Figure 1** Most infiltrating TILs in the mesenchyma of the tumor, few in the parenchyma. HE×100

**Figure 2** Strong positive signals of perforin in the TILs of hepatoma tissue. ABC×100

**Figure 3** Strong positive signals of fas-L in the TILs of hepatoma tissues. ABC×100

## DISCUSSION

TILs, a heterogeneous group of lymphocytes consisting largely of T lymphocytes, are located mainly in the tumor mesenchyma. As early as 1907, TILs were found in tumor tissues and the phenomenon that lymphocytes infiltrated in tumor tissues was supposed to be the result of the resistance of the

host to the tumor. Later, it was found that the greater the number of TILs in the tumor tissue, the better outcome the patient would get[4].

Early studies on TILs focused on their phenotypes. Recent investigations showed that T lymphocytes killed the tumor cells mainly under the perforin and fas-L pathways. Perforin and fas-L expression in TILs of tumor tissues is directly related to the killing activity of TILs in tumor tissues. In 1994, Leger-Ravet *et al*[5] found perforin and granzyme B expression in TILs in 10 follicular lymphoma cases. Of all 20 HCC cases we studied, TILs expressed perforin a nd fas-L in varing degrees in 16 patients, and negatively in 4. This indicates that cytotoxic T lymphocytes existed in most of the HCC patients. Furthermore, in one case there was a large number of TILs expressing fas-L and perforin in both the mesenchyma and the parenchyma of the tumor tissues. This patient did not sustain relapse within the 1.5 year follow-up period. This implies that large quantities of T lymphocytes with killing activities existing in the tumor tissues are beneficial for the prognosis of HCC patients. Except the case presen ted above, TILs expressing perforin or fas-L in the other 19 cases of HCC were below 10%, showing that most of the TILs were in an immunosuppressive state. The mechanism of this phenomenon might be ①the HCC could not activate T lymphocytes due to its deficient expression of the second signal B7; ② T lymphocytes expressing high levels of fas and fas-L resulted in self-apoptosis[6,7]. Therefore, amplification of the T lymphocytes with killing activities *in vivo* or *in vitro* may improve the therapeutic effect for the patients with tumors.

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# Expression and significance of proapoptotic gene *Bax* in gastric carcinoma *

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**Subject headings** stomach neoplasms/ pathology; Bax gene; gene expression; immunohistochemistry

## Abstract

AIM **To study the expression of proapoptotic gene Bax in human gastric carcinoma and its significance.**

METHODS **Using immunohistochemistry methods, the Bax protein expression in 57 specimens of gastric carcinoma and its relationship with clinical status and pathomorphological parameters were observed.**

RESULTS **Thirty-three (57.9%) cases were positive for Bax pr otein staining which was mainly located in the cytoplasm of tumor cells. The rate of Bax protein expression was not correlated with the tumor size, lymph node metastasis, depth of invasion, clinical stages of tumors and age and sex of patie nts ($P<0.05$), but strongly associated with the morphological type and diff erentiation degree of tumors. It was significantly higher in intestinal type and well or moderately differentiated gastric carcinoma than in diffuse type and poorly differentiated gastric carcinoma ($P<0.05$ and $P<0.01$).**

CONCLUSION **The proapoptotic gene Bax is differently expressed in most of gastric carcinoma and may take part in the modulation of apoptosis i n gastric carcinoma. The expression of Bax might be associated with the occurrence of intestinal type gastric carcinoma and the differentiation of gastric carci noma.**

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## INTRODUCTION

Recent investigations have demonstrated that apoptosis plays a significant role in the pathogenesis of tumors[1,2]. Emphasis has been laid on the mechanisms that regulate apoptosis pathways. Bcl-2 associated X protein (Bax), which has extensive amino acid homology with Bcl-2, can form heterodimers with Bcl-2 *in vivo*. Overexpressed Bax can counter the death repressor activity of Bcl-2, and accelerate apoptotic cell death[3]. To determine whether proapoptotic gene Bax plays a role in the regulation of apoptosis in gastric carcinoma , an immunohistochemical study of Bax protein expression in gastric carcinoma an d its relation to clinical status, pathomorphological parameters were carried out.

## MATERIALS AND METHODS

### *Histological specimens*

Fifty-seven cases of surgically resected gastric carcinomas (male 39, female 16; mean age 58.6 years) were collected from the files of the Department of Pathology of our hospital. All blocks were fixed in 10% formalin and embedded in paraffin. Serial sections were cut from each block in 4μm, stained with hematoxylin and eosin and confirmed pathologically.

### *Immunohistochemical methods*

Immunohistochemical staining for Bax protein was performed using SP technique wi th the following procedure: ① slides were deparaffinized in 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 30 mL/L $H_2O_2$ in methanol for 5 minutes, and then incubated for 1 0 minutes at room temperature in normal goat serum (1:20). ② Slides were incubated with a 1:50 dilution of the primary rabbit antihuman Bax polyclonal antibody (Santa Cruz, USA) for 30 minutes at 37℃. A biotin-streptavidin detection system was employed with diaminobenzidine as the chromogen. ③ Slides were washed twice wit hphosphate-buffered saline and incubated with the linking reagent (biotinylate d antiimmunoglobulin) for 10 minutes at 37℃. After rinsing in phosphate-buff ered saline, the slides were incubated with the peroxidase-conjugated streptavidin

label for 10 minutes at37℃,and incubated with diaminobenzidine and $H_2O_2$ 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 prepar ed for each sample using an irrelevant antibody of the same isotype as the prima ry antibody.

The immunostaining of Bax protein was visually classified into negative and positive groups by observing 1000 tumor cells in the areas of the sections: no staining present in any of tumor cells or less than 10% tumor cells with staining (-); more than 10% tumor cells with positive staining. 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 Bax protein in gastric carcinoma*

Thirty-three (57.89%) of the fifty-seven gastric carcinomas showed immunoreac tivity for Bax protein in gastric carcinoma cells. The Bax protein immunoreactivity appeared brown or dark brown, which was mainly located in the cytoplasm (Figure 1), and a few specimens simultaneously expressed Bax protein in the cell nuclear of tumor cells. Some of the mature lymphocytes infiltrating in the stroma of gastric carcinomas also had Bax protein expression.

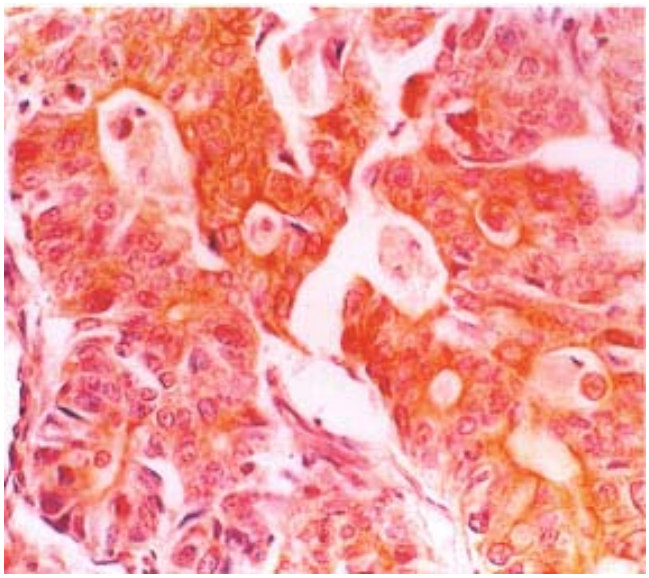

**Figure 1** Bax immunoreactivity was detected in cytopl asm of gastric carcinoma cells. SP×200

### *Correlation between Bax protein expression and clinicopathological parameters of gastric carcinomas*

Correlation between Bax protein expression and clinical pathological data of gastric carcinoma is illustrated in Table 1. The rate of Bax protein expression was not correlated with patient age, sex, tumor size, lymph node metastasis, depth of invasion and clinical stages ($P>0.05$). The immunoreactivity of Bax wa s significantly associated with morphologic phenotype and grades of differentiation of gastric carcinoma. 20 (73.3%) of 30 gastric carcinomas of intestinal morphologic phenotype were immunoreactive versus 11 (40.7%) of 27 diffuse gastric carcinomas ($P<0.05$). 17 (81.0%) of 21 well and moderately differentiated gastric carcinomas were immunoreactive versus 10 (38.5%) of 26 poorly differentiated gastric carcinomas($P<0.01$).

**Table 1 Correlation between Bax protein expression and clinicopathological parameters of gastric carcinomas**

| Parameters | *n* | Bax protein expression - | Bax protein expression + | Positive rate (%) |
|---|---|---|---|---|
| Age (year) | | | | |
| ≤59 | 39 | 22 | 17 | 56.41 |
| ≥60 | 18 | 11 | 7 | 61.11 |
| Sex | | | | |
| M | 39 | 24 | 15 | 61.54 |
| F | 18 | 9 | 9 | 50.00 |
| Type | | | | |
| Intestinal | 30 | 22 | 8 | 73.33[a] |
| Diffuse | 27 | 11 | 16 | 40.74 |
| Grade of differentiation | | | | |
| Well/moderate | 21 | 17 | 4 | 80.95[b] |
| Poor | 26 | 10 | 16 | 38.46 |
| Mucoid | 10 | 6 | 4 | 60.00 |
| Tumor size | | | | |
| <5cm | 35 | 21 | 14 | 60.00 |
| ≥5cm | 22 | 12 | 10 | 54.55 |
| Lymph-node metastasis | | | | |
| Negative | 23 | 13 | 10 | 56.52 |
| Positive | 34 | 20 | 14 | 58.82 |
| Serosal invasion | | | | |
| Absent | 27 | 14 | 13 | 51.85 |
| Present | 30 | 19 | 11 | 56.67 |
| Clinical stages | | | | |
| I and II | 34 | 20 | 14 | 58.82 |
| III and IV | 23 | 13 | 10 | 56.52 |

[a]$P < 0.05$, $\chi = 6.193$, *vs* diffuse-type gastric carcinoma, [b]$P < 0.01$, $\chi = 8.580$, *vs* poorly differentiated gastric carcinoma.

## DISCUSSION

Apoptosis is a highly regulated form of programmed cell death defined by distinct morphological and biochemical features. Apoptosis plays a major role in development, embryogenesis, regulation of the immune system, and carcinogenesis, as well as in the

maintenance of tissue homeostasis. Various protein molecules or oncogenes and suppressor genes are involved in the process of apoptosis, including *p53*, *myc*, *ras*, Bcl-2, Bax and the Fas/Fas ligand system[4]. In recent studies, Bax protein expression has been identified in various human malignant tissues, including the prostate, colon, breast, testis and ovary[5-8]. But, little is known about Bax protein expression and its relationship with the biological behavior of human gastric carcinoma.

In this study, we found that the positive rate of Bax protein staining in gastric carcinoma was 57.9%. The proapoptotic gene Bax can express to various degrees in most kinds of the gastric carcinoma and may take part in the regulation of apoptosis of gastric carcinoma. Our findings concerning the relationship between Bax protein expression and the pathological characteristics of gastric carcinoma showed that Bax expression was associated with morphologic phenotype and grades of differentiation of gastric carcinomas. The difference in the Bax protein expression in the intestinal and diffuse types demonstrated that aberrant Bax protein expression was preferentially associated with development of intestinal type gastric carcinoma, indicating once more the different biologic mechanisms involved in the development of these two histologic subtypes. The difference in the Bax protein expression between poorly differentiated and well/ moderately-differentiated gastric carcinomas demonstrated that aberrant Bax protein expression was associated with differentiation or growth speed of gastric carcinomas. There was no significant relationship between Bax protein expression and tumor size, lymph node metastasis, serosal invasion or clinical stages. Therefore, Bax prote in expression might play an important role in the early development and phenotypic differentiation of gastric carcinomas, but not in tumor progression.

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# Antisense to cyclin D1 reverses the transformed phenotype of human gastric cancer cells *

CHEN Bing[1], ZHANG Xue-Yong[2], ZHANG Yu-Jing[3], ZHOU Ping[3], GU Yan[4] and FAN Dai-Ming[2]

**Subject headings** stomach neoplasms; cyclin D1; RNA, antisense; gene therapy

## Abstract

**AIM To further investigate the effect of cyclin D1 on the bio logic behavior of cancer cells and its potential role in gene therapy of tumor.**

**METHODS A cyclin D1 subcloning plasmid termed BKSD1 was const ructed by subcloning the human cyclin D1 cDNA into Bluescript-KS, a plasmid vec tor with a pair of T7 and T3-promoters, with recombinant DNA technology of molecular biology. So, it is easy to generate digoxigenin (DIG)-labeled RNA probes of antisense and sense to cyclin D1 using RKSD1 as a template vector. PDORD1AS, an eukaryotic expression vector containing the full-length human cyclin D1 cDNA in its antisense orientation cloned into the retroviral vector pDOR-neo, was succ essfully constructed with BKSD1 to change restriction sites. A gastric cancer cell line, SGC7901/VCR, was transfected with pDORD1AS by Lipofect Amine-mediate d introduction and a subline termed SGC7901/VCRD1AS, which had stable overexpr ession of antisense RNA to cyclin D1, was obtained by selection in G418. The sub line, control subline transfected pDOR-neo and SGC7901/VCR were evaluated by methods of immunohistochemistry, flow cytometry, molecular hybridization, morpho logy and cell biology.**

**RESULTS Compared with control cell lines, SGC7901/VCRD1AS had a reduced expression of cyclin D1 (inhibition rate was about 36%), increased ce ll size and cytoplasm to nucleus ratio, increased doubling time (42.2 h to 26.8 h and 26.4 h), decreased saturation density ($18.9\times10^4$ to $4.8\times10^5$ and $4.8\times10^5$), increased percentage of cells in the G1/G0 phase (80.9%-64.6% and 63.8%), reacquired serum dependence, and a loss of tumorigenici ty in nude mice (0/4 to 4/4 and 4/4).**

**CONCLUSION Stable overexpression of antisense RNA to cyclin D1 can reverse the transformed phenotype of human gastric cancer cells and may provide an approach of gene therapy for gastric cancer.**

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## INTRODUCTION

Studies on the functions of cellular proto-oncogenes and tumor suppressor genes indicate that most of these genes mediate signal transduction pathways that play a critical role in cell proliferation and differentiation as well as cell cycle control[1]. This has led to the realization that cycle regulatory proteins can also be directly involved in oncogenesis. Critical transitions in the eukaryotic cell cycle are regulated by the sequential activation of a series of cyclins and cyclin-dependent kinases (CDKs)[2]. Since the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G1 phase of the cell cycle[3], the deregulated expression of G1 phase cyclins or their related CDKs might cause loss of cell cycle control, thus enhancing oncogenesis. Cyclin D1 has been strongly implicated in controlling the G1 phase of the cell cycle. So, cyclin D1 gene is regarded as one of the best oncogene candidates[4]. Indeed, rearrangement, amplification, and overexpression of the cyclin D1 gene, which is located on the human chromosome 11q13 region, have been found in several types of human cancer[5]. Overexpression of cyclin D1 in rat fibroblasts enhanced their growth and tumorigenicity and cyclin D1 collaborated with an activated *ras* oncogene[6] or a defective adenovirus E1A oncogene[7] to increase the transformation of primary rodent fibroblast. The present study was undertaken to obtain more direct evidence th at cyclin D1 plays a critical role in establishing and maintaining the transformed phenotype of these tumor cells. To this end,

an antisense cyclin D1 cDNA was stably expressed in human gastric cancer cell line SGC7901/ VCR that kept high expression of cyclin D1. This led to decreased levels of the endogenous cyclin D 1 protein, marked inhibition of cell proliferation, and loss of tumorigenicity. These findings provide direct evidence that the cyclin D1 gene plays an essential role in the increased proliferation and oncogenesis of the gastric cells.

## MATERIALS AND METHODS

### *Cell culture*

The human cell lines used in the study, SGC7901/ VCR gastric cancer cell line[8], T24 bladder carcinoma cell line and K562 leukemia cell line, were obtained from Digestive Diseases Institute and Stomatological Biology Center of the Fourth Military Medical University, Xi'an, China. Cells were maintained in RPMI 1640 medium plus 100mL/ L fetal calf serum (FCS). The medium for the cell lines contain ing the *neo* resistant gene was supplemented with G418.

### *Construction of antisense cyclin D1 expression plasmids*

The RKSD1 that contains the 1.1kb-human cyclin D1 cDNA in *Hind* III site is a present of Columbia-Presbyterian Cancer Center. A cyclin D1 subcloning plasmid termed BKSD1 was constructed by subcloning the human cyclin D1 cDNA into a subcloning vector Bluescript KS with recombinant DNA technology of molecular biology. pDORD1AS, an eukaryotic expression vector containing the 1.1 kb human cyclin D1 cDNA in its antisense orientation cloned into the retroviral vector pDOR-neo, was successfully constructed by the method as described before[9].

### *Lipofectamine mediated transduction*

The pDORD1AS and vector control pDOR-neo plasmids were transfected into SGC7901 /VCR gastric cancer cells using standard lipofectamine transfection procedure. A stable expression of cyclin D1 antisense RNA subline termed SGC7901/VCRD1AS was obtained by selection in G418.

### *Immunohistochemistry*

Exponentially proliferating cells grown on glass slides were subjected to immuno histochemical staining by using a monoclonal antibody DSC-6 against cyclin D1. T24 and K562 cell lines were used as positive and negative controls of cyclin D1 overexpression, respectively[6,10].

### *In situ hybridization*

Using BKSD1 containing a pair of promoters for T7 and T3 RNA polymerase as a tem plate vector, DIG-labeled, antisense and sense RNA probes of cyclin D1 was made by *in vitro* transcription of DNA. Exponentially proliferating cells grown on glass slides were subjected to *in situ* hybridization by a standard nonradioactive *in situ* hybridization procedure. Control cell lines included T24, K5 62, SGC7901/ VCR and SGC7901/VCRneo which is a pDOR-neo transfected subline of SGC7901/VCR.

### *Flow cytometric analysis*

Cells were cultured in complete medium. When they were exponentially dividing, the cells were collected and analyzed for the cell cycle distribution (PI dyeing) and the expression level of cyclin D1 (immunofluorescence) by flow cytometry. All experiments were repeated three times.

### *Doubling times and saturation density*

Cells were plated in triplicate at a density of $2.5\times 10^4$ per well in 24-well plates in 1mL of RPMI 1640 plus-100mL/L-FCS. The number of cells per well was counted every day for 10 days. The doubling times and saturation dens ities of each cell line were calculated.

### *Assessment of serum dependence*

The growth rates of the two control cell lines and SGC7901/VCRD1AS cell line were measured at different concentrations of FCS. All cells were seeded initially of $2.5 \times 10^4$ cells/ well. Growth of each of the three cell lines was determi ned at three different serum concentrations (10%, 2.5% and 0.5%).

### *Soft agar assay*

Growth in 3g/L-Noble agar was assayed. In brief, cells were suspended in RPMI1640 plus 200mL/L-FCS containing 3g/ L-agar and plated in triplicate in 24-well plates. After 2 weeks of growth, the cells were counted by microscopy. All experiments were repeated two times and similar results were obtained.

### *Tumorigenicity assays*

Cells of $5\times10^5$ were injected subcutaneously into multiple sites in athymic (nude) mice. The animals were monitored for tumor formation every week and sacrificed one month later.

## RESULTS

### *Expression of antisense RNA of cyclin D1 in SGC7901/VCR cells*

We introduced an antisense cyclin D1 cDNA sequence into the SGC7901 cell line, whose cyclin D1 gene was overexpressed. Following G418 selection, the drug resistant (neo+) cell SGC7901/VCRD1AS was randomly collected from the cultures infected with the pDORD1AS construct. As controls, neo+ clone SGC7901/ VCRneo was selected from SGC7901/VCR culture infected with pDOR-neo vector lacking the antisense cyclin D1 cDNA sequence.

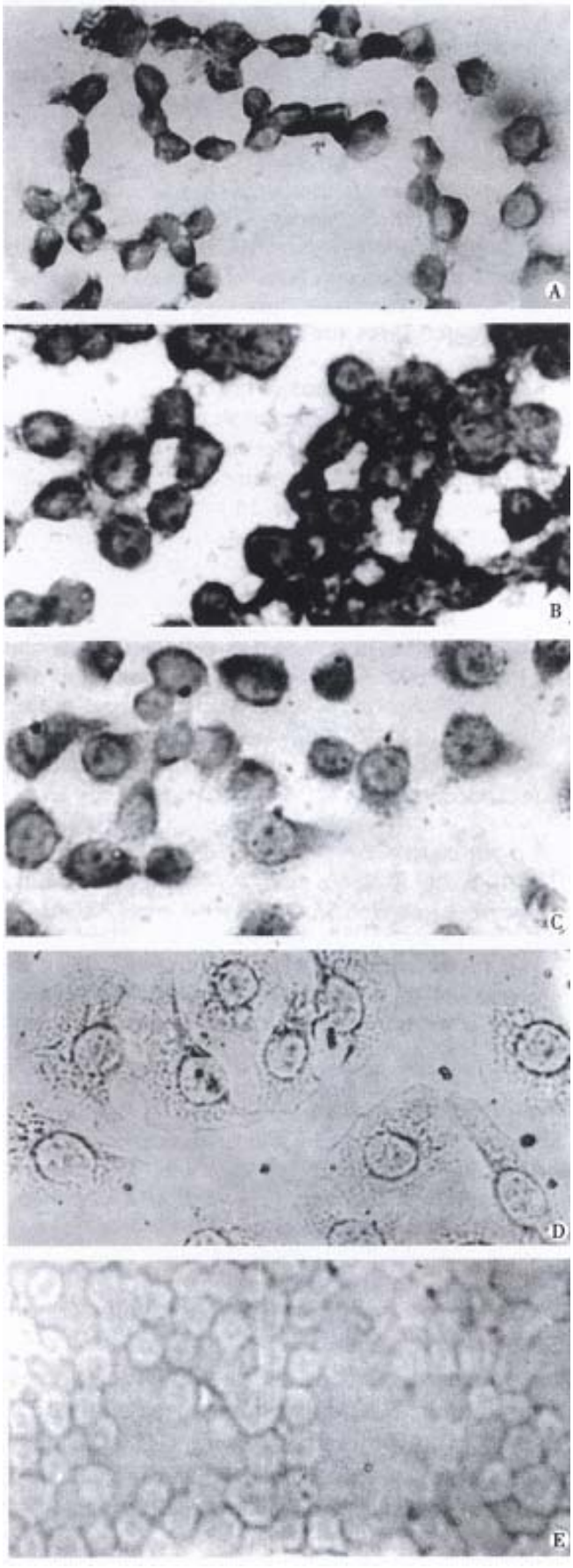


**Figure1** The result of *in situ* hybridization of cyclin D1 mRNA. A T24; B. SGC7901/ VCR; C. SGC7901/ VCRneo; D. SGC7901/VCRD1AS; E. K562

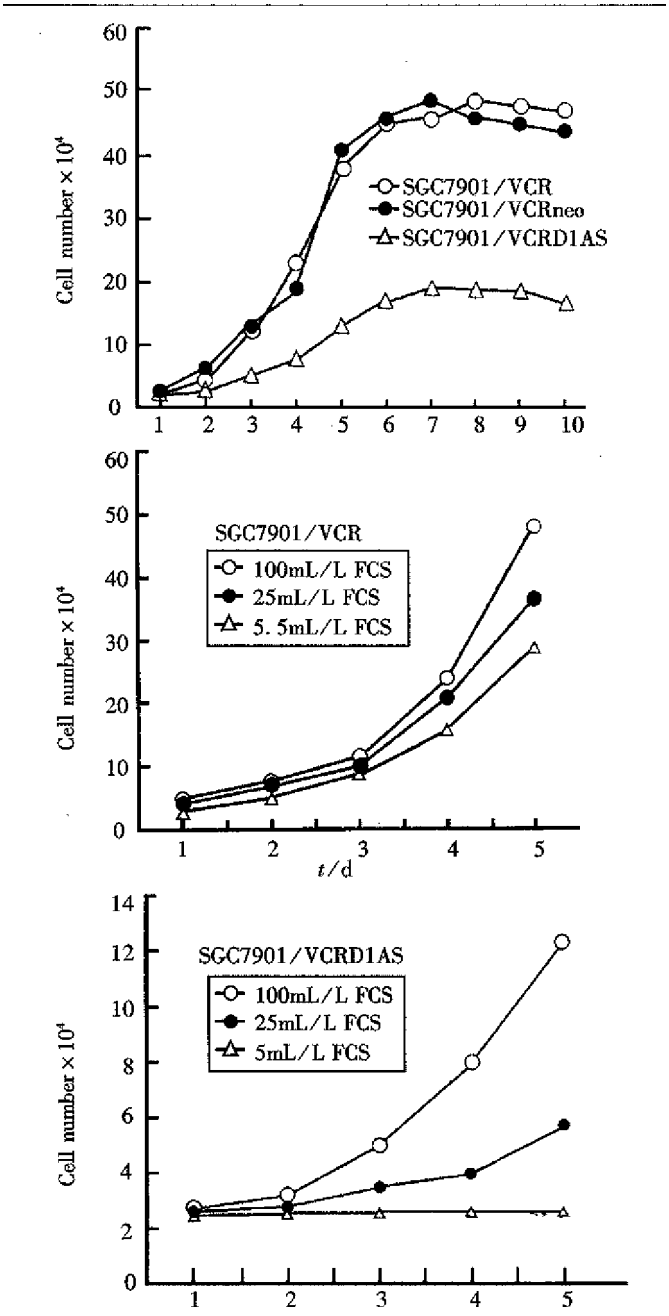


**Figure2** Growth curve of SGC7901/VCRD1AS and control cell lines.

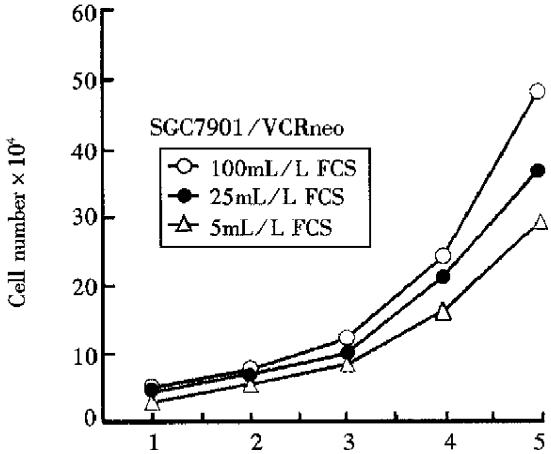


**Figure3** Serum dependence of SGC7901/VCRD1AS and control cell lines.

It turned out by immunohistochemistry, flow cytometry and *in situ* hybridization that the expression levels of cyclin D1 protein and mRNA were much lower ($P < 0.01$, inhibition rate of cyclin D1 protein expression level by flow cytometry assay was 36%) in SGC7901/ VCRD1AS than in SGC7901/ VCR and SGC7901/VCR n eo control cells.

### *Characterization of morphology, growth properties and cell cycle progression of antisense cyclin D1 expressing cells*

The observation results of morphology. The SGC7901/ VCR and SGC7901/ VCR neo control cells displayed a loss of contact inhibition and formed dense foci when they grew to a high saturation density. The SGC7901/ VCRD1AS cells grew at monolayer and low cell density, manifested an increased cytoplasm to nucleus ratio, and wer e much flatter and larger in cell size than the control cells (Figure 1).

The analysis of cell biology. As Figures 2, 3 and Table 1 show, compared with control cells, the SGC7901/ VCRD1AS cells displayed a much longer doubling time ($P < 0.01$), an increased percentage of cells in the G1/G0 phase, reac quired serum dependence to some extent, a much lower saturation density ($P < 0.01$), and a marked inhibition on tumorigenicity in nude mice and soft agar clo ning efficiency ($P < 0.01$).

**Table 1 Growth properties and tumorigenicity of SGC7901/ VCRD1AS and control cell lines**

| Cell line | SGC7901/VCR | SGC7901/VCRneo | SGC7901/VCRD1AS |
|---|---|---|---|
| Doubling time (h) | 26.8 | 26.4 | 42.2 |
| Saturation density ($\times 10^5$) | 4.8 | 4.8 | 1.9 |
| Cell cycle distribution (%) | | | |
| G1/G0 | 64.6 | 63.8 | 80.9 |
| S | 25.9 | 27.8 | 13.0 |
| G2-M | 9.4 | 8.3 | 6.1 |
| Colony forming efficiency (%) | 5.47 | 5.50 | 0.03 |
| Tumor formation in nude mice | 4/4 | 4/4 | 0/4 |

## DISCUSSION

In previous studies, the anti-cyclin D1 antibodies or cyclin D1 antisense plasmids were microinjected into fibroblast[10] or B-cell lymphoma cell line s[11] during the G1 interval. The cells were prevented from entering the S phase. These results indicated that cyclin D1 is required for cells to undergo the G1-S transition. However, because of the transient nature of these microin jection studies, they did not address the role of cyclin D1 overexpression in continuously dividing cells, in growth control and tumorigenesis. Overexpressi on of cyclin D1 in fibroblasts shortened the G1 phase[6]. In our study, stable overexpression of antisense RNA to cyclin D1 can inhibit the cell growth and reverse the transformed phenotype of gastric cancer cells besides a marked decrease in the mRNA and protein level of cyclin D1. For example, a much longer doubling time and increased percentage of G1/G0 cells reveal G1 phase arrest, a much lower saturation density and reacquired serum dependence announce restored adjustability by exogenous signals, a monolayer growth feature in low density and increased cytoplasm to nucleus ratio manifest reacquired anchorage depen dent and somewhat epithelial feature. Our present studies provide the evidence t hat inhibition of the expression of cyclin D1 in tumor cells that overexpress this gene can reverse their transformed phenotype. It is of clinical significance because large numbers of human tumors display overexpression of cyclin D1. Our findings may provide a potential approach of gene therapy fo r gastric cancer[12].

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# Effect of cell fusion on metastatic ability of mouse hepatocarcinoma cell lines *

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**Subject headings** carcinoma, hepatocellular; cell lines; cell fusion; neoplasm metastasis; lymphatic metastasis

## Abstract

**AIM To study the effect of cell fusion on metastatic ability of mouse hepatocarcinoma cells and the factors involved in the process of metastasis.**

**METHODS By the method of successively increasing the conc entrations, cell fusion and limit dilution, 8-Ag resistant cells were selected, and HGPRT-Hca-P cells and eight cloned hybridoma cells were obtained. To observe their metastatic ability, they were inoculated into mice foodtaps and the drainage lymph nodes were examined under microscope.**

**RESULTS The end concentration of 8-Ag which was used to select HGPRT deficient Hca-P cells was 30mg/L. All the cells selected died in HAT culture medium in one week. Fused cells appeared approximately 9 days later. They were round, transparent and a little larger than their parental cells. Eight clones of hybridoma cells were obtained and named as PSH1-PSH8. The metastatic rate of HGPRT-Hca-P cells and PSH7 cells was 28.6% and 71.4% respec tively, the difference being significant ($P < 0.05$). The metastatic rate of other clones was no more than 20% and there was no significant difference from HGPRT-Hca-P cells ($P>0.05$).**

**CONCLUSION In normal mice splenic lymphocytes, there are some factors that could inhibit tumor metastasis, however, there are some other factors accelerating tumor cells to metastasize. The e stablishment of PSH7 provides an experimental model which could be used to study the factors involved in metastasis.**

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## INTRODUCTION

It was known during the 1970s that the malignancy of hybridoma cells decreased when tumor cells were fused with normal cells and the malignant phenotype was suppressed obviously or diminished. But since 1980, in many laboratories, the increased invasive and metastatic abilities of hybridoma cells have been found when tumor cells were fused with lymphocytes or macrophages which had ambulant ability[1-5]. These results implied that there were some factors that could increase the metastatic ability of tumor cells and raised a hypothesis that tumor cells used the ambulant mechanism of normal cells[6]. In this study, cell fusion was used to study the factors involved in the process of metastasis.

## MATERIALS AND METHODS

### *Animals*

A total of 615 inbred mice were provided by the Department of Pathology of Dalian Medical University.

### *Tumor cell lines*

Mouse hepatocarcinomaceu lines Hca-P(P) with low lymphatic metastatic ability were established and stored as described previously[7].

### *Hypoxanthine-guanine phosphor ibosyltrasferase deficient (HGPRT-) cells*

In order to select HGPRT- cells, P cells were converted into 8-Ag resistant by growing in successively increased concentration of 8-Ag as described previously[8]. The initial concentration was 3mg/L. If alive tumor cells had the dominant position, the concentration was increased successively, from 3 mg/L to 6 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L, and 30 mg/L. To test the sensitivity of selected cells to HAT, 8-Ag resistant cells which had been cloned by limit dilution method were inoculated into HAT culture medium. A week later, Trypan blue repelling test was used to judge the vitality of cells and the 100% dead cells which kept alive in normal culture medium were proliferated. The cells were taken as parental cells in fusion. To measure their metastatic ability, HGPRT - Hca-P cells were inoculated into foodtaps of 615 mice[7]. The animals w ere sacrificed 28 days later, and original tumor, the ipsilateral popliteal, inguinal and axillary lymph nodes were removed, fix ed in 10% formalin, made into paraffin section, stained with HE

and observed und er microscopy.

### *Fusion*

Spleen lymphocytes of normal mice were prepared routinely. HGPRT--Hca-P cells and normal spleen lymphocytes were taken as parental cells of fusion. Solution of fusion was 50% PEG-4000. The average cell numbers of each well in 96 well plate used for fusion were 3×105 and the ratio of lymphocytes to tumor cells was 8:1. While fused cells appeared, they were suspended in 2×HAT culture medium, then were cultured at 37℃ in a humidified atmosphere (950 mL/L air, 50 mL/L $CO_2$). The medium was changed every three days and two weeks later it was substituted by normal medium. Subsequently, the obtained hybridoma cells were cloned by lim it dilution method. To measure the metastatic ability of hybridoma cells, Hca-P cells and HGPRT-Hca-P cells were inoculated into foodtaps of 615 mice resp ectively as described above.

## RESULTS

The end concentration of 8-Ag which was used to select HGPRT deficient Hca-P cells was 30mg/L. All of the cells proliferated after being cloned died in HAT culture medium within one week, suggesting that they were HGPRT- cells.

Fused cells appeared approximately 9 days later. They were round, transparent and were a little larger than their parental cells. Eight clones of hybridoma cells were obtained by using limit dilution method and were named PSH1-PSH8. It was shown by the histological examination that the metastatic ability of PSH7 incre ased but the rest decreased. The metastatic ability of PSH7 was significantly higher than that of HGPRT--Hca-P cells, but there was no significant difference between HGPRT--Hca-P and Hca-P which were not treated with 8-Ag ($P$<0.05, Table 1). Because the sample size was small, exact probabilities in 2×2 table of Chi-square test was used to analyze the data.

**Table 1 Metastatic rate of cells**

| Cells | Number of experimental animals | Number of metastatic animals | Metastatic rate (%) | *P* |
|---|---|---|---|---|
| HGPRT--Hca-P | 14 | 4 | 28.6 | |
| Hca-P | 16 | 3 | 18.8 | 0.24>0.05 |
| PSH1 | 8 | 1 | 12.5 | |
| PSH2 | 8 | 0 | 0 | |
| PSH3 | 8 | 1 | 12.5 | |
| PSH4 | 10 | 2 | 20 | |
| PSH5 | 8 | 0 | 0 | |
| PSH6 | 10 | 1 | 20 | |
| PSH7 | 14 | 10 | 71.4 | 0.027<0.05 |
| PSH8 | 8 | 0 | 0 | |

## DISCUSSION

The method of successively increasing concentration was often used to select resistant cells. In this study, it was used to select 8-Ag resistant Hca-P cells. The critical concentration was 30 mg/ L-under which most cells died. As the concentration was increased, the survived and proliferated cells were 8-Ag resistant cells. These cells could be transplanted into normal culture medium. In order to prevent HGPRT$^-$ cells from turning into HGPRT$^+$ cells, it was necess ary to treat them with 8-Ag now and then or always keep them growing in culture medium containing 8-Ag.

Cell fusion has been extensively used in the study of phenotypic expression and regulation of malignant cells. It has been known that the metastatic ability of hybridoma cells could decrease or increase while certain normal cells were fused with nonmetastatic or low metastatic cells. In those experiments, the metastatic ability of hybridoma cells increased only when the parental cells were ambulant cells, such as lymphocytes or macrophages and the hybridoma cells obtained certain characters.

Both Hca-F and Hca-P cells isolated from mouse hepatocarcinoma cells had diffe rent metastatic ability only to lymph nodes but not to other o-gans. Because the meta static rate of P cell was 18.8% and metastatic phenotype was stable, it is of great advantage to study the changes and related mechanism of metastatic ability ?of hybridoma cells which were obtained from the fusion of Hca-P and spleen cells of mice.

The metastatic rate of HGPRT-Hca-P cells was still lower than 30% and there was no significant difference from that of Hca-P cells. The metastatic ability of most hybridoma cells kept stable or decreased, in contrast, that of PSH7 increased up to 71.4%, being significantly different from that of HGPRT$^-$-Hca-P cells. Because hybridoma cells had the nature of their parental cells, it is umportant to study the factors which affect tumor metastatic ability by cell fusion. Hca-P as one of the parental cells had the character of stable metastatic ability to lymph nodes. Therefore no matter how the metastatic ability changed, it was caused by lymphocytes. The metastatic ability of seven hybridoma cell lines decreased, while only one increased. These results suggested that the lowering trend was dominant, which was probably due to tumor suppressive gene existing in normal cells, furthermore, there were some other factors that could enhance tumor metastatic ability.

There were many similar aspects between metastasis of tumor cells and ambulance of lymphocytes. Both of them could enter circulation by passing endothelia of vessels, proliferate in drainage lymph nodes, and enter peripheral tissue by immi-

gration. Furthermore, organ preference of tumor metastasis was similar to the homing of lymphocytes. The relationship between homing receptor and tumor metastasis was discovered recently[9]. Here comes the question: do the hybridoma cells with increased metastatic ability use some special mechanism of lymphocytes or macrophages, such as ambulant mechanism and homing receptor It is suspected that probably tumor cells metastasize to special organs by means of some structures like homing receptor and a certain mechanism like the homing of lymphocytes. The results of our study show that there must be something existing in spleen lymphocytes that accelerates tumor cells to metastasize. The establishment of PSH7 has provided an experimental model which could be used to study the factors involved in metastasis.

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# Effect of HCV infection on expression of several cancer-associated gene products in HCC *

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**Subject headings** carcinoma; hepatocellular/etiology; hepatit is C-like viruses/pathogenicity; oncogenes/genetics; genes, suppressor; tumor/genetics; immunohistochemistry/methods

## Abstract

**AIM To study hepatocarcinogenesis of hepatitis C virus (HCV).**

**METHODS Expression of HCV antigens (CP10, NS3 and NS5) and several cancer-associated gene products (ras p21, c-myc,c-erbB-2, mutated p53 and p16 protein) in the tissues of hepatocellular carcinoma (HCC, *n* = 46) and its surrounding liver tissue were studied by the ABC (avidin-biotin complex ) immu nohistochemical method. The effect of HCV infection on expression of those gene products in HCC was analyzed by comparing HCV antigen-positive group with HCV antigen negative group.**

**RESULTS Positive immunostaining with one, two or three HCV antigens was found in 20 (43.5%) cases, with either of two or three HCV antigens in 16 (34.8%) cases, and with three HCV antigens in 9 (19.6%) cases. Deletion rate of p16 protein expression in HCC with positive HCV antigen (80%, 16/20) was significantly higher than that in HCC with negative HCV antigen. Where as no significant difference of the other gene product expression was observed between the two groups.**

**CONCLUSION HCV appears related to about one-third of cases of HCC in Chongqing, the southwest of China, and it may be involved in hepatocarcinogen esis by inhibiting the function of p16 gene, which acts as a negative regulator of cell cycle.**

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## INTRODUCTION

Our previous studies by seroepidemiological, molecular epidemiological and immunopathological methods have revealed that hepatitis C virus (HCV) infection is closely linked to development of hepatocellular carcinoma (HCC) and HCV may be the second important factor in association with HCC- etiology in Chongqing, the southwest of China[1-5]. But the molecular mechanisms involved in hepatocarcinogenesis of HCV remain poorly understood. Up to now, many authors believe that HCV can not directly change the structure of the host genes like hepatitis B virus by integration because HCV is a RNA virus. Therefore, the effect of HCV on factors of controlling cell growth and development is an important field in the hepatocarcinogenesis studies. In this study, expression of several oncogene and tumor suppressor gene products in HCV-associated and non-HCV-associa ted HCC was investigated, so as to identify if HCV infection can affect expression of these gene products.

## MATERIALS AND METHODS

### *Specimens*

HCC specimens of 46 cases were randomly selected from partial hepatectomy in 1994 in this hospital. Of them, 38 cases contained pericancerous liver tissues. All specimens were fixed in 100mL/L-formalin, embedded in paraffin and sequentially sectioned with a thickness of 5 μm.

### *Reagents*

Mouse monoclonal antibodies (mAb) to HCV NS3 and NS5 were kindly provided by Professor TAO Qi-Min (The Institute of Hepatology, Beijing Medical University). Mouse mAb against HCV CP10 was kindly presented by Professor LI Meng-Dong (Department of Infectious Diseases, the Southwest Hospital). Mouse mAbs to human ras p21, C-myc, C-erbB-2 and mutated p53 protein were purchased from Fuzhou Maxim Biotechnical Company. Mouse McAb to human p16 protein was purchased from Beijing Zhongshan Biotechinical Company. Avidin-biotin complex (ABC) kits were purchased from Fuzhou Maxim Biotechnical Company and Vector company.

### *Immunostaining*

Immunostaining of HCV antigens CP10, NS3, NS5 and cancer-associated gene produc ts ras p21, c-myc,

c-erbB-2, mutated p53 and p16 proteins was performed by the ABC method in each case. The procedures of ABC staining were taken according to the manufacturer's recommendations as previously described[5]. The color was developed with diaminobenzidine and hematoxylin. Positive and negative controls were simultaneously used to ensure specifity and reliability of the staining.

## RESULTS

### Expression of HCV antigens

In the 46 cases of HCC, positive HCV antigen was found in 20 (43.5%) cases, of which 4 cases with one positive HCV antigen, 7 cases with two positive HCV antig ens, and 9 cases with three positive HCV antigens. The positive staining of HCV antigen CP10, NS3 and NS5 in the cancer tissues was observed in 10 (21.7%), 10 (21.7%) and 7 (15.2%) cases, respectively, while in its pericancerous liver tissues in 14 (36.8%), 13 (34.2%) and 12 (31.6%) cases. Although the expression rates were higher in the pericancerous tissues than in the cancer tissues, no statistical significance was obtained ($P$>0.05) (Table 1). The immunostaining of each HCV antigen was mainly seen in HCC and hepatocyte cytoplasms, seldom in the cell membranes, none in the nuclei. The positive-staining cells were distributed mostly in scattered or focalized patterns, seldom in diffused pattern.

**Table 1 Expression of HCV antigens in HCC tissue and its surrounding liver tissue (%, positive rate)**

| HCV antigens | Cancer | Non-cancer |
|---|---|---|
| CP10 | 21.7(10/46) | 36.8(14/38) |
| NS3 | 21.7(10/46) | 34.2(13/38) |
| NS5 | 15.2(7/46) | 31.6(12/38) |

### The effect of HCV infection on expression of the gene products

On the one hand, positive rates of ras p21 and mutated p53 in HCC (58.7%, 27/46; 28.3%, 13/46) were significantly higher than in the pericancerous tissues (34.2%, 13/38; 7.9%, 3/38, $P < 0.05$), whereas the positive rate of p16 in HCC (41.3%, 19/46) was significantly lower than in the pericancerous tissues (63.2%, 24/38, $P < 0.05$). But the expression rates of c-myc and c-erbB-2 did not show significant difference between the cancer and pericancerous groups ($P > 0.05$). On the other hand, it attracted our attention that the positive rate of P16 protein in HCV antigen-positive HCC (20%, 4/20) significantly lower than in HCV antigen-negative HCC (57.7%, 15/ 26, $P < 0.025$), even though the expression rates of ras p21, C-myc, C-erbB-2 and mutated p53 showed no significant difference between HCV-associated and non HCV-associated HCC (Table 2).

**Table 2 Relationship of HCV antigens with expression of cancer-associated gene products(CAGP) (*n*, positive cases)**

| HCV antigens | *n* | CAGP expression | | | | |
|---|---|---|---|---|---|---|
| | | p21 | C-myc | C-erbB-2 | p53 | p16 |
| Positive | 20 | 11 | 11 | 9 | 5 | 4 |
| Negative | 26 | 15 | 20 | 13 | 8 | 15 |

## DISCUSSION

In the previous studies, we found that HCV RNA could be detected in 36.6% (34/93) serum samples of patients with primary hepatic carcinoma and 37.5% (21/56) cases of HCC tissues[1,3]. In this study, using three McAbs to different HCV antigens and immunohistochemical ABC method, we found that the positive immunostaining with either one, two or three HCV antigens was found in 20 (43.5%) cases, with either two or three HCV antigens in 16 (34.8%) cases and with three HCV antigens in 9 (19.6%) cases among the 46 cases of HCC. The present data are consistent with our previous studies and further indicate that about one-third of HCC seems to be related to HCV infection in Chongqing, the southwest of China. Up to now, a lot of affirmative evidences in seroepidemiology, molecular epidemiology and immunopathology have been obtained concerning the association of HCV infection with HCC development in this area.

Recent studies have shown that the molecular mechanisms of hepatocarcinogenesis are involved in oncogene activation and anti-oncogene inactivation like many other tumors. The role of ras, c-myc, c-erbB-2, p53 and p16 gene in the development and progression of HCC have been noted by many workers. To understand the potential hepatocarcinogenesis of HCV, we studied the expression of these gene products in HCV-associated and non-HCV associated HCC tissues. The results showed that the expression of ras p21, c-myc, c-erbB-2 and mutat ed p53 was not significantly different between HCV antigen-positive and HCV antigen-negative groups, but the deletion rate of p16 protein expression in HCV antigen-positive HCC (80%, 16/20) was significantly higher than in HCV antigen-negative HCC (42.3%, 11/ 26, $P < 0.025$). It implicates that the molecular mechanisms involved in HCV hepatocarcinogenesis seems to be connected with the repression of p16 gene function.

The p16 gene is a new negative regulator of cell

cycle and tumor supressor gene found recently, which is located in chromosome 9p21 with 8.5kb long and encoding for a nucleus phosphoprotein with 16kD-P16 protein. P16 protein can bind to cycle-dependent kinase 4 (CDK4), preventing their interaction with cyclin D and thereby preventing cell cycle progression from G1 to S phase. Many authors proposed that when p16 gene function is repressed, the activity of cyclin D/ CDK4 complex will increase because of the CDK4 being free from the inhibition of P16 protein, thereafter cell proliferation will be out of control and tumor may develop at last[6,7]. Recently, Ray *et al* reported that HCV core protein can act as an effector in the promotion of cell growth by repression trancription of the another negative regulator of cell cycle and inhibitor of cyclin D/CDK4 complex p21 (WAF1/ Cip1/ Sid1) gene through unknown cellular factors[8]. Therefore, the role of p16 gene in molecular mechanisms of HCV hepatocarcinogenesis deserves further studies.

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# Experimental research on phospholipids variation of halothane on liver mitochondria

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**Subject headings** halothane; sevoflurane; liver mitochondria; HPLC; hepatotoxicity

## Abstract

**AIM To study the pathogenesis of hepatotoxicity of halothane.**

**METHODS The effect of different concentration of halothane and sevoflurane on mitochondrial membrane phospholipids composition of rat liver were analyzed using high performance liquid chromatography (HPLC) technology.**

**RESULTS Halothane at low concentration could degrade mitochondr ial membrane major phospholipids and increase lysophosphatidylcholine.**

**CONCLUSION The pathogenesis of halothane hepatotoxicity was the phospholipids variation on liver mitochondria.**

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## INTRODUCTION

The effect of traditionally inhalational anesthetic halothane and new drug sevof lurane on mitochondrial membrane is reported below in an attempt to study the pa thogenesis of halothane hepatotoxicity.

## MATERIALS AND METHODS

### *Preparation of liver mitochondria and pretreatment of specimen*

According to modified Estabook's velocity gradient method[1], the mitochondria of male rat weighing 150g-200g was separated. Seventy mmol sucrose and 220mmol bovine serum albumin were used as isolation medium. Albumin was assayed by biuret reaction. The mitochondria concentration was adjusted to 10g/L-30g/L. Phospholipids except for ganglioside and acetal phospholipid were extracted using improved Higgins' method[2]. The mito chondrial suspension was mixed well with the extraction solvent (1:10, V/V ), and stood for 15min. The albumin was removed by centrifugation. $CaCl_2$ 0.05mL/L was added to the supernatant, and stood for centrifugation (3000 r/ min). The lower layer was evaporated to dryness under nitrogen at 40℃ - 50℃. After added with diluent accurately to the residue, the solution was sealed to protect from light and stored at -20℃ for HPLC analysis. The whole procedure was carried out at 4℃ in the airtight ice-bath.

### *Preparation of solvent*

The standard control phospholipids of phosphatidylethanolamine (PE), phosphatidy linositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), cardiolipin (CL), sphingomyclin (SPH) and lysophosphatidylcholine ( LPC ) were purchased from Sigma Co.

Extracting solution: chloroform:methanol:hydrochloric acid (2:1:0.01,V/V/V).

Moving phase: n-hexane:isopropanol:ethanol: potassium dihydrogen phosphate (25 mnol/ L) : glacial acetic acid (370 : 485 : 100 : 562 : 0.1 V/V/V/V). The solution was evenly mixed and stood overnight for separating phosphoric acid crystal. After ultrafiltration and deoxygenation the supernatant was used as moving phase.

Standard solution: the standard control phospholipids were dissolved in the mixture of n-hexane: isopropanol (6 : 8, V/V). The concentration was 2g/L.

### *HPLC analytical method*

ISUZU LC-6A liquid chromatograph, ISUZU Shim-Pack CLC-SIC column (6 mm × 15 cm), Guarc PAKTM prepared column were used. The detection was performed at 206nm. After reaction of low concentration and high concentration of halothane or sevoflurane with mitochondrial membrane phospholipids, 10ml reaction solution was taken out for repeated injection[3]. Each sample was repeated for 8 times, and linear velocity (mm/min) was recorded. Qualitative analysis was made by identification of the retention time with standard control samples. The eluting sequence referred to Patton sequence[4]. Quantitative ana lysis was made by calculating the peak area and the relative content of phospholipids was expressed by the ratio between peak area and albumin.

## RESULTS

### *Qualitative analysis*

By comparing the HPLC chromatograph peak of phospholipids affected by halothane at low and high concentration with that of the normal liver mitochondria phospholipids, it could be seen that the main phospholipid peak decreased to some degree and LPC peak increased, especially when at high concentration. Sevoflurane at low concentration had no influence on phospholipid peak, but at high concentration it could decrease the main phospholipid peak and increase the LPC peak. However, the effect was not so obvious as that caused by halothane.

### *Quantitative analysis*

The change of liver mitochondrial phospholipids caused by halothane and sevoflur ance is shown in Table 1. Halothane at both high and low concentration could decrease the main liver mitochondrial phospholipids and increase LPC significantly. The change of phospholipids had no significant difference between sevofurane at low concentration and the control while at high concentration the difference was marked. At high concentration, the change of phospholipids in liver mitochondria caused by halothane was much more obvious than that caused by sevoflurane.

### *Time-phase change*

The effect on the liver mitochondrial phospholipid principle started and went up rapidly as soon as halothane contacted with mitochondria and reached the peak at 4h. At low concentration it could recover to the level of the control group at 6h-8h while at high concentration it could not even within 24h. In each phase there had no significant difference between low concentration of sevoflurane and the control. At high concentration the effect caused by sevoflurane reached the peak at 4h and recovered to the control level at 8 h - 12 h. The time-phase change on LPC by halothane and sevoflurane at high concentration is demonstrated in Figure 1.

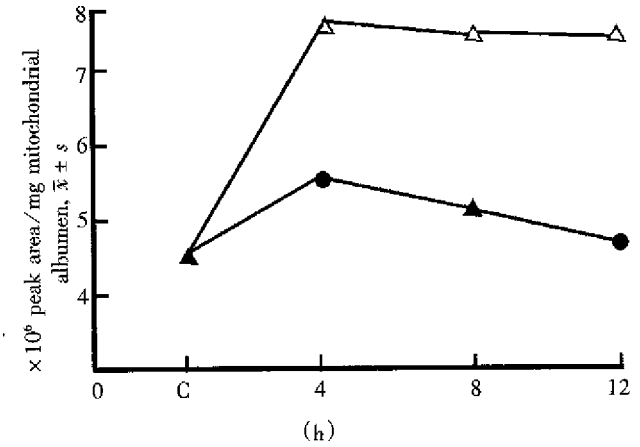


**Figure 1** The time phase change on LPC affected by ha lothane and sevoflurane in high concentration.

**Table 1 The variation of phospholipid in liver mitochondrial membrane affected by halothane and sevoflurane (phospholipid unit: $\times 10^9$ peak area/g mitochondrial albumin $\bar{x} \pm s$)**

| Group | PE | PI | PS | CL | PC | SPH | LPC |
|---|---|---|---|---|---|---|---|
| Control | 1.48±0.26 | 1.16±0.19 | 0.84±0.09 | 1.02±0.11 | 2.93±0.28 | 3.98±0.59 | 4.54±0.42 |
| At low concentration | | | | | | | |
| Sevoflurane | 1.37±0.19 | 1.08±0.09 | 0.78±0.07 | 0.95±0.11 | 2.72±0.17 | 3.76±0.33 | 4.89±0.33 |
| | (-7.62%) | (-7.12%) | (-6.93%) | (-6.55%) | (-7.19%) | (-5.52%) | (+7.81%) |
| Halothane | 1.19±0.12 | 0.96±0.06 | 0.70±0.80 | 0.85±0.16 | 2.50±0.26 | 3.37±0.29 | 5.90±0.12 |
| | (19.60%)[a,b] | (-17.24%)[a,b] | (-16.94%)[a,b] | (-16.25%) | (-14.38%)[a,b] | (15.43%)[a,b] | (+30.1%)[a,b] |
| At high concentration | | | | | | | |
| Sevoflurane | 1.30±0.12[a] | 1.02±0.04a | 0.63±0.04[a] | 0.89±0.70[a] | 0.64±0.16[a] | 3.57±0.22[a] | 5.69±0.32[c] |
| | (11.89%) | (12.02%) | (-11.26%) | (-12.55%) | (-10.36%) | (-10.40%) | (+25.35%) |
| Halothane | 1.06±0.09[c] | 0.81±0.07[c] | 0.78±0.09[c] | 0.75±0.09[c] | 2.09±0.16[c] | 2.95±0.24[c] | 7.81±0.67[c] |
| | (-28.38%)[b] | (-2.41%)[b] | (-25.24%)[b] | (-26.59%)[b] | (-2867%)[b] | (-26.61%)[b] | (+41.87%)[b] |

[a]$P<0.05$, [c]$P<0.01$ *vs* control; [b]$P<0.05$ *vs* sevoflurane.

## DISCUSSION

This study indicated that halothane at low concentration could degrade mitochondrial membrane major phospholipids and increase LPC, at high concentration it could damage mitochondrial membrane irreversibly. Although sevothane had action on mitochondria, the effect was reversible. Probably due to its molecular stru cture halothane soluble in liver mitochondria easily and destroy phospholipids obviously. Halothane had the similar result in the study on the inhalational anesthetic effect on liver mitochondria intimal fluidity[5].

Phospholipase A (PLA1, PLA2) is universal in liver membrane. Characterized by intramembranous mode of action, PLA2 has a high activity in mitochondria and the highest catalytic speed toward PE (twice that of PC, ten times of CL). PLA2 could be excited by Ca equilibration of liver cell caused by poison *in vivo* and *in vitro*. The phospholipid structure variation greatly influences biomembrane function and physical property including membrane congugase and receptor kinetics[6]. Some other studies showed that lipid variation such as mitochondrial phospholipids degradation and lipid peroxidation is an important original cause of liver cell damage. Destruction of the integration of mitochondria is the result of mutual function of the above-mentioned two mechanisms while degradation of membrane phospholipid caused by activation of mitochondria probably plays a more important role in the early damage of overall function of liver cells[7].

Besides hypoxia and low volume of blood flow the study also showed that mitochondrial phospholipids variation in the unorganized test is the main factor of halothane hepatotoxicity. Inhabition of $PLA_2$ activity and antilipid peroxidation may be the important measure of antihalothane hepatotoxicity[8].

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# Multifactorial analysis of recurrence of cholecystolithiasis in Shanghai area

CHEN Pei, WANG Bing-Sheng and HE Lian-Qi

**Subject headings** cholecystolithiasis/ therapy; recurrence; follow-up studies

## Abstract

**AIM To explore the risk factors of gallbladder stone recurrence.**

**METHODS A multifactorial analysis was made for 1058 patients in Shanghai area whose gallbladder stones disappeared after different kinds of nonsurgical therapy, including oral litholytic therapy, extracorporeal shock wave lithotripsy and percutaneous choledocholithotripsy. Serum level of in sulin and total bile acid were determined in 122 patients.**

**RESULTS After 1-8.8 years of follow-up, the recurrence rate of gallbladder stone was 11.6%, 22.4%, 29.5%, 36.4%, 39.3% and 39.7% respectively within 1, 2, 3, 4, 5 and over 5 years. The risk factors for the recurrence are: primary multiple gallstones ($P<0.05$); family history of cholecysto lithiasis ($P<0.05$); greasy food intake ($P<0.01$); low mean value of serum insulin ($P<0.01$); and high mean value of total bile acid ($P<0.01$).**

**CONCLUSION The recurrence of cholecystolithiasis is related to overintake of high fat and high cholesterol food, and might also be related to low level of serum insulin.**

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## INTRODUCTION

With the clinical application of different kinds of nonsurgical therapy, such as oral dissolution of gallstone (ODG), extracorporeal shock wave lithotripsy (ESWL), percutaneous transhepatic gallbladder catheterization (PTGC) and contact dissolution, and percutaneous choledocholithotripsy (PCCL), the recurrence and anti-recurrence of cholecystolithiasis come as a problem now. The clinical value of these methods mostly depends on the recurrence rate of this desease. Discover y of the risk factors of the recurrence of cholecystolithiasis, and interference procedures make it possible to lower the recurrence rate of gallstone. A multifactorial analysis of the recurrence of cholecystolithiasis in Shanghai area was carried out by the Shanghai Gallstone Research Coordination Group.

## MATERIALS AND METHODS

### *Research subjects*

A total of 1058 patients whose gallbladder stones had disappeared after different kinds of non-surgical therapy in Shanghai area entered this study, including 454 cases after ESWL, 594 cases after PCCL and 10 cases after ODG.

### *Collection of follow-up materials*

**Formulation of follow-up table** Let patients mark the items in the table and put the database into computer. The table includes sex, age of gallstone incipient occurrence, height, weight, diet hobby, symptom and medical treatment, related diseases (such as diabetes mellitus, coronary heart disease, liver disease), family history of gallstone, size and number of gallstone, recurrence and recurrence time of cholecystolithiasis.

**Examination of patients** One hundred and twenty-two patients were randomly selected from gallstone patients. Stone recurrence was found in 48 patients and non recurrence in 74. Venous blood of 5ml was drawn before breakfast in the morning. After standing still for half an hour, serum was sealed after centrifugation. Serum insulin level was determined with Coat-Acount insulin kit, and serum total bile acid (TBA) level by Ausbile Auto kit at the same time. Ultrasound examination was performed to evaluate the condition of gallbladder (length, width, height, stones), the degree of the liver lipid infiltration and the condition of the common bile duct (CBD), about 1 hour after greasy food. Ultra-

som ography was repeated to reveal the width, length and height of the constricted gallbladder. With the formula $V = (3.1416 \times L \times H \times W)/6$, the volume of gallbladder when starve or after diet, and the contraction ratio of the gallbladder volume were calculated.

### *Statistical method*

① There were 792 pieces of subjective materials and 122 objective materials when database was set up. ② The follow-up rate, recurrence rate and loss to follow-up rate of patients after ESWL, PCCL and ODG were calculated. ③ To find out the statistical difference between the recurrence and non-recurrence groups (Table 1). ④ To find out the statistical difference between the multiple and solitary stones groups(Table 2). ⑤ With the help from Epidemiology Teaching and Research group of Shanghai Medical University, the Epi-info Version 5.01a software was used to process the data.

**Table 1 Difference between recurrence and non-recurrence groups**

| | Odds ratio | M-H Chi square | *P* value |
|---|---|---|---|
| Incipient stone | 1.52 | 6.43 | <0.05 |
| Sex proportion | 0.91 | 0.31 | 0.58 |
| Diet hobby | 0.66 | 6.02 | <0.05 |
| Related disease | 0.85 | 0.87 | 0.35 |
| Clinical symptom | 12.51 | 190.97 | <0.01 |
| Medical treatment | 1.70 | 9.03 | <0.01 |
| Family history | 1.55 | 4.54 | <0.05 |
| Mean thickness of gallbladder wall | 1.95 | 2.75 | 0.10 |
| Adipose infiltration of liver | 1.01 | 0.00 | 0.98 |
| | Recurrence | Non-recurrence | *F* test | *P* value |
| Mean age | 43.85±11.3 | 43.15±11.9 | 0.534 | 0.528 |
| Mean weight/height | 0.387±0.05 | 0.380±0.05 | 2.701 | 0.097 |
| Mean contraction ratio of gallbladder | 0.500±0.264 | 0.522±0.277 | 0.196 | 0.663 |
| Mean value of serum insulin | 0.611±0.320 | 0.753±0.261 | 7.289 | <0.01 |
| Mean value of serum TBA | 5.963±1.883 | 5.00±1.955 | 7.545 | <0.01 |

**Table 2 Difference between multiple stones and solitary stone groups**

| | Odds ratio | M-H Chi square | *P* value |
|---|---|---|---|
| Recurrence | 1.52 | 6.43 | <0.05 |
| Sex proportion | 1.08 | 0.21 | 0.65 |
| Diet hobby | 1.26 | 2.10 | 0.15 |
| Related disease | 0.95 | 0.09 | 0.76 |
| Medical treatment | 1.42 | 4.35 | <0.05 |
| Clinical symptom | 1.21 | 1.38 | 0.24 |
| Family history | 1.08 | 0.13 | 0.72 |
| Mean thickness of \gallbladder wall | 0.55 | 2.15 | 0.14 |
| Adipose infiltration of liver | 0.91 | 0.05 | 0.83 |
| | Multiple stones | Solitary stone | *F* test | *P* value |
| Mean age | 45.52±12.02 | 43.98±11.47 | 2.438 | 0.115 |
| Mean weight/height | 0.382±0.048 | 0.383±0.052 | 0.108 | 0.742 |
| Mean contraction ratio of gallbladder | 0.538±0.251 | 0.50±0.28 | 0.528 | 0.524 |
| Mean value of serum insulin* | 0.586±0.331 | 0.739±0.266 | 7.526 | <0.01 |
| Mean value of serum TBA | 5.590±1.788 | 5.349±2.063 | 0.391 | 0.540 |

*Because of abnormal distribution of the value of ser um insulin, all data were changed into log value.

## RESULTS

### *Total follow-up rate and stone recurrence rate*

From January 1988 to October 1995,there were 1058 patients whose gallbladder stones had disappeared after different kinds of non-surgical therapy. Seven hundred and ninety-two patients were followed up for 1-8.8 years with a rate of 74.8%. The stone recurrence rate was 11.6%, 22.4%, 29.5%, 36.4%, 39.3% and 39.7% respectively within 1, 2, 3, 4, 5 and over 5 years. The total recurrence rate was 30.8%.

### *Follow-up rate and recurrence rate after EWSL*

Among 454 patients treated with ESWL, 413 patients are followed up, with a rate of 91.0%. There were 285 cases with non- recurrence and 128 with recurrence (17 were treated surgically, the others received conserrative treatment). Fourty-one patients were lost to follow-up. The recurrence rate of gallstone was 11.9%, 20.2%, 34.8%, 35.7%, 37.2% respectively within 1, 2, 4, 5 and over 5 years. The total recurrence rate was 31.0%.

### *Follow-up and stone recurrence rate after PCCL*

Among 594 patients treated with PCCL, 370 were followed up, the follow-up rate being 62.3%. There were 262 cases with non-recurrence and 108 with recurrence. Seven of them were treated surgically, the others received consecutive treatment, and 224 patients were lost to follow-up. The recurrence rate of gallstone was 8.8%, 22.4%, 29.5%, 36.7%, 47.4% respectively within 1, 2, 3, 4 and 5 years. The total recurrence rate was 29.9%.

### *Follow-up and stone recurrence rate after ODG*

Nine of 10 patients treated with ODG were followed up. The stone recurrence occurred in 8 patients. One case was lost to follow-up. Average follow-up length was 5 years and 4 months. The stone recurrence rate was 88.9%.

## DISCUSSION

### *Recurrence rate of gallbladder stone*

According to the literature, the recurrence rate of cholecystolithiasis is about 7%-11.8% after ODG, ESWL, PTGC and PCCL treatment[1-3]. In this study, the 1-year stone recurrence rate was 8.8%-11.9%, similar to the literature. It has been reported that the stone recurrence rate increases by about 10% each year, and by the fifth year it reaches 50%. After 5 years, a plateau with no further recurrence is usually seen[4]. The recurrence rates after ESWL, PCCL and ODG in the fifth year were 35.7%, 47.7% and 88.9% in this study. The lower gallstone recurrence rate after ESWL was probably related to strict selection of cases and higher ratio of solitary stone. More research should be done about

the relatively high recurrence rate of gallstone, otherwise the non-surgical therapy of gallbladder stone will lose their clinical application value.

### *Risk factors for recurrence of gallstone*

The occurrence and the recurrence of the gallbladder stone probably have similar physiopathologic mechanism. It is related to many factors such as sex, age, weight index, diet hobby, labor strength, endocrine and metabolism, the size, number and character of the gallstone. In our study, no difference exists between recurrence and non-recurrence groups on such items as sex, average age, average weight/height, thickness of gallbladder wall, average ratio of gallbladder const riction, average degree of liver lipid infiltration and related diseases (diabetes, coronary heart disease, etc). Some items have significant difference between the two groups. The following in the recurrence group were significantly different from the non-recurrence groups: more clinical symptoms, more patients receiving medical treatment, low mean value of serum insulin and high mean value of serum TBA. The group with multiple gallstones, family history of gallstone and intake of greasy food has a higher recurrence rate. All of these differences are sta tistically significant ($P<0.05$).

Multiple gallstones seem to recur more often than solitary stone probably because ① most of solitary stones are cholesterol caculus, and lithotripsy and litholysis are effective treatment. The proportion of combined caculus is quiet higher in multiple stones. The insoluble bile sludge after lithotripsy and dissolution might become the nuclear of the recurrence stone. ② Solitary stone is easier to be hit during the lithotripsy. The treatment takes less time and the broken stones are easier to be removed. There were less fine stones left and less injury to the gallbladder, while results were different for multiple stones.

Patients with family histories of gallstone had higher recurrence rates probaly because of similar component and hobby of the diet, and hereditary factors.

Most literature reports that the serum insulin level in patients with gallstone is high[5]. The mechanism might be that insulin activates the cholesterol synthesis reductase of liver, causing the increase of cholesterol synthesis and accelerating gallstone formation. Some authors have found no statistical difference in serum insulin level between diabetes patients with or without gallstone. In our cases, the mean serum insulin level in stone recurrence and multiple stone groups is significantly lower than in non-recurrence and solitary stone gro ups. When insulin is deficient, most glucose produced by glyconeogenesis was consumed, and the amount of pyruvate used to synthesize acetyl coenzyme A decreased. Most of acetyl coenzyme A is derived from lipose. A great quantit y of acetyl coenzyme A provides the material for cholesterol synthesis. At the mean time, the deficiency of insulin reduces the capability of cholesterol utilization of liver, resulting in the hypercholesterolemia. This abnormal metabolism of lipid is often related to the formation of gallstone.

It has been proven that food is closely related to gallbladder stone. Epidemiological investigation also indicated that the high morbidity of cholecystolithiasis is correlated with the intake of low fiber and refined food in some developed countries. With the changing of the food components and reduction of the labor intensity, the morbidity of cholecystolithiasis is rising progressively.

Content and hobby of diet, over intake and low consumption of high fat and cholesterol food, relative deficiency of serum insulin, abnormal metabolism of glucose and lipose, liver disease and dysfunction of gallbladder might be all related to the formation of gallstone. Effective propaganda and education, reasonable diet structure, constant physical exercise and a certain amount of labor might help control the occurrence and recurrence of cholecystothiasis.

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# Consequence alimentary reconstruction in nutritional status after total gastrectomy for gastric cancer *

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**Subject headings** stomach neoplasms; gastrectomy; nutritional status; nutrition disorders; esophagitis

## Abstract

**AIM To investigate the effect of gastroenteric reconstruction on the nutritional status of patients with gastric cancer after total gastrectomy.**

**METHODS From 1989-1994, nutritional status was studied in 24 patients, including 12 patients with the gastric reservoir and pyloric sphincter reconstruction (GRPS), 7 with Braun's esophago-jejunostomy (EJ) and 5 with Lawrance's Roux-en-Y reconstruction (RY). The ability of these patients to ingest and absorb the amount of nutrients was examined and compared, and metabolic balance test was performed to compare the efficiency of those patients to accumulate and use the absorbed nutrients.**

**RESULTS In the controlled hospital situation, the amount of food ingested by all the patients was greater than that required for maintenance of ideal body weight. In direct contrast, food intake in most patients with EJ or RY reconstruction significantly decreased when the patients returned home and that in EJ patients it was the lowest. The overgrowth of anaerobic bacteria was found in the jejunum in the patients with EJ and RY, due mainly to food stasis in the duodenum or in the Roux limb, caused by the operative procedure itself. In patients with GRPS, because of restoring of the alimentary continuity according to the normal digestive physiologic characters, all the nutritional parameters c ould fall in the normal range.**

**CONCLUSION The most common mechanism responsible for postoperative malnutrition was inadequate food intake. Having solved the problem of alkaline reflux esophagitis, it is imperative to preserve the duodenal food passage to reduce malabsorption and other complications after total gastrectomy.**

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## INTRODUCTION

To investigate the nutritional consequences of gastroenteric reconstruction in patients with gastric cancer after total gastrectomy, nutritional status was studied among patients undergoing the gastric reservoir and pyloric sphincter reconstruction (GRPS), Braun's esophago-jejunostomy (EJ) and Lawrance's Roux-en-Y reconstruction (RY) from 1989 to 1994, and the metabolic balance test was performed to compare the patients' efficiency to accumulate and use the absorbed nut rients.

## MATERIALS AND METHODS

### *Subjects*

All the patients studied were free from malignant recurrence or metastasis confirmed by CT for more than 6 months after the study. They were divided into 3 groups: (I) those with GRPS (12 patients, 9 men and 3 women, mean age, 47 years, range, 32 -61 years); (II) those with EJ (7 patients, 5 men and 2 women, mean age, 51 years, range 42-60 years); and (III) those with RY (5 patients, 3 men and 2 women, mean age, 48 years, range 35-57 years).

### *Methods*

Each patient stayed in hospital for 18 days which were divided into 4 periods.

**The smorgasbord period** From 1-3 days, according to "the Table of the Nutrition amount Supplied in Meals per Day" (published in "Food Elem ents Table" by the China Nutrition Research Institute in

1997), the standard diet was supplied to patients based on each one's dietary habits, and total caloric intake and the proportion of calories from protein, fat and carbohydrate were recorded accurately and calculated.

**The equilibration period** From 4 - 6 days, all patients were sup plied the balance diet of 80g protein, and 100g fat, except the fat amount for those with steatorrhea reduced to 50g in the last 3 days.

**The metabolic balance period** From 6-12 days, the intake-ou tput balance test period consisted of two consecutive 3-day periods, stool and 24h urine samples were collected for fat, nitrogen, $Na^+$, $K^+$, $Cl^-$, $P^{2+}$, $Ca^{2+}$ and $Mg^{2+}$ analyses.

**The special tests period** From 13-18 days, the Schilling test, D-xylose absorption test, glucose tolerance test and barium small intestinal transit time were made respectively. On the day of admission, while no treatment applied, serum specimens were drawn for various biochemistry examinations, and gastroscopy was performed to examine the esophagus carefully to discover if reflux esophagitis occurred. On the morning of the forth day, via the guidance of fluoroscopy, a sterile tube was inserted through nose to jejunum to collect jejunal aspirate for culture and identification of anaerobes under sterile and anaerobic conditions. The aspirate was cultured and the anaerobic organisms were further classified according to procedures stipulated by "Bergey's Manual of Determin ation Bacteriology".

**Follow-up** Upon leaving the hospital, the patients were given the format designed according to "Nutritional Manual for Hospita lized Patients"[1], and food intake was recorded accurately for 7 consecutive days at home environment for analysis later.

**Statistical analysis** The results were expressed as $\bar{x}\pm s$, and statistical analyses were made using Student's *t* test.

## RESULTS

### *Clinical data*

**Body weight** The average preoperative body weight of 3 groups all reached their ideal body weight (IBW). On the day of admission, group I patients achieved IBW, groups II and III weighted 10% and 20% less than their IBW respectively. The individual body weight of group I patients exceeded more than 5% - 10% of their pre-operative weight with one exception, in group III only 2 patients achieved their pre-operative weight, the others weighed 5% - 15% less than their pre-operative weight, in group II all the patients weighted 10% - 20% less than their pre-operative weight.

**Dietary history** In the controlled hospital situation, the average caloric intake by all the patients reached or exceeded the Recommended Dietary Allowance (RDA). After returning to the home environment, the average daily caloric intake in group I was 100% of the RDA for the maintenance of IBW, and 75% in group II and 85% in group III, the largest decrease was noted in one patient of group II, only 63% of the RDA.

### *Absorption studies*

**Glucose tolerance and D-xylose absorption tests** Early hyperglycemia (> 11.01 mmol/L at 30min) and delayed hypoglycemia (< 3.92 mmol/L) were found by glucose tolerance test in 7 patients of group III and 4 patients of group III. Low D-xylose value in urine specimen was lowered in 2 patients of group II and 1 patient of group III.

**Fecal nitrogen examination** The nitrogen intake-output balance tests showed that the average value for fecal nitrogen in group I was less than 0.14mmol/d, and more than 0.14mmol/d in 4 patients of group II and 3 patients of group III, the most serious nitrogen wasting was noted in the azotorrhea patients of group II, whose average value was more than 0.16mmol/d. The loss rate for fecal nitrogen was 18.5% ± 3.2% in 4 patients of group II, and 17.4% ± 4.1% in 3 patients of group III. Low values of serum albumin were noted in 3 patients of group II and 2 patients of group III whose fecal nitrogen exceeded 0.15mmol/d.

**Fecal fat examination** Steatorrhea occurred in 6 patients of group II and 4 patients of group III. In those patients, the fecal fat loss rates averaged 16.1%±4.5% in 6 of group II and 17.5%±3.8% in 4 of group III. When the fat intake was reduced to 50g, the steatorrhea condition showed no alleviation. Fecal fat excretion of group I was less than 6g/d, while that in steatorrhea patients of group II and group III was more than 6g/ d (range 8g/ d - 21g/ d). Serum carotene was low in steatorrhea patients (< 0.711 mmol/L), and serum cholesterol was low (< 2.84mmol/L) in 5 of group III steatorrhea patients and 3 of group III. Low values of serum albumin, serum carotene, serum cholesterol and D-xylose occurred only in the patients suffering from malabsorption of fat or protein.

**Caloric loss** In the patients with malabsorption of

protein and fat, the caloric loss was 351KJ on a standard diet due to fat and protein malabsorption. The highest caloric loss of 1966KJ occur red in one patient of group II.

**Water soluble vitamins** Normal serum values of Na, K, Cl, Mg, Ca, alkaline phosphatase, and prothrombin time, hemorrhagic phenomena and tetany and osteomalacia were not noted, all these serve as indirect evidence of adequate levels of vitamins D and K. Shelling test showed declined B12 absorpti on in all the patients.

**Gastroscopic examination and small intestinal transit time** Gastroscopic evidence of reflux esophagitis was noted in 7 patients of group II, and none in groups I and III. Barium small bowel transit time in group I was 3.2 h ± 1.22 h (normal time 3.4 h ± 2.3 h). There was no significant difference, while there were significant differences between 1.6 h ± 1.2 h of group II, and 2.3 h ± 1.3 h of group III and the normal time.

**Bacterial culture** Anaerobes presented in the jejunal aspirate of one patient in group I, its count being $10^7$/L. Anaerobes were also found in the jejunal aspirate of 6 patients in group II and 4 patients in group III. Those were identified mainly as lactobacilli, yeasts, bacteroides, veillonella and clostrida.

**Balance studies** In the controlled hospital period, the data collected from the intake-output tests and repeated tests of serum samples showed that each element of N, P, $Cl^-$, $Ca^{2+}$, $Mg^{2+}$, $Na^+$, and $K^+$ was in positive average daily balance, and there were no significant differences among the 3 groups.

## DISCUSSION

Protein, fat, carbohydrate, vitamins and minerals are the 5 major food elements required for proper nutrition. So it is important to ingest and absorb these 5 elements to keep good nutritional state of the post-operative patients.

### *Effect of reconstruction on body weight*

The major clinical manifestation of malnutrition is weight loss. Previous studies reported that the average postoperative weight loss was 24% as compared with preoperative one and only one-third patients achieved IBW[2]. Some studies indicated that a major contributing factor to weight loss and failure to gain weight was inadequate caloric intake of food[3]. The most serious complication leading to such state was alkaline reflux esophagitis[4]. The most serious cli nical symptoms caused by reflux esophagitis were found in group II patients in this study, and caloric intake was the lowest among the three groups after returning to home environment. In group III patients, although the Roux-en-Y reconstruction has solved the problem of esophagitis, the Roux-en-Y syndrome occurring in most post-operative patients also affects normal intake of food. Caloric lo ss is another factor contributing to malnutrition in groups II and III patients suffering from malabsorption of fat and protein.

### *Effect of reconstruction on digestion and absorption*

Besides adequate intake of the 5 food elements, good digestion and absorption are important as well. The duodenum plays an important role in the process of food digestion and absorption, and in controlling chyme emptying through a mechanism of immediate brake[5], being the main site of cholecystokinin and gastric secretion stimulated by food after total gastrectomy. When the duodenum passage of digested food was excluded, secretion of bile and pancreatic enzymes could not coordinate and syn-chronize with emptying of chyme, therefore proper mixing of them could not precede within the time necessary for physiologic digestion. Without emulsi-fication and specific hydrolysis of pancreatic pepti-dase and lipase, and without adequate biological re-action of conjugated bile salts, malabsorption of fat and protein would occur, and azotorrhea and steat-orrhea ensued. In II and group III patients whose reconstruction excluded the passage of food through the duodenum, the barium small intestinal transit time was faster than that of normal control group, the glucose tolerance tests were abnormal in 7 pa-tients of group II and 4 patients of group III, 6 pa-tients of group II and 4 patients of group III experi-enced steatorrhea, and azotorrhea occurred in 4 and 3 patients of the two groups respectively. Because malabsorption of fat would result in malabsorpti on of some fat-soluble vitamins, the serum carotene level was low in those patients with steatorrhea. In group I patients, those parameters mentioned above could fall within normal biological range due to the maintenance of duodenal passage of food.

### *Effect of reconstruction on bacterial overgrowth*

The results of this study showed that anaerobes were cultured out of the jejunal aspirate in one patient of group I, 6 of group II and 4 of group III. Six hours after barium examination, barium residue was found in the jejunal loop and Roux limb in the corresponding patients of groups II and III respectively, imply-

ing that the ingested food would stay in the segment of reconstruction for rather a long time after intake of food stuff by those patients. The residual food would be anideal place for microorganism overgrowth without sterilization of gastric acid after total gastric resection. Based on the results of this study, there is direct correlation between the reconstruction and bacterial overgrowth in the small bowels. Anaerobes proliferating in the small intestine, especially bacteroides, areable to change the structures of bile salts, to reduce water-soluble fat absorption impaired with inadequate concentration of conjugated bile salts. Anaerobes are also able to diversify ingested protein nitrogen to urea by deamination, resulting in impaired protein a bsorption. Meanwhile, bacterial consumption and toxins produced by bacterial met abolism would aggravate B12 deficiency of the postoperative patients.

***Effect of reconstruction on nutritional balance and dietary habits.***

Patients in the controlled period of hospitalization, the food caloric intake by groups II and III could exceed RDA. The results of balance studies showed that all these patients could maintain positive balance of N, P and electrolytes. It was observed in this study that the abnormal nutritional status was mainly caused by gastrointestinal continuity altered by reconstruction after total gastrectomy for gastric cancer, inducing abnormal changes of gastro intestinal dynamics and digestive environment, but the reconstruction exerted little influence on the absorption capacity of the small intestine, and the nutritional status could be improved by strict control. But in the home environment, especially for those with financial difficulties, it would not be easy, thus leading to malnutrition. However, the patients of group I could achieve normal nutritional state in daily life without any dietary control. Therefore, for maintenance of good nutritional status of postoperative patients, it is imperative to preserve the duodenal food passage, on the basis of having solved the problem of alkali ne reflux esophagitis.

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# Detection of serum TNF-α,IFN-γ,IL-6 and IL-8 in patients with hepatitis B *

WANG Jing-Yan, WANG Xue-Lian and LIU Pei

**Subject headings** hepatitis B; TNF-α; IFN-γ; IL-6; IL-8

## Abstract

**AIM To assess the possible roles of cytokines (TNF-α, IFN-γ, IL-6 and IL-8) in liver damage of hepatitis B.**

**METHODS The serum TNF-α, IFN-γ, IL-6 and IL-8 were detected by ELISA in 66 patients with hepatitis B and 20 healthy blood donors.**

**RESULTS TNF-α and IL-6 in all types of clinical hepatitis B were significantly higher than those in healthy blood donors ($P<0.05$ ); meanwhile the levels of TNF-α, IFN-γ, IL-6 and IL-8 in the patients with fulminant hepatitis B were much higher than those in the patients with acute hepatitis B($P<0.05$); the level of TNF-α was positively correlated with the levels of IFN-γ, Il-6 and IL-8 in all types of hepatitis B ($r_{IFN} = 0.24$, $r_{IL-6} = 0.35$, $r_{IL-8}=0.44$) and the TNF-α, IFN-γ, IL-6 and IL-8 were positively correlated with serum bilirubin ($P<0.05$). Dynamic changes of these cytokines were observed in the course of acute and fulminant hepatitis. The level of IFN-γ peaked in the initial period of acute hepatitis and early stage of hepatic coma in fulminant hepatitis; TNFα, IL-6 and IL-8 increased with exacerbation, and reached a peak when the liver damage was most serious, then decreased when patient conditions were improved.**

**CONCLUSION The increased cytokines were related to the inflamm ation of liver cells and multiple factors may play certain roles in liver damage.**

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## INTRODUCTION

Since Muto[1] reported that TNF-α and IL-1 were related to fulminant hepatitis, the studies on the relationship between cytokines and liver damage have been paid more and more attention especially in recent years. Most scholars now agree that TNF and IFN are related to liver damage, so are IL- 6 and IL-8. We detected the serum TNF-α, IFN-γ, IL-6 and IL-8 in the patients with different clinical types of hepatitis B by ELISA for asse ssing the relationship between the cytokines and the liver damage.

## MATERIALS AND METHODS

### *Samples*

A total of 66 patients with HBV infection and 20 healthy blood donors were studied. They were admitted to this college between 1993 and 1997. The patients (48 men and 18 women) ranged in age from 21 to 56 years. There were 22 cases of acute hepatitis (AH), 25 cases of chronic hepatitis (CH) and 19 cases of fulminant hepatitis (FH). The serological markers of HAV, HCV, HEV, CMV and EBV were negative, and HBsAg and other markers of HBV were positive in all the patients.

### *Healthy blood donors*

Healthy blood donors, aged from 25 to 43 years, included 16 men and 4 women. They had no serological markers of HAV-HEV, CMV, EBV infection and liver functions were normal.

### *Detection of cytokines*

The kits of the four cytokines were produced by the Genzyme Company, U. S. A. No. 1 9970214. The four cytokins were detected by ELISA according to the manufacturer's instructions. The first antibody was biotin-labelled and the second one was connected with horse radish peroxidase.

## RESULTS

The serum TNF-α, IFN-γ, IL-6 and IL-8 in patients with different types of hepatitis B are shown in Table 1.

**Table 1 TNF-α, TNF-α, IL-6 and IL-8 in various types of hepatitis B** ($\bar{x}\pm s$, ng/L)

| | *n* | TNF-α | IFN-γ | IL-6 | IL-8 |
|---|---|---|---|---|---|
| FH | 19 | 359.0±17.2[ab] | 234.7±16.5[ab] | 347.5±31.3[ab] | 181.1±19.6[ad] |
| AH | 22 | 220.6±8.9[c] | 174.9±12.0 | 285.8±16.5[c] | 118.4±5.1 |
| CH | 25 | 322.1±13.0[c] | 200.0±15.7[c] | 329.5±25.2[c] | 133.1±6.7[c] |
| CI | 20 | 146.7±9.4 | 165.0±7.7 | 231.1±16.4 | 110.2±2.9 |

CI: healthy blood donor. [a]$P$<0.01, *vs* CI; [c]$P$<0.05, *vs* CI; [b]$P$<0.01, *vs* AH;[d]$P$<0.05, *vs* AH.

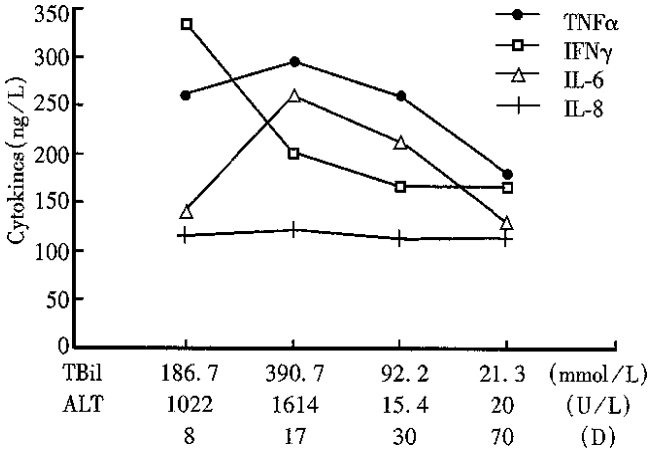


**Figure 1** Dynamic changes of four cytokines in the course of AH.

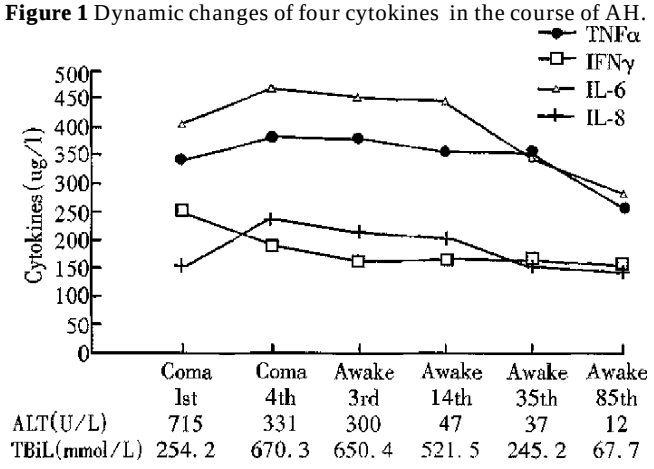


**Figure 2** Dynamic changes of four cytokines in the co urse of FH.

The TNF-α and IL-6 in various clinical types of hepatitis B were significantly higher than those in healthy blood donors ($P$<0. 05). Except acute hepatitis B, the levels of IFN-γ and IL-8 were obviously higher in other types than those in healthy blood donors. The levels of four cytokines of patients with FH were much higher than those of patients with AH ($P$<0.05).

### *Correlation between TNF-α and IFN-γ, IL-6 and IL-8*

The correlations between TNF-α and IFN-γ, IL-6 and IL-8 in HBV infections were analyzed. The results suggested that TNF-α and the other cytokines were positively correlated ($r_{IFN}$ = 0.24, $r_{IL\text{-}6}$ = 0.35, $r_{IL\text{-}8}$ = 0.44).

### *Correlation between serum bilirubin and four cytokines*

The correlation between serum billirubin and four cytokines was analyzed in HBV infections. The results suggested that bilirubin was positively related to the levels of four cytokines ($P$<0.05).

### *Dynamic changes of four cytokines in AH and FH*

The dynamic changes of four cytokines in the course of AH and FH are shown in Figures 1, 2. At the early stage of AH, IFN-α peaked and decreased rapidly. TNF-α and IL-6 increased with exacerbation, reached a highest level when jaundice became most severe, and then decreased gradually. IL-8 level did not change during the course.

On the first day of hepatic encephalopathy of FH, IFN-γ reached a peak, then decreased rapidly. TNF-α, IL-6 and IL-8 increased when condition of the patients worsened. They reached the highest level during the peak of jaudice, then decreased gradually, and maintained abnormal for a long time.

## DISCUSSION

Since it was reported that TNF and IL-1 in supernatant of cultured monocytes of peripheral blood in patients with FH were obviously higher than those with AH, the studies on cytokines related to liver damage advanced rapidly. Pei Liu[2] proved that TNF could cause liver necrosis in corynebacterium sensitized animals and the necrosis could be blocked by anti-TNF monoclonal antibody[3]. At present, the studies of hepatitis focused on the roles of cytokines in cell-mediated injury of tissues. Ferluga *et al*[4] reported that the liver injury of animal model induced by corynebacterium-endotoxin could be caused by the soluble factors produced by monocytes gathering at the hepatic lobules. Luca[5] proved that activation of CTL induced by IFN-γ resulted in CTL-mediated hepatocytic injury in the study of HBV transgenic mouse. IL-6 may induce the activation, differentiation and maturation of NK cells[6] and expression of monocytic IL-8 gene[7]. The IL-8 may cause degranulation of nutrophile granulocyte, leading to DIC within the l iver[8].

The serum levels of TNF-α, IFN-γ, IL-6 and IL-8 in patients with HBV infection were higher than those in healthy blood donors. The difference was obvious between the levels of cytokines in FH and those in AH. TNF- α and IFN-γ, IL-6 and IL-8 were positively correlated in various types of hepatitis B. The bilirubin was also positively related to the four cytokines. In the course of AH and FH, IFN-γ peaked in the early stage of AH and the 1st day of hepatic coma of FH. TNF-α, IL-6 and IL-8 in-

creased with exacerbation of condition of the patients with AH and FH except for IL-8 in AH. These suggested that the four cytokines were related to liver injury.

The roles of cytokines in the liver damage are complex. They affect each other to form a cytokine network, in which IFN-γ may be the chief cytokine and induce the immune cells to release other cytokines and improve cytotoxic activities mediated by immune cells.

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# Gastroesophageal reflux disease is uncommon in Asia: evidence and possible explanations

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**Subject headings** gastroesophageal reflux; esophagitis; Barrett's esophagus; hiatus hernia; *Helicobacter pylori*; gastric acid

## DEFINITIONS

Gastroesophageal reflux that predisposes an individual to the risk of physical complications, or produces symptoms leading to significantly impaired quality of life, is termed gastroesophageal reflux disease (GERD)[1]. Clinically, GERD encompasses a broad spectrum of separate, though related conditions that are sometimes conveniently grouped under two broad categories: endoscopic esophag itis and endoscopy negative reflux disease. Endoscopic esophagitis is considered to be present when there is endoscopically visible breakage of the mucosa[2], regardless of whether the patient has symptoms. The term "endoscopic negative reflux disease" refers to GERD that is not associated with Barrett's esophagus or esophageal mucosal breaks. It includes such conditions as esoph ageal mucosal acid sensitivity, which is symptomatic reflux induced by acid reflux and proven by objective means; abnormal esophageal acid exposure, which is excessive acid reflux confirmed by objective measures; and reflux-type symptoms (heartburn and/or acid regurgitation) that clearly dominate the patient's complaints[3]. Barrett's esophagus is the eponym applied to the columnar epithelium-lined lower esophagus that is acquired as a consequence of chronic gas troesophageal reflux[4]. Hiatus hernia, on the other hand, has been defined as a displacement of the gastric mucosa 1.5cm or more above the diaph ragmatic hiatus[5].

## EVIDENCE FOR A LOW PREVALENCE OF GERD IN ASIA

### *Prevalence of reflux-type symptoms in general population*

Until recently, there has been no systematic study on the prevalence of reflux type symptoms in the general population of Asia. A cross-sectional survey of a race-stratified sample of adults in a Singaporean town provides some of the first evidences, that reflux-type symptoms are uncommon in the East[6]. Of 696 persons evaluated, only 2% had heartburn and/ or acid regurgitation for more than once a month. This prevalence is much lower than those (29%-44%) of Wester n populations[7,8].

### *Prevalence of GERD in pregnant women*

The individuals with the highest prevalence of heartburn are often said to be pregnant women. A prospective study, using a reliable questionnaire, on a consecutive series of pregnant women in Singapore, provides the second piece of evidence that reflux-type symptoms are uncommon among Asians[9]. Of the 35 pregnant women evaluated, 23% had heartburn some time during their pregnancy. This percentage is lower than those (48% - 96%)reported previously in the West[10,11].

### *Frequency of GERD in outpatient clinics*

In a large clinical series from Singapore, Kang *et al* from Singapore noted a 2% frequency of GERD among 2141 consecutive patients investigated[12]. The diagnosis of GERD was established on the basis of an abnormal endoscopy, a positive acid perfusion test and/ or an abnormal 24-hour pH monitoring. The frequency was lower as compared with a similar series from the West[13].

### *Prevalence of endoscopic esophagitis*

Very few epidemiological data on reflux esophagitis in Asians are available in the literature. However, Chang *et al* found 5% with reflux esophagitis[14] in an endoscopic series of 2044 patients who underwent self-paid medical check-ups. Esophagitis, when present, was often mild. The prevalence of endoscopic esophagitis among symptomatic subjects has not been well studied and the available data are conflicting. In a study from Taiwan, a 15% prevalence of erosive esophagitis was found in 455 consecutive patients evaluated for various upper gastrointestinal tract symptoms[15]. Most of the patients presented with mild esophagitis. The expected high frequency of erosive esophagitis is not supported by other endoscopic series from Asia. Erosive esophagitis was uncommon in both indigenous Fijians and Indians, being detected in only 2% of a total of 693 endoscopic examinations[16]. This contrasts with the higher prevalence (11%) of reflux esophagitis noted by the same author among New Zealanders[17]. Esophagitis is likewise uncommon in Japan; a preva-lence rate of 3% was recorded among 240 consecutive outpatients with dyspepsia[18]. Our own retro-

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spective series from Singapore showed that of 11943 patients undergoing diagnostic upper endoscopy for various complaints, 4% had esophagitis[19]. This frequency was lower than those reported from Western centers[20,21]. Thus, with the exception of the Taiwanese series, the proportion of patients with endoscopic esophagitis in Asian series appears lower than that in reports from Western countries. The severity of esophagitis also appears mild, unlike that in Western populations[20,21].

### *Prevalence of hiatus hernia*

Hiatus hernia, as seen on barium studies, appears rare in the Far East with a < 1% prevalence[22]. Recent endoscopic series from Asia confirm this impression. Chang *et al* from Taiwan found hiatus hernia in 2% of patients endoscoped as part of an annual medical examination[14]. In another Taiwanese study in patients endoscoped for gastrointestinal complaints, hiatus hernia was found in 7% of the cases[15]. In our retrospective series from Singapore, the proportion of hiatus hernia among patients seen for gastrointestinal complaints was 3%[19]. Thus, the available data show that the prevalence of hiatus hernia is lower than that in Western series (17%-22%)[20,21].

### *Prevalence of GERD complications*

The prevalence of Barrett's esophagus varies, depending on the population being studied. In a series from Taiwan, 2% of patients endoscoped for a variety of upper gastrointestinal symptoms were found to have Barrett's esophagus[15]. When evaluating only those with erosive esophagitis, this rate increased to 14%. The corresponding figures from the West are 4%-20%[23,24] and 36%, respectively[23]. Reports from a Taiwanese center, and our own center showed a frequency of benign (presumably reflux-related) esophageal stricture of only 0.4% and 0.2% respectively, among patients endoscoped for various gastrointestinal indications[16,19]. These frequencies are lower in comparison with those in reports from the West[25].

## POSSIBLE REASONS FOR THE LOW FREQUENCY OF GERD IN THE EAST

The pathogenesis of reflux esophagitis can be considered in terms of excessive acid load overwhelming mucosal defense. The degree of acid load is in turn determined by the anti-reflux barrier of the gastroesophageal junction[26], the quantity of acid refluxed[27], and the ability of the esophagus to clear any refluxate back into the stomach[28]. The latter depends on the integrity of peristaltic function[29] and the neutralizing ability of swallowed saliva[28]. More recently, an inverse relationship between *Helicobacter pylori* (*H. pylori*) and GERD has been suggested[30]. By examining the potential pathogenetic factors, it is hoped that the lower frequency of GERD in the East than in the West could be explained.

### *Anti-reflux barrier*

An increase in intra-abdominal and intragastric pressure overcomes the gastroes ophageal pressure gradient maintained by the lower esophageal sphincter (LES). Such an increase may occur through obesity[31] and delayed gastric emptying by fatty meals[32]. Alcohol, smoking and fat can lower the LES pressure and esophageal peristalsis, thus favoring the occurrence of gastroesophageal reflux[33-35]. A large hiatus hernia traps gastric contents in its pouch above the diaphragm. This leads to free retrograde flow of acid into the esopha gus.

Increased body mass index and presence of hiatus hernia were found to be the most important factors associated with the occurrence of esophagitis in a recent study from Taiwan[14]. The authors suggested that the lower prevalence of hiatus hernia and smaller body mass index in the Chinese population might account for the lower prevalence of reflux esophagitis in Taiwan. The low prevalence of hiatus hernia in the East has previously been attributed to the consumption of high residue diets in the developing world[22]. Another report from Taiwan found erosive esophagitis to be associated with smoking, and alcohol consumption[15]. The authors suggested that the recent increase in smoking, alcohol use, and fat consumption among Taiwanese were contributed to the observed rise in the prevalence of GERD in Taiwan.

### *Gastric acid output*

Since acid secretion correlates with body surface area, Asians in general are ch aracterized by a smaller parietal cell mass and a lower acid output as compared with Caucasians[36]. Except for the striking example of Zollinger-Ellis on syndrome, however, the association between the amount of acid output and the occurrence or severity of reflux disease has remained unproven[37].

### *Acid clearance*

While evaluating the consecutive Singaporean patients who underwent esophageal manometry, we found that poor esophageal clearnance was more common among those with esophagitis than among those without. The results were identical to Western studies[38]. It is possible that this clearance mechanism has an inherited basis, and is more efficient in Asians than in Caucasians. Data to support this is, however, lacking.

### *Mucosal defence*

Presently, there is no risk factor known to disrupt tissue resistance, except for nonsteroidal anti-inflammatory drugs[39]. Such drugs cannot be an important factor underlying the geographical variation in the prevalence of GERD, because they are con-

sumed by Asians no more than by Westerners. However, it is possible that inborn differences in tissue resistance, due to yet unrecognized factors, may account for some of the geographical differences.

### *H. pylori infection*

There is circumstantial evidence to suggest that *H. pylori* infection is rela tively protective for the occurrence of GERD[30]. It has been suggested that Hong Kong Chinese are protected against reflux esophagitis by their high prevalence of *H. pylori* associated gastritis[40]. Such gastritis, when becoming chronic, can lead to gastric atrophy and hypochlorhydria, thereby reducing the likelihood of GERD. If this hypothesis is correct, the effects of *H. pylori* induced gastritis may be an important factor determining the lower prevalence of reflux esophagitis in this part of the world, in which *H. pylori* infection is especially common. No data, however, exists to support this hypot hesis.

### *Genetic factors*

It is unlikely that the lower frequency of GERD in Asian populations can be explained simply by the known extrinsic risk factors, such as obesity, smoking habits, and alcohol consumption, being less frequent in Asians as compared with Caucasians. It is likely that genetic factors are involved. If that was the case, the mechanisms through which they confer protection against GERD are poorly understood. It may be that LES function is truly more competent in Asians compared with Westerners. Alternatively, the esophageal mucosa in Asians is inherently more acid resistant. Differences in gastric acid output and esophageal clearance ability between Asian and Western patients are further possibilities. Comparative studies into these parameters in Eastern and Western populations may shed more light on this question, and may lead to formulation of appropriate- therapeutic strategies.

In summary, most reports from Asia have suggested that GERD is an uncommon condition in this part of the world. The reasons for the lower frequency compared with the West are not known, and further studies are required.

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# Protective effects of polydatin against $CCl_4$-induced injury to primarily cultured rat hepatocytes *

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**Subject headings** polydatin; injury, hepatocyte; $CC1_4$

## Abstract

**AIM To investigate the protective effects of polydatin (PD) against injury to primarily cultured rat hepatocytes induced by CCl4.**

**METHODS Rat hepatocytes were separated by methods of liver infusion in vivo and cultured medium ($7.5\times10^5$ cells/mL). Two mL or 0.2mL was added into 24-well or 96-well plates respectively. Twenty-four hours after cell preculture, PD at concentrations of $10^{-7}$ mol/L-$10^{-4}$ mol/L was added into each plate. At the same time injury to hepatocytes was induced by adding 10mmol/L-$CCl_4$. Then, 0.1mL or 1mL-culture solution was removed from the 96-well or 24-well plates at 6h, 12h, 2 4h and 48h after $CCl_{14}$ intoxication respectively for the determination of GPT, GSH and MDA. At 48h, the survivability of rat hepatocytes was assayed by the MTT colormetric method.**

**RESULTS After CCl4 challenge, the release of GPT and the form ation of MDA in rat hepatocytes markedly increased and maintained at a high level in 48h, whereas PD with different concentrations could markedly inhibit this elevation with $10^{-5}$mol/L PD having the strongest effects and inhibiting rate was over 50%. PD could also improve the decreased content of GSH caused by $CCl_4$ in accordance with the doses used. $CCl_4$ evidently decreased the he patocyte survivability from 91.0%±7.9% to 35.4%±3.8%. On the other hand, PD at $10^{-7}$mol/L-$10^{-4}$mol/L could reverse this change and improve t he cell survival rates to 56.1%±5.2%, 65.8%±5.0%, 88.7%±6.8% and 75.2%±7.3%, respectively.**

**CONCLUSION PD at $10^{-7}$ mol/L-$10^{-4}$ mol/L could protect primarily cultured rat hepatocytes against CCl4 induced injury.**

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## INTRODUCTION

*Polygonum cuspidatum* Sieb. et Zucc. (Polygonaceae) is a traditional Chinese herbal drug, with bitter taste and cold nature. It mainly acts upon the liver, gallbladder and lung meridians. It is well known that *P. cuspidatum* has various activities such as promoting blood circulation, relieving swelling and pain, eliminating phlegm, alleviating cough, clearing away heat, and removing dampness and toxin. The drug has been widely used for cardiovascular and liver diseases. Its active compounds mainly consist of free anthraquinones which include emodin, physcion and chrysophanol. Another important compound is resveratrol[1].

Polydatin (PD), 3, 4', 5-trihydroxystibene-3-β-mono-D-gluc oside, also named piceid, is the glycoside of resveratrol[1]. Some previous studies demonstrated that PD could lower the level of blood lipid, inhibit the platelet aggregation, dilate blood vessels, protect cardiocytes, reduce cerebral ischemic damage and inhibit lipid peroxidation[2-6]. However, the effects of PD on hepatocytes and its mechanisms have not been reported up to date. In this paper we report the details of protective effects of polydatin against injury to primarily cultured rat hepatocytes induced by $CCl_4$.

## MATERIALS AND METHODS

### *Materials*

Collagenase (type IV), 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), dexamethasone, *N*-2-hydroxyethyl-piperazine-*N*'-2'-ethane sulfonic acid (HEPES), insulin, penicillin and streptomycin were purchased from Sigma Chemical Corp (St. Louis, USA). RPMI 1640 was

a product of Gibco Life T echnologies INC (Grand Island, NY). Fetal calf serum was obtained from Institute of Hemopathy, Chinese Academy of Medical Sciences (Tianjin). PD (Purity>90%), which was isolated from the root and rhizome of *P. cuspidatum*[7], provided by the Department of Chemistry, the First Military Medical University.

### Animals

Wistar rats, male, 6 weeks old, weighing 160 g-180 g, were used for hepatocyte isolation. They were provided by Laboratory Animal Center, Guangzhou Un iversity of TCM.

### Isolation and culture of rat hepatocytes

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Then the liver parenchymal cells of rat were isolated by the collagenase perfusion method following the procedure of Seglen and Koji[8,9]. Simply, the portal vein of rat liver was exposed and cannulated with a teflon catheter. The liver was perfused with $Ca^{2+}$ free solution containing NaCl 142, KCl 6.7, HEPES 10, NaOH 5.5 (mmol/L), pH 7.4, at 37℃, with a flow rate of 40 mL/min. Twelve minutes later, recirculation started with collagenase solution composed of NaCl 67, KCl 6-7, $CaCl_2 \cdot 2H_2O$5, HEPES 100, NaOH 66, collagenase 0.2 g/L, pH 7.6. Isolated cells were cultured in RPMI 1640 containing 100mL/L-fetal calf serum, 10mmol/L-HEPES, 100kU/L-penicillin and streptomycin, 10mmol/L insulin and 10mmol/L- dexamethasone. The content of hepatocytes was adjusted to $7.5 \times 10^8$ cells/ L with the above medium. Cultured medium 2mL and 0.2mL were added into 24-well and 96-well plates respectively. The cells were incubated for 4h at 37℃ under 50mL/L $CO_2$ in air. Non-adherent hepatocytes were eliminated by replac ing the medium, and adherent hepatocytes continued to be incubated, and the medium was changed every 24 h.

### $CCl_4$-induced hepatocytes injury

After pre-culture for 24 h, the hepatocytes were exposed to fresh medium containing 10mmol/L-$CCl_4$ and various concentrations of PD. At 6, 12, 24 and 48 h after $CCl_4$ intoxication, 0.1 mL and 1 mL culture solution were removed from 96-well and 24-well plates respectively for determination.

### Measurement of glutamic pyruvic transaminase (GPT)

The kits of GPT analysis, provided by the Shanghai Institute of Biological Products of Ministry of Health, were used to measure the activity of GPT in 0.1 mL- culture medium.

### Determination of reduced glutathione (GSH) and malondialdehyde (MDA)

Utilizing the kits of GSH analysis and the kits of MDA analysis, all purchased from Nanjing Jiancheng Bio-engineering Institute, the content of GSH in 1mL culture medium and the level of MDA in 0.1mL culture medium we re measured.

### Cell survivability assay

The survivability of rat hepatocytes was assayed by the MTT colormetric method[10]. At 48h after $CCl_4$ challenge, 20 μL/well- MTT stock solution (5 g/L) was added into each well of 96-well plates. The cells were continuously incubated for another 4 h before 0.1 mL/well dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the brown-black crystals. The plates were read on microplate reader, using a test wavelength of 570 nm with a reference wavelength of 655 nm.

### Statistical analysis

The results were expressed as $\bar{x} \pm s$ and significant difference was a ssessed by Student's *t* test.

## RESULTS

### Effects of PD on GPT activity in culture medium

The concentration of GPT in culture medium significantly increased after $CCl_4$ challenge, and maintained at a high level in 8 h (Table 1). Furthermore, a progressively elevated trend existed with time-dependence. PD could significantly inhibit the level of GPT in accordance with the doses used. Especially, PD 10 μmol/L had the strongest effects and the inhibiting rate was over 50%.

**Table 1 Effect of PD on GPT activity in culture medium** ($\bar{x} \pm s, n = 8$)

| Group | c/(mol/L) | GPT(U) 6 h | 12 h | 24 h | 48 h |
|---|---|---|---|---|---|
| Normal | | 13.5±2.5[b] | 13.8±3.1[b] | 13.7±5.6b | 14.1±3.3b |
| Control | | 72.3±14.1 | 79.7±10.3 | 85.4±9.2 | 88.3±19.6 |
| PD | $10^{-7}$ | 60.3±17.1[a] | 62.0±15.6[a] | 68.8±17.5[a] | 71.4±20.5[a] |
| PD | $10^{-6}$ | 55.0±10.3[a] | 58.3±16.7[a] | 64.1±13.6[a] | 69.1±19.2[a] |
| PD | $10^{-5}$ | 30.6±10.6[b] | 38.3±5.5[b] | 42.5±7.0[b] | 45.0±7.6[b] |
| PD | $10^{-4}$ | 42.1±7.8[a] | 47.5±9.8[a] | 56.8±11.3[a] | 59.2±10.7[a] |

[a]$P<0.05$, [b]$P<0.01$, *vs* $CCl_4$-treated control group.

### Effects of PD on GSH content in culture medium (Table 2).

The content of GSH in culture medium decreased obviously as compared with that in normal hepatocytes after 6 h incubation with $CCl_4$ (Table 2). On the other hand, PD of various concentrations could

improve GSH in a dose-dependence manner, and 10 μmol/L PD showed a most signifi cant activity.

**Table 2 Effects of PD on GSH content in culture supernatant ($\bar{x}\pm s$, $n$ = 8)**

| Group | c/(mol/L) | GSH (ng/L) after $CCl_4$ challenge | | | |
|---|---|---|---|---|---|
| | | 6 h | 12 h | 24 h | 48 h |
| Normal | | 9.8±0.8[b] | 10.1±0.8[b] | 10.4±0.7[b] | 10.6±1.2[b] |
| Control | | 4.2±0.6 | 4.1±0.7 | 4.1±0.3 | 3.8±0.6 |
| PD | $10^{-7}$ | 5.0±0.3 | 5.4±0.5 | 5.6±0.9 | 6.1±1.0[a] |
| PD | $10^{-6}$ | 5.3±0.8 | 5.6±0.9 | 6.4±0.6[a] | 6.8±1.1[a] |
| PD | $10^{-5}$ | 8.4±1.2[b] | 5.9±1.3[a] | 7.7±0.8[a] | 9.0±1.2[b] |
| PD | $10^{-4}$ | 6.7±0.4[a] | 6.1±1.0[a] | 6.8±0.7[a] | 7.6±0.9[a] |

[a]$P$<0.05, [b]$P$<0.01, *vs* $CCl_4$ treated control group.

### *Effects of PD on MDA formation in rat hepatocytes*

$CCl_4$ challenge obviously elevated the MDA formation in rat hepatocytes, with a marked rise in time-dependence manner, whereas MDA formation of rat hepatocytes decreased significantly at various concentrations of PD as compared with that in $CCl_4$ control group, and it reached minimum value at $10^{-5}$ mol/L and slightly elevated when PD concentration was up to $10^{-4}$-mol/L (Table 3).

**Table 3 Effects of PD on MDA formation in rat hepatocytes ($x\pm s$, $n$ = 8)**

| Group | c/(mol/L) | GSH (ng/L) after CCl4 challenge | | | |
|---|---|---|---|---|---|
| | | 6 h | 12 h | 24 h | 48 h |
| Normal | | 4.0±0.4[b] | 4.5±0.6[b] | 4.8±0.4[b] | 4.6±0.7[b] |
| Control | | 15.5±1.8 | 16.0±2.7 | 17.5±2.1 | 19.0±2.4 |
| PD | $10^{-7}$ | 13.1±2.0 | 13.8±3.3 | 13.0±4.3[a] | 14.5±1.8[a] |
| PD | $10^{-6}$ | 11.4±1.7[a] | 12.0±1.8[a] | 12.1±3.1[a] | 12.5±2.0[a] |
| PD | $10^{-5}$ | 6.5±1.2[b] | 6.7±1.2[b] | 7.5±2.3[b] | 8.2±2.7[b] |
| PD | $10^{-4}$ | 8.7±3.5[b] | 8.9±2.8[b] | 9.8±2.6[b] | 10.3±3.0[b] |

[a]$P$<0.05, [b]$P$<0.01, *vs* $CCl_4$ treated control group.

### *Effects of PD on cell survivability in primary culture rat hepatocytes*

The results of MTT assay showed that normal hepatocytes had high level of cell viability (91.0% ± 7.9%) and $CCl_4$ induced marked decrease of hepatoc ytes survivability (35.4% ± 3.8%, $P < 0.01$ *vs* normal group), whereas the level of cell survivability could be significantly enhanced by PD at the concentrations of $10^{-7}$ mol/L - $10^{-4}$ mol/L to 56.1% ± 5.2% ($P < 0.05$, *vs* $CCl_4$-treated control group), 65.8% ± 5.0% ($P < 0.05$), 88.7% ± 6 .8% ($P < 0.001$) and 75.2% ± 7.3% ($P < 0.01$) respectively. It reached a maximum value at $10^{-5}$ mol/L and slightly declined when the concentration of PD was up to $10^{-4}$ mol/L (Figure 1).

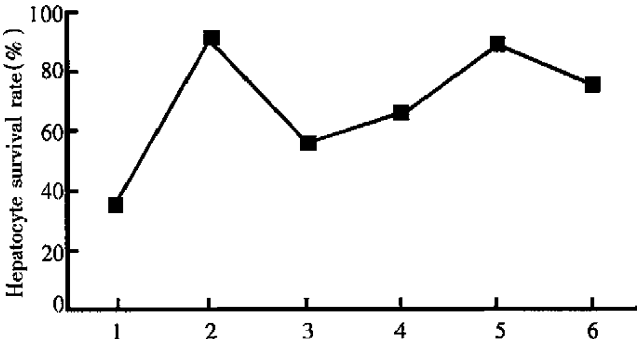


**Figure 1** Effects of PD on cell survivability in primary culture rat hepatocytes.
1. $CCl_4$-treated control group; 2. normal hepatocytes; 3. PD $10^{-7}$ mol/ L; 4. PD $10^{-6}$ mol/ L; 5. PD $10^{-5}$ mol/ L; 6. PD $10^{-4}$ mol/ L.

## DISCUSSION

*P. cuspidatum* has been used to treat some chronic liver diseases such as hepatitis and hepatocirrhosis. We have been trying to search for hepatoprotective compounds of *P. cuspidatum*. Our previous *in vitro* studies showed that emodin, another active compound, had a hepatoprotective effect[11]. The present *in vitro* study also indicated that PD had a protective effect against $CCl_4$ induced injury to primarily cultured rat hepatocytes. Since the extraction and isolation of PD are relatively simple and have a high content of 1.23% in the root of *P. cuspidatum*[7], we may take these advantages to further study its mechanisms of hepatoprotective effect and develop a new drug from it.

$CCl_4$ is a well-known example of a chemical that produces free radical-mediated liver injury. It generates $CCl_4$ by the activation of liver cytochrome P-450, initiating lipid peroxidation of bio-membranes[12]. In the presente xperiment, it was found that $CCl_4$ induced both the increase of GPT in supernat ant and the elevation of MDA in rat hepatocytes. However, administration of $10^{-7}$ mol/ L - $10^{-4}$ mol/L PD could partly reduce GPT and MDA. Therefore, there may be two possible mechanisms contributing to the hepatoprotective actions of PD. One is that PD inhibits further production of lipid peroxidation in rat hepatocytes, and the other is that it inhibits the destructive action of lipid peroxidation on liver cells.

GSH is an important endogenous anti-oxidant substance. The decrease of GSH content may be due to increased GSH consumption as it participates in the detoxifica tion system for the metabolism of $CCl_4$, and results in an enhanced susceptibility of hepatocytes to $CCl_4$ toxicity[13]. Our results showed that $CCl_4$ obviously decreased GSH content in the

hepatocytes, but PD could partly reverse it. This suggested that the nature of PD protecting-SH compounds (such as GSH ) from CCl4 injury may be the third mechanism of its hepatoprotection.

It is interesting that PD of $10^{-5}$ mol/L was more effective than that of $10^{-4}$ mol/L, at the same time, the hepatoprotective action of PD was in dose dependence at concentrations of $10^{-7}$ mol/ L - $10^{-5}$ mol/ L. Its mechanisms of action need to be further studied.

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# Effect of HCV $NS_3$ protein on *p*53 protein expression in hep atocarcinogenesis *

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**Subject headings** liver neoplasms; oncogenes; hepatitis virus; *p*53 protein

## Abstract

**AIM To investigate hepatocarcinogenesis by detecting the effect of HCV $NS_3$ protein on *p*53 protein expression in hepatocellular carcinoma (HCC) and pericarcinomatous liver tissue (PCLT).**

**METHODS The expression of HCV $NS_3$ and *p*53 protein was det ected with immunohistochemical technique (SP method) in specimens of HCC and PCLT from 47 patients with negative HBV.**

**RESULTS The positive rate of HCV $NS_3$ protein was lower in HCC (62%) than in PCLT (83%) ($P < 0.025$). The better differentiaton of cancer cells, the stronger expression of HCV $NS_3$ protein ($P < 0.025$). The positive rate of *p*53 protein in HCC (81%) was higher than in PCLT (47%) ($P < 0.025$). The worse differentiaton of cancer cells, the stronger expression of *p*53 protein ($P < 0.05$). The *p*53 protein expression was not correlated with the HCV $NS_3$ protein expression in HCC ($P > 0.5$), whereas their expression was closely related to PCLT ($P<0.01$), and the expression rate of p53 protein in the cases of positive HCV $NS_3$ protein was higher than that in the cases of negative HCV NS-3 protein.**

**CONCLUSION HCV $NS_3$ protein may exert its hepatocarcinogenic effect in early stage on host cells by endogenous pathway which may bring about mutation of *p*53 gene and transformation of hepatocytes.**

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human cancers in the world. Recently, the HCV infection was found to be an etiological factor to HCC. HCV is a RNA virus bearing no reverse transcriptase activity. Therefore, in stead of "promoter insertion", or "insertion mutagenesis", the HCV $NS_3$ expression of HCV gene may play an essential role in transfomation of hepatocytes[1]. *p*53 gene, an oncogene, has been intensively investigated recently. Mutation of this gene was found to be related to HCC in a variety of studies. Inorder to understand the relationship among *p*53 protein, HCV $NS_3$ protein and HCC, we studied the effect of HCV $NS_3$ protein on expression of *p*53 protein in HCC and pericarcinomatous liver tissue (PCLT).

## MATERIALS AND METHODS

### *Tissue samples*

HCC and PCLT were obtained by surgical resection from 47 patients in Xiangya Hospital and the Second Affiliated Hospital of Hunan Medical University, China. Forty patients were males and 7 females. Their age ranged 33 to 67 years (mean, 52 years), and all patients were negative for HBsAg serological marks. The tissues were fixed in 10% formalin and embedded in paraffin.

### *Reagents*

Anti-HCV $NS_3$ protein MAb was purchased from GIB Comp. (Beijing, China), anti-*p*53 protein MAb and SP detection kit from Maixing Comp. (Fuzhou, China).

### *Immunohistochemistry*

Five μm tissue sections were deparaffinized and washed in 0. 05mol/L PBS, handled with 20g/L $H_2O_2$ and treated with microwave. According to SP method, the tissues were detected with immunohistochemical technique. HCV RNA(+) biopsy liver tissues and breast cancer tissues were used as positive control of HCV $NS_3$ protein and *p*53 protein respectively. PBS was used as substitutes of Mabs for negative control groups.

### *Histological assessment*

Semi-quantity analysis was performed as Formonitz[2] described.

### *Statistical analysis*

The difference between each group was analyzed by Chi-square test.

## RESULTS

### *Expression and distribution of HCV NS3 protein in HCC and PCLT*

Among 47 cases, the positive rate of HCV $NS_3$ protein in HCC was 62% (29/47), and the positive cells were clustered or diffused in HCC. The positive signal was localized in cytoplasm. The expression strength of HCV $NS_3$ protein in HCC was related to the degree of carcinoma cell differentiation ($P < 0.05$). The better differentiaton of cancer cells, the stronger expression of HCV $NS_3$ protein ($P < 0.05$). The positive rate (83%) and the expression strength of HCV $NS_3$ protein in HCC were higher than those in PCLT. The distribution of positive cells in PCLT appeared large patchy or diffused.

### *Expression and distribution of p53 protein in HCC and PCLT*

Of the 47 cases, the positive rate of *p*53 protein in HCC was 81% (38/47), and the positive cells appeared focal or patchy in HCC. The positive signal was in the nuclei. The expression strength of *p*53 protein in HCC was correlated with the degree of carcinomatous cell differentiation ($P < 0.05$). The worse differe ntiaton of cancer cells, the stronger expression of *p*53 protein ($P < 0.05$). The positive rate (47%) and the expression strength of *p*53 protein in HCC were lower than those in PCLT. The positive cells in PCLT was scattering in distribution.

**Table 1 The expression strength of HCV NS protein and *p*53 protei n in HCC and PCLT**

| | *n* | HCV $NS_3$ protein | | | | | *p*53 protein | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | - | + | ++ | +++ | Positive rate(%) | - | + | ++ | +++ | Positive rate(%) |
| HCC | | | | | | | | | | | |
| I | 8 | 2 | 0 | 3 | 3 | 6/8 | 4 | 2 | 2 | 0 | 4/8 |
| II | 10 | 2 | 1 | 2 | 5 | 8/10 | 2 | 3 | 4 | 1 | 8/10 |
| III | 18 | 8 | 3 | 6 | 1 | 56 | 2 | 0 | 6 | 10 | 89 |
| IV | 11 | 6 | 3 | 2 | 0 | 46 | 1 | 1 | 3 | 6 | 91 |
| PCLT | | | | | | | | | | | |
| Normal | 3 | 2 | 1 | 0 | 0 | 1/3 | 3 | 0 | 0 | 0 | 0 |
| Hepatitis | 23 | 4 | 2 | 5 | 12 | 83 | 13 | 8 | 2 | 0 | 44 |
| Cirrhosis | 21 | 2 | 2 | 6 | 11 | 91 | 9 | 7 | 5 | 0 | 57 |

### *Relationship between p53 protein expression and HCV NS3 protein*

In HCC, the expression rate of *p*53 protein in 29 cases of positive HCV $NS_3$ protein was 83% (24/29), and in 18 cases of negative HCV $NS_3$ prote in was 78% (14/18), the difference between the former and the latter being not significant ($P>0.1$). In PCLT, the expression rate of *p*53 protein in 39 cases of positive HCV $NS_3$ protein was 54% (21/39), and in 8 cases of negative HCV $NS_3$ protein was only 13% (1/8), the difference between the former and the latter being significant ($P<0.05$).

## DISCUSSION

Chronic infection with HCV is strongly associated with the development of HCC. HCV causes HCC by expressing protein, especially HCV $NS_3$ protein[1,3]. Nevertheless, the exact molecular mechanism remains quite unknown. Our results showed that the positive rate and expression strength of HCV $NS_3$ protein in PCLT were higher than those in HCC, and the expression strength of HCV $NS_3$ protein in HCC was related to the degree of carcinoma cell differentiation. The better differentiaton of cancer cells, the stronger expression of HCV $NS_3$ protein. It is indicated that the cellular internal environment for HCV replication is disturbed with cancer growth. HCV may be eliminated at the final stage of hepato cytes transformation because the virus without reverse transcriptase is unable to integrate into the host hepatocytes genome. On the other hand, the positive rate and expression strength of *p*53 protein in PCLT were lower than those in HCC. The expression strength of *p*53 protein in HCC was related to the degree of carcinoma cell differentiation. The worse differentiaton of cancer cells, the stronger expression of *p*53 protein. It is suggested that *p*53 protein may play a role in the morphological change and the differentiated degree of cancer cells in HCC, thus detecting *p*53 protein expression is of benefit to HCC prognosis.

Effect of HCV $NS_3$ protein on mutation of *p*53 gene was not confirmed, although HCV infection may result in mutation of *p*53 gene[4]. This study revealed that there was no relationship between *p*53 protein and HCV $NS_3$ prote in in HCC. In PCLT, *p*53 protein expression was positive in 21 of 39 cases of pos itive HCV $NS_3$ protein while only one case of *p*53 protein expression in 8 cases of negative HCV $NS_3$ protein was positive. HCV $NS_3$ protein may exert its hepatocarcinogenic effect in early stage on host cells by endogenous pathway which may bring about mutation of *p*53 gene and transformation of hepatocytes.

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# Expression of C*x* genes in liver and stomach of different embryonic stages *

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**Subject headings** liver/embryology; liver/cytology; stomach/embryology; stomach/cytology; Cx genes; gene expression

## Abstract

**AIM To explore the relationship between the rules of Cx gene expression and cellular differentiation in organs of different embryonic stages.**

**METHODS A series of Cx gene serving as molecular probes and the Northern blot hybrydization were employed to study the Cx gene expression.**

**RESULTS Cx31, Cx31.1, Cx46 did not express while other Cx genes expressed in the embryonic liver and stomach. The Cx gene expression in the liver and stomach showed different state at different embryonic stages. The Cx gene expression had organic diversity. The expression of Cx26 gene was overlapping in the above organs. Cx43 did not express in the human liver after birth, but it expressed in the embryonic stage.**

**CONCLUSION The expression state of Cx genes is concordant with cellular differentiation. It might be a key candidate gene to regulate some differentiational events associated with cellular differentiation, proliferation, and morphogenesis in the early embryo.**

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## INTRODUCTION

Cellular connexin genes are a multigene family consisting of more than 10 members[1-4] which encode the gap junctional channel assembled protein, connexin (Cx). The latter is the key composite of gap junctional intercellular commun ication (GJIC). The expression of Cx genes determines the formation, pattern, amount and the degradation of GJIC channel. The specific connexin proteins transcribed and translated by different members of Cx gene family contribute to the diversity of gap junctional channel within different tissue cells or the same type of cells but in different functional states, leading to the differences in patterns and structures of gap junction. The members of connexin gene are related to the type and size of communication channel and communication ways of gap junction[5]. Gap junction is present in the early stage of embryonic de velopment. The morphological study has verified that gap junction is detected within morula and blastocyst, and dense GJICs appear in cells of embryonic ectoderm at 20 h - 30 h of developing chicks[5]. In order to investigate the relationship between the expression law of Cx genes and cellular diffe rentiation in the liver and stomach during embryonic development, the Cx gene expression state was studied using C*x*26, C*x*31, C*x*31.1, C*x*32, C*x*37, C*x*40, C*x*43 and C*x*46 as molecular probes and the Northern blot hybridization.

## MATERIALS AND METHODS

### *Materials*

The liver and stomach derived from the fetals of mothers receiving natural abortion with conceptional age of 5 weeks, 2, 3, 4 and 5 mouths.The tissues or organs were stored in fluid nitrogen.

### *Methods*

**Preparation of plasmids containing Cx cDNA** A small amount of bacteria containing 8 kinds of pCx plasmids and inner control pGAPDH plasmid was recovered. An inoculum of bacteria was streaked on an agar plate and incubated overnight at 37℃. The next day single bacterial colonies were picked for amplification; plasmids were extracted with alkaline lysis procedure and digested and identified with appropriate restrictive endonuclease. The termini were then isolated by electrophoresis in 10g/L agarose gel and retried and purified with Glass Max kit (Table 1).

**Table 1 A list of plasmids containing Cx cDNA**

| Plasmids | Vectors | Restriction enzymes | Inserts (kb) | Species |
|---|---|---|---|---|
| pCx26 | pSG5 | *Bam*H I | 0.68 | human |
| pCx31.1 | SP63T | *Bgl* II | 0.85 | mouse |
| pCx32 | pGEM3 | *Eco*R I | 1.5 | rat |
| pCx33 | SP64T | *Bgl* II | 0.9 | rat |
| pCx37 | SP64T | *Hind* III / *Xba* I | 1.25 | mouse |
| pCx40 | pGEM4z | *Eco*R I / *Xba* I | 1.1 | mouse |
| pCx43 | pSG5 | *Bam*H I | 1.11 | human |
| pCx46 | BSK+ | *Eco*R I | 1.6 | human |
| pGAPDH | | *Eco*R I / Hind III | 0.6 | human |

**Table 2 Expression of Cx genes in different organs at different fetal ages**

| Connexin genes | 2 month | | 3 month | | 4 month | | 5 month | |
|---|---|---|---|---|---|---|---|---|
| | Liver | Stomach | Liver | Stomach | Liver | Stomach | Liver | Stomach |
| Cx26 | ++ | + | +++ | + | ++ | + | + | + |
| Cx31 | - | - | - | - | - | - | - | - |
| Cx31.1 | - | - | - | - | - | - | - | - |
| Cx32 | +++ | - | +++ | + | +++ | ++ | +++ | +++ |
| Cx37 | - | ++ | - | +++ | - | - | - | ++ |
| Cx40 | - | + | - | ++ | - | - | - | +++ |
| Cx43 | ++ | - | +++ | - | ++ | ++ | + | ++ |
| Cx46 | - | - | - | - | - | - | - | - |

+++: high, ++: moderate, +: low, -: no or weak

### *Preparation and purification of total RNA*

Total RNA was extracted by acid guanidinium-phenol chloroform procedure from the liver and stomach in different stages of embryo respectively.

### *Northern blot*

Total RNA (20μg in each well) was separated by electrophoresis through formal dehyde denaturing gels and transferred onto nylon membranes by capillary transfer for 24 h in 20 × SSC. The RNA was cross-linked to the membranes by exposure to UV light.

### *Northern hybrydization between Cx gene probes and RNA*

Connexin-specific cDNA probes were random-prime labeled with [α-$^{32}$P] dCTP. The membranes were prehybridized at 42℃ for 4h in Northern hybridization solution (500 g/L-formamide, 5 × SSPE, 5 × Denhardt' s reagent, 1 g/ L-SDS solution and 100 mg/L salmon sperm DNA) and then hybridized with the labeled probe for 16 h - 24 h in the same solution.

### *Autoradiograph (ARG)*

The membranes were washed twice at 37℃ in 2×SSC/ (1 g/L)-SDS for 40 min and three times at 65℃ in 0.1 × SSC/ (1 g/L) SDS for 1 h. Exposure of X-ray film to the blots was conducted at -70℃ with intensifying screens. To remove probes from the membranes, the membranes were immersed in 1 g/L- SDS solution in rotating platform from boiling to RT. The process was repeated. Then the membranes were rehybridized with GAPDH probe.

## RESULTS

C*x*31, C*x*31.1 and C*x*46 did not express while other C*x* genes expressed in the liver and stomach of embryonic stage. The expression of Cx genes showed different states in the liver and stomach at different embryonic stages. The expression of Cx genes was of organic diversity, e. g. , the expression of C*x*32 in the liver and C*x*37 in the stomach. The C*x* gene expression was overlapping in the above organs, and C*x*26 presented different states of expression. C*x*32 gene had high expression from the 5th week to the 5th month in the liver and the 4th to 5th month in the stomach. C*x*43 did not express in human liver after birth, but it expresse d in the fetal stage. C*x*37 gene in the stomach of fetals highly expressed from the 3rd month to the 5th month (Table 2).

## DISCUSSION

C*x* genes showed different expression states, e.g., C*x*26, C*x*32, Cx43 in the liver, and C*x*26, C*x*32, C*x*37 and C*x*43 in the stomach. The diversity of expression indicated that the various gap junctional channels between different cells or the same cells in different functional state was due to the expression of different members of C*x* gene family[6].

C*x*26 gene in the fetal liver had weak expression in the 5th week, and high expression in the 3rd month, which lowerd gradually during the 4th to 5th month. The expression and nonexpression of C*x*26 gene are believed to be relevant to morphogenesis of hepatic lobule, central vein and portal area. There was no structure of hepatic lobule, central vein and portal area in the liver in the 5th week of embryo. The structures formed gradually during the 2nd month, and hepatic lobule was recognized in the 3rd month. C*x*26 was in its high expression state during this stage. The structure of portal area became clear in the 4th month. Up to this stage, the C*x*26 expression gradually reduced when the basal morphological structure of liver was established. The C*x*26 gene expression was overlapping in the stomach, which might be related to the morphogenesis of stomach. It is suggested that the C*x*26 expression plays an important role in the morphogenesis and structural building of the liver and stomach during embryogenesis.

The expression state of C*x* genes is conformed to the cellular differentiation. It may be related to its own differentiation and proliferation of liver cells. This is compatible with Zeng's report in which the cells of primeval region will acquire further differentiative capacity, which is closely related to a high level of expression of C*x* genes among the same cells[7]. C*x*32 gene expression in the stomach is related to development of gastric gland, it was low during gastric gland bud stage in the 3rd month, and increased gradually during the developing stage of primordial gastric gland in the 4th month, and reac hed a peak in the 5th month when the gastric gland grew completely.

Eghbali[8] transfected cDNA of whole length C*x*32 gene into the hepat ocellular cancer lacking C*x*32, and found mRNA expression level, and gap junction were increased markedly, and ion coupling and metabolite coupling reappeared in the tests of dye transfer and electric current of intercellular communicati on, meanwhile the growth rate of carcinoma cells transfected with C*x*32 decreased rapidly, and cellular structure of neoplastic cells differentiated towards normal cells. It is indicated that the expression of C*x*32 gene plays a significant role in the cellular differentiation.

C*x*43 does not express in the human liver after birth[9], but it expresses in the fetal stage. It may be related to hematopoietic function of the liver during the fetal stage. Hematopoietic stem cells migrate to liver from yolk sac at early embryonic stage; at this time, C*x*43 expression is low and turns high when the weight of hematopoietic tissues amounts to 30%-40% of the liver by the 3rd to 4th month of embryonic development. With the formation of the spleen, thymus and bone marrow, most of the hematopoietic stem cells in the liver migrate to the above organs, and the C*x*43 gene expression lowers distinctly in the 5th month of the fetal liver, indicating the lowered hematopoietic function of the liver. It may be related to the smooth muscle development in stomach[4].

Wang SQ[10] held that gap junction appeared in the early embryonic stage made it possible the intercellular transfer of substances which regulated cellular differentiation, morphological formation and growth control. For example, in archiga-strula and nuerula, gap junctions made notochord-induced ecoderm develop into neural tubes. During embryonic development, the expression state of C*x* genes is basically concordant with the formation and degradation of gap junctional communication channel. It is suggested that Connexin gene may be a key candidate gene to regulate some differentiational events associated with cellular differentiation, proliferation and morphogenesis and organ devolopment.

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# Expression of IL 1β converting enzyme in 5-FU induced apoptosis in esophageal carcinoma cells

DENG Li-Ying[1], ZHANG Yun-Han[2], XU Ping[2], YANG Su-Min[1] and YUAN Xue-Bin[1]

**Subject headings** esophageal cancinoma; cell line; apoptosis; 5-fluorouracil; interleukin 1β-converting enzyme

## Abstract

**AIM To study the role of interleukin 1β-converting enzyme (ICE) in antitumor drug-induced apoptosis in tumor cells.**

**METHODS Morphological changes in human esophageal carcinoma Eca-109 cells after treated with 5-fluorouracil (5-FU) were observed under light and electron microscope. Expression of ICE in the tumor cells exposed to 5-FU was examined by the immunocytochemical method.**

**RESULTS The cells treated with 5-FU displayed disappearance of nucleoli, chromatin gathering under nuclear envelope, karyorrhexis, budding and the formation of apoptotic bodies. The expression of ICE was negative in control cells, and 5-FU could induce the ICE expression in Eca-109 cells undergoing apoptosis. The number and the staining intensity of positive cells increased with the extension of action time.**

**CONCLUSION 5-FU may induce apoptosis in human esophageal carcinoma Eca-109 cells; ICE gene may be involved in the regulation of 5-FU-induced apoptosis; and ICE protein may mediate apoptosis induced by 5-**FU.

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## INTRODUCTION

Apoptosis is an important physiological form of cell death. It is strictly controlled by genes. It has been shown in experiments that external and internal signals of cells may start the process of apoptosis, e. g., anticancer drugs[1]. The genes involved in apoptosis include the family of *bc1*-2, *p*53, *c-myc* and so on. Recent studies showed that interleukin-1β-converting enzyme (ICE) gene was a mammalian homologue of the C. elegans cell death gene ced-3[2] ant that overexpression of ICE gene could induce apoptosis in Rat-l fibroblast[3]. Thus, the expression of ICE protein in human esophageal carcinoma Eca-109 cells treated with 5-fluorouracil (5-FU) was examined to investigate the role of ICE in anticancer drug induced apoptosis.

## MATERIALS AND METHODS

### *Tumor cell culture*

Human esophageal carcinoma Eca-109 cells were cultured in RPMI 1640 (Gibco) medium supplemented with 10% heat-inactivated new-born calf serum 100U/mL penicilin, and 100μg/mL streptomycin, and kept in a controlled atmosphere (5% $CO_2$) incubator at 37℃.

### *Drug treatment*

Exponentially growing cells were seeded in plates for 24 h and treated with 5-FU (10, 50 and 100mg/L) for 48 h, 72 h and 96 h, respectively. The cells not treated with the drug served as control cells.

### *Preparation of specimens for light and electron microscopy*

The tumor cells of experimental and control groups were collected, made into smears and fixed. Some smears were HE stained and observed for morphological features under light microscope. some smears were stained by the immunocytochemical method in order to examine the expression of ICE protein. The cells were fixed in 25 g/L-glutaraldehyde for 1h at room temperature, and postfixed for 1 h in 10g/L osmium tetroxide, and dehydrated through gradient ethanol, stained and observed under electron microscopy (EM) (Hitachi 600A model).

### *Immunocytochemical staining*

Rabbit anti-human ICE (p20) multiclonal antibody

(Santa Cruze) was used at 1:100. The immunocytochemical staining was performed using the LSAB (Dako) method. The enzyme was developed with DAB in conjuntion with hydrogen peroxide. A negative control for non-specific Ig binding was employed in which the first antibody was substituted with PBS. Results were judged from the number of positive cells, i. e. , (-) was positive cells<25%; (+): positive cells 25% - 50%; (+ +): positive cells 51%-70%; and (+++): posi tive cells>70%.

## RESULTS

### *Morphological changes*

Most of the floating cells were presented with nuclear fragmentation, nuclear disappearance, budding and the formation of apoptotic bodies. Many cells detached with trypsin-EDTA from the plates showed that chromatin was gathered under the nuclear membrane in mass or ring-shape with the disappearance of nucleoli. Under EM, these changes were observed more apparently. In addition, some of the tumor cells displayed budding with several circular or semicircular protrusive vesicles, some of which were detached from the main body (Figure 1). Apoptotic cells were not easily observed in the group of low co ncentration (10 mg/L), and they increased with higher concentration (>50 mg/L) and longer action time (>48 h).

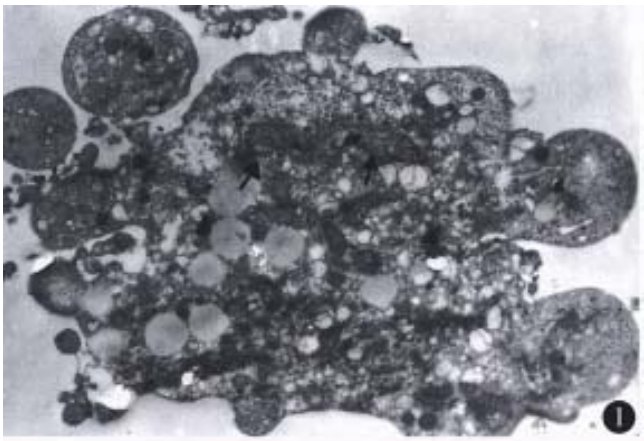
### *ICE protein expression*

ICE protein was expressed negatively in control tumor cells, but positively in the tumor cells treated with 5-FU. The number of positive cells increased with the 5-FU exposure-time (Table 1).

**Table 1 ICE expression in Eca-109 cells treated with 5-FU**

| Doses (mg/L) | Degrees of ICE expression | | |
|---|---|---|---|
| | 48 h | 72 h | 96 h |
| 0 | - | - | - |
| 50 | + | ++ | +++ |
| 100 | ++ | ++ | •+++ |

Although individual control cells were sometimes stained brown, they were small in number and positive granules existed only near the cellular membrane. Brown granules in the cells treated with 5-FU were distributed all over the cytoplasm and cellular membrane. Most of apoptotic cells expressed ICE protein, however, a few cells with obvious features of apoptosis did not expresse ICE. In addition, some cells without features of apoptosis expressed ICE protein (Figure 2).



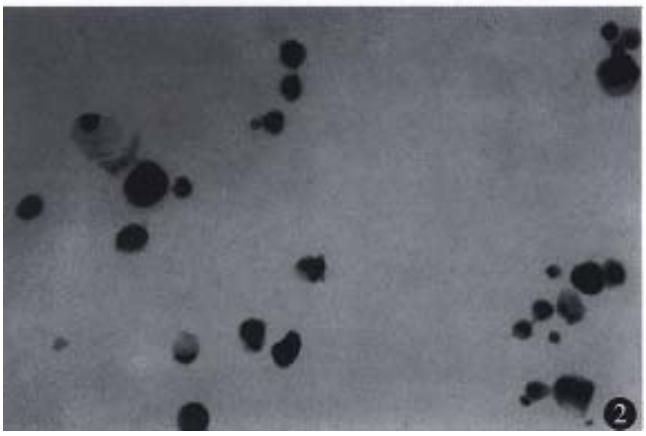


**Figure 1** An apoptotic cell with feature of budding. Nuclear fragments were shown by arrows. ×6000

**Figure 2** ICE protein expression in esophageal carcinoma cells after exposure to 5-FU. LSAB ×400

## DISCUSSION

5-FU may induce apoptosis in human esophageal carcinoma Eca-109 cells. 5-FU is one of the commonly used antitumor drugs. One of the principal mechanisms of action of 5-FU is inhibition of the enzyme thymidylate synthase (TS) and affecting DNA synthase. In recent years, it has been shown that 5-FU may induce apopt osis in fibroblast and acute leukaemic T-lymphocytes[4,5]. The present study indicated that 5-FU may induce apoptosis in human esophageal carcinoma Eca-109 cells. Chromatin gathering under nuclear, the disappearance of nucleoli and the formation of ring-shaped nucleus may be the early changes of apoptosis. Apoptotic cells became visible late at 24h and apparent by 48h. It may be related to the characteristic action of time-dependence.

ICE may mediate apoptosis induced by 5-FU. Ced-3 is known as one of the essential genes for cells to undergo apoptosis in C. elegans. ICE gene, a homologue of the ced-3, was recently identified as inducer of apoptosis in Rat-1 fibroblasts[3]. Kondo *et*

*al* reported that cisplatin increased the mRNA expression of ICE and induced apoptosis in malignant glioma cells[6]. In our study 5-FU increased the expression of ICE protein in Eca-109 cells undergoing apoptosis, suggesting that ICE protein may play a role in apoptosis induced by 5-FU. The expression of ICE protein was also detectable in some cells without features of apoptosis. From the phenomenon that the expression of ICE protein appeared at the early stage of apoptosis even before development of apoptosis, we inferred that ICE protein may be involved in the development of apoptosis. T he induction of ICE does not necessarily trigger cell death, but the manifestation of cells which were seriously injured. If the injury can not be repaired, ICE may activate its downstream target molecules such as apopain, one member of the ICE family, and in the end, target proteins necessary for the existence of cells are cleaved and apoptosis becomes irreversible. The reason why negative expression of ICE protein was observed in some cells with typical features of apoptosis is not clear. The antibody used in this study was specific to ICEp20 subunit. There is no cross-reaction between ICEp20 and ICEp10 subunits. Other member(s) of ICE family may be also involved in apoptosis. It is known that at least six members of the ICE family are expressed in mammalian cells. However, whether ICE acts directly or through its action product interleukin-1β is not fully understood. The recent studies found that ICE may directly cleave actin which makes up cytoskelet on and that alteration in actin was implicated in the formation of plasma membrane budding and disintegration of cells[7].

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# Congenital expression of *mdr*-1 gene in tissues of carcinoma and its relation with pathomorphology and prognosis

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**Subject headings** neoplasm; multidrug resistance; gene expression/ *mdr*-1 gene; surgery; esophageal neoplasms

## Abstract

**AIM To detect the congenital expression patterns of *mdr*-1 gene in commonly encountered malignant tumors in clinic, and the relationship between the expression of *mdr*-1 gene and the prognostic morphology in esophageal carcinomas.**

**METHODS A total of 151 resected samples of malignant tumors without preoperative treatment were taken from Anyang City Tumor Hospital. The congenital expression of their *mdr*-1 gene was detected with reverse transcripti on polymerase chain reaction (RT-PCR) and was compared with each other. The positive incidence of *mdr*-1 gene in 46 samples of esophageal carcinoma was compared with their differentiated grades, TNM stages and macroscopic types, and the precautions and advantages of RT-PCR were evaluated.**

**RESULTS All the 151 samples were confirmed to be malignant histopathologically, including cancers of stomach and gastric cardia ($n$ = 51), esophagus ($n$ = 46), colorectum ($n$ = 16), breast ($n$ = 15), thyroid ($n$ = 10), lung ($n$ = 9) and uterine cervix ($n$ = 24). The positive expression rate of their *mdr*-1 gene was 33.3%, 37%, 31.3%, 13.2%, 40%, 55%, and 0% respectively. All the 46 samples of esophageal carcinoma were pathologically confirmed to be squamous cell carcinoma. The total expression rate of their *mdr*-1 gene was 37% (17/46), 35% (6/17), 40% (8/20), and 33% (3/9) for differentiation grade I, II and III respectively. The expression rate of TNM classification was 33% (6/18), 40% (5/12) and 37% (6/16) in stage IIa, IIb and III. The expression rate was 33% (3/9) in ulcerous type, 37% (3/8) in constrictive types, 33% (5/15) in fungoid types, and 40% (6/14) in medullary types. No statistically significant difference was found.**

**CONCLUSION Compared with other methods, RT-PCR is more simple, reliable and accurate in detecting *mdr*-1 gene expression in tissues of tumor. The overexpression of *mdr*-1 gene in these neoplasms suggested that cases should be handled differently for chemotherapy with rational use of drugs. Excision is the chief treatment for carcinoma of esophagus. The expression of *mdr*-1 gene in tissues of esophageal cancer is correlated with the parameters of tumor molecular biology which are independent of histopathological mor phology.**

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## INTRODUCTION

Multidrug resistance (MDR) of malignant tumor cell has aroused widespread interest. It has been shown that MDR is present in many malignant tumors. One of its molecular bases is *mdr*-1 gene amplification and its expression product. Failure of chemotherapy was chiefly due to drug resistance of tumor cells[1-9]. It is very important to detect-MDR *in choosing reasonable* treatment, espec ially in using effective chemotherapeutic drugs for a specific patient. Others[10] believe that *mdr*-1 gene expression in cancer tissue is a malignant biological indicator for neoplasms. Few research reports have been found in the literature on *mdr*-1 gene expression in tissues of esophageal carcinoma, and on its relation with the morphological parameters of esophageal carcinoma. For these reasons, a primary study was made.

## MATERIALS AND METHODS

### *Specimens and clinical data*

One hundred and fifty-one specimens were taken

from the Surgical Department of Anyang City Tumor Hospital, Henan Province soon after they were excised. Of the 151 cases, 78 were male and 73 female. They were aged from 21 to 80 years, averaging 52.1. All the cases were confirmed to be malignant tumors without preoperative treatment. Of them, 51 were cancers of stomach and gastric cardia, 46 cancers of esophagus, 16 cancers of colorectum, 15 cancers of breast, 10 cancers of thyroid, 9 cancers of lung and 4 cancers of uterine cervix. The 46 patients of esophageal carcinoma were all permanent residents of Anyang citizenship, 26 male and 20 female. The site of cancer was found in the upper, middle and lower thor acic segment in 8, 30, and 8 cases respectively according to 1987 UICC criteria. Eighteen cases were in stage IIa, 12 in stage IIb, and 16 in stage III according to TNM classification. All the cases were squamous cell carcinomas. Of them, 17 were in grade I, 20 in grade II, and 9 in grade III according to SUN Shao-Qian's grading system for squamous cell carcinoma. Nine were found to be ulcerous type, 6 constrictive type, 15 fungoid type, and 16 medullary type according to WU Ying-Kai's gross pathological typing method.

### *Main instrument and reagents*

Reverse Transcription Polymerase Chain Reaction (TT-PCR) Reagent Kit was supplied by Beijing Jinghai Biological Engineering Company. Bio-RAD Gene CyclerTM (Gene Amplifier) was made in Japan. LG15-w high-speed centrifuge was made by Beijing Medical Centrifuge Factory. SA-U94.11 ultraviolet transilluminator was made by Shanghai Zhongya Biological Institute.

### The sequences of *mdr*-1 gene primers

5'ACCCATCATTGCAATAGCAG3'
5'TGTTCAAACTTCTGCTCCTG3'

### The sequences of inner control β2-microglobulin gene primers

5'ATGGCTCGCTCGGTGACCCTAC3'
5'TCATGATGCTTGATCACATGTCTCG3'

## METHODS

### *Methods for determining mdr-1 gene expression*

Methods for determining *mdr*-1 gene expression[1] was used with minor modifications. Major steps were extraction of total tumor RNA by guanidine isothiocyanate, synthesis and amplification of complementary DNA (cDNA) to *mdr*-1 gene by RT-PCR. The products were separated by electrophoresis on agarose gel containing EB. DNA bands were made visible by transillumination with ultraviolet.

### *Assessment criteria*

Only one 300bp band was found in the negative results. Inner control band and *mdr*-1 gene 170 bp band were found in the positive results. Gene expression was calculated on a concentration scanner by the relative yield of the *mdr*-1 gene to the $\beta_2$ inner control gene. Its formula is expressed as:

$$\text{Mdr-1 expression ratio} = \frac{\text{mdr-1 band absorption}}{\text{Inner control band absorption}}$$

The ratio < 0.1 means negative expression, > 0.4 high expression, 0.1 - 0.4 moderate expression. The observed parameters included *mdr*-1 gene expression positively in macro- and micro-scopicmorphology of the specimens, and comparison *mdr*-1 expression in various groups.

### *Statistical analysis*

Chi-square test was used, and *P* value less than 0.05 stands for statistica l significance.

## RESULTS

The expression of mdr-1 gene in the studied samples was over 30%, except that in cancer of uterine cervix and breast (Table 1).

The total expression rate of *mdr*-1 gene was 37% (17/46) in the 46 cases of esophageal carcinoma with no relation to the morphological parameters of the tumor. It was an independent molecular biological characteristic of the tumor (Tables 2-4).

**Table 1 *mdr*-1 gene expression in 151 specimens (%)**

| Site of tumors | *n* | H | M | L/N | H&M |
|---|---|---|---|---|---|
| Esophagus | 46 | 10.9 | 26.1 | 63.0 | 37.0 |
| Gastric cardia | 35 | 17.1 | 11.9 | 71.0 | 29.0 |
| Stomach | 16 | 25.0 | 12.5 | 72.5 | 27.5 |
| Colorectum | 16 | 18.8 | 12.5 | 68.7 | 31.3 |
| Lung | 9 | 22.0 | 33.0 | 45.0 | 55.0 |
| Breast | 15 | 6.6 | 6.6 | 86.8 | 13.2 |
| Thyroid | 10 | 20.0 | 20.0 | 60.0 | 40.0 |
| Uterine cervix | 4 | 0.0 | 0.0 | 100.0 | 0.0 |
| Total | 151 | 15.2 | 18.6 | 66.2 | 33.8 |

H: high expression, M: middle expression, L(N): lower / no expression

**Table 2 *mdr*-1 gene expression in TNM stages of esophageal carcinoma**

| TNM stage | *n* | No. of positive cases | Positive rate (%) |
|---|---|---|---|
| IIa | 18 | 6 | 33 |
| IIb | 12 | 5 | 40 |
| III | 16 | 6 | 37 |

*P*>0.05, among the three stages.

**Table 3 *mdr*-1 gene expression in SUN Shao-Qian's grading system of squamous cell carcinoma of esophagus (*n* = 17)**

| Grade | No. of positive cases | Positive rate (%) |
|---|---|---|
| II | 6 | 35 |
| II | 8 | 40 |
| III | 3 | 33 |

*P*>0.05, among the three grades.

**Table 4 *mdr*-1 gene expression in WU Ying-Kai's macroscopical typing system of esophageal carcinoma**

| Types | *n* | No. of positive cases | Positive rate (%) |
|---|---|---|---|
| Ulcerative | 9 | 3 | 33 |
| Constrictive | 8 | 3 | 37 |
| Fungoid | 15 | 5 | 33 |
| Medullary | 14 | 6 | 40 |

*P*>0.05, among the four types.

## DISCUSSION

It is believed that mdr-1 gene is one of the normal sequences of human genome. Nevertheless, its expression and expressive level are decided by different cell type and environmental factors. The *mdr*-1 gene expression can be investigated with several molecular methods including evaluation of protein expression and mRNA. Protein may be detected by Western blot analysis and immunohistochemical techniques. Immunohistochemical staining is commonly used, but it is not suitable for quantitative determination of protein due to its complicacy and influence of experimental conditions. Since all organisms store their genetic information in nucleic acid, methods of direct detection of *mdr*-1 at mRNA level have advantages of high efficiency, sensitivity, and specificity. Traditional methods for mRNA such as $S_1$ nuclease test, RNA slot blots, RNA protection assays, *in situ* hybridization and Northern blot analysis are greatly limited due to their overlaborate procedure and poor sensitivity. RT-PCR was used in present study. After cDNA of *mdr*-1 gene was synthesized according to the transcribed mRNA of *mdr*-1, it was detected by PCR *in vitro*. Compared with other gene measure ments, RT-PCR is one of the most sensitive, specific, reproducible, effective, simple, and time-saving methods[2]. We believe that the prospects of its application in clinic are quite broad.

One of the mechanisms of drug resistance to cancer cells is called MDR which is known as the resistance to lipophilic drugs such as daunorubicin, adriamycin, vincristin, and colchicin. It is very unfavourable to chemotherapy, because once tumor cells develop resistance to one of the these drugs, they will develop resistance to all lipophilic drugs[5-7]. How to evaluate multidrug- resistant tumor cells is a problem demanding prompt solution. Recently, there were several papers on the mechanism, evaluation and reversion of MDR[5-8]. Most of them focused on *mdr*-1 and its products, p-170[2-6]. p-170 (P-gp) was considered as an ATP-dependent drug molecular pump, which would lead to failure of chemotherapy as a result of drugs being pumped out from cells. This fact has been proved in many researches of diseases such as leukocythemia, breast can cer and melanoma. These recearches were of no significance in clinical practice because they were usually focused on a certain kind of tumors and their results were obtained from malignant cell line with different methods. In the present study, the expression of *mdr*-1 gene in commonly encountered malignant tumors was synchronically studied by the same technicians with same instruments, experi mental methods and reagents. The results showed that all the detected neoplasms except cervical carcinoma, expressed *mdr*-1 gene in different degrees (Table 1). Breast cancer also had a low expression of *mdr*-1. The positive number of *mdr*-1 gene in the other tumor tissues was more than 1/3 except that in breast and cervical cancers. This kind of expression was congenital because these tumors had not received chemotherapy when they were detected. It indicated tumor carried *mdr*-1 gene and its product-Pgp from the development of tumor. Clinicians should pay great attention to the mechanisms of its drug resistance if they are tenable. They should differentiate the subgroups of malignant tumors from molecular level in addition to taking other clinical indexes such as tissue differentiation, TNM staging system, and tissue type into consideration, in order to avoid unsuitable chemotherapy and use of MDR-drugs especially lipophilics. Combined treatment with reverser of *mdr*-1 gene and supressor of P-gp should be used to improve curative effect if it has been proved to be effective. Some researchers held that MDR of tumors could not be completely explained by *mdr*-1 gene and its P-gp system[10], and further research should be made. In our phase II study, a control study will be made on the difference of *mdr*-1 gene expression in cancer and normal tissues as well as in those before and after chemotherapy. We believe that the theory of MDR will be an important reference index for chemotherapy of tumors as a result of our better understanding of it.

Surgical treatment is the commonly used treatment for esophageal carcinoma. Its curative effect is chiefly decided by TNM stages, histological differentiation and types. Generally speaking, it is better for the early and well differentiated squamous cell carcinoma than the late and poorly differentiated adenocarcinoma or the undifferentiated one. However, exceptions in clinic suggest that further research at the molecular level of gene is needed in esophageal carcinoma which has its unique biological characteristics independent of its morphological parameters as other malignant tumors.

Since the finding of *mdr*-1 gene and its product P-gp (P-glycoprotein, p17 0), they have been ap-

plied to researches on their relations to cytotoxic chemotherapeutic drugs, especially to the lipophilic drugs. Many of these researches were in the field of hematic malignancies, and new ways were explored to reverse MDR. However, attention was seldom paid to the congenital expression of *mdr*-1 gene in tissues of esophageal carcinoma and its relation with morphological parameters. It was found in this study that the congenital expression of *mdr*-1 gene was 37% in tissues of the 46 cases of esophageal carcinoma without chemoth erapy before operation. It was much higher than that reported in leukemia[4,9-13], melanoma[6], and breast cancer[1,3,5,7]. This is one of the possible reasons why no progress has been achieved in the curative effect of chemotherapy for esophageal carcinoma in the past years. It indicates that only by attaching importance to the selection of chemotherapeutic drugs and suitable chemotherapy for esophageal carcinoma, can its curative effect be achieved. It also suggests that surgical treatment for it at present should be stressed. Many researches have demonstrated that cancer is a genetic disease. Besides traditional morphological indexes, the following factors were found to be related with the prognosis of esophageal carcinoma such as antioncogene, oncogene and their abnormal products as well as others at the level of gene molecules, and have been taken into account in clinic. Overexpression of *mdr*-1 gene was believed to be an index of drug resistance and further malignization of the histogical behavior of cancer cells[10]. The theory of drug resistance of tumors was advanced by Goldie and Codman[8] in 1979 in the light of gene change. It held that drug resistance was resulted from gene mutation of tumor cells produced in frequency of the tumor, and that the larger the tumor, the more the frequency of proliferation and the stronger the drug resistance. This theory also indicates that drug resistance of tumors has a positive correlation with the stage of tumors. No significant difference was found in the expression of *mdr*-1 gene in tissues of esophageal carcinoma on the basis of its morphology, differentiati on and TNM. It was suggested that expression of *mdr*-1 gene is a molecular parameter independent of surgical pathomorphological indexes. This results show that surgical treatment is the first choice for esophageal carcinoma at present due to its drug resistance.

It was held that expression of *mdr*-1 gene was a protective mechanism of cells, which was found in some normal tissue cells in addition to cancer cells[10]. The results in our study seemed to support it. Long follow-up study is needed to decide whether overexpression of *mdr*-1 gene in esophageal carcinoma is an index of its further malignization, because no comparison was made for its difference in cancer and normal tissues as well as before and after its chemotherapy.

Although surgical treatment of esophageal carcinoma is destructive and will exert some influence on the quality of life of the patients, it remains the first choice before a breakthrough is made in chemotherapy. In order to improve the curative effect of chemotherapy, the reverse mechanism of MDR should be further studied while the drug-resistant mechanism is comprehensively researched.

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# Preparation and distribution of 5-fluorouracil $^{125}I$ sodium alginate-bovine serum albumin nanoparticles

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**Subject headings** 5-fluorouracil (5-FU); sodi-um alginate-al bumin; preparation of nanoparticle (NP); distribution

## Abstract

**AIM To prepare 5-FU sodium alginate $^{125}I$ bovine serum albumin nanoparticles (BSA NP), to determine the radioactive count in different organs of rats at different time points after oral administration of 5-FU $^{125}I$ sodium alginate-BSA NP and to calculate the kinetic parameters of its metabolism.**

**METHODS Emulsion solidification method was used to prepare 5-FU $^{125}I$ sodium alginate-BSA NP, and to determine its diameter under transmission electronic microscope (TEM). Then the rate of NP and external drug releasing velocity were measured. Radioactive counting in different organs of rats wa s made after oral administration of the NP by GAMA Counter, and the kinetic para meters of drug metabolism were calculated by handling the data with the two-department model.**

**RESULTS The average arithmatic diameter of the NP was 166nm±34nm, the rate of 5-FU was 32.8% and the cumulative external releasing ratio amounted to 84.0% within 72 hours. The NP was mainly distributed in the liver, spleen, lungs and kidneys after NP oral administration to rats. The mic ro-radioautographic experiment showed that NP was distributed in the Kupffers cells of liver, liver parenchymal cells and the phagocytes of spleen and lungs. The kinetic parameters of matabolism were: $T_{1/2}$ = 9.42h, $C_{max}$ =2.45×10$^7$Bq, $T_{max}$=2.18h, AUC=148×10$^9$Bq.**

**CONCLUSION NP is difficult to pass through the blood-cerebral barrier,and $^{125}I$ sodium alginate -BSA NP enters the body circulation by gastroin testinal passage.**

## INTRODUCTION

Nanoparticle (NP) is an colloidal dispersion system, with diameters ranging from 10nm to 1000nm. The particles exist mainly in the organs rich in phagocytes after absorption, such as liver, spleen and lymph system. Since its introduction in the 1980s, scientists have done a lot of researches on its preparation, stability and targeting[1,2]. Now most materials used to prepare NP are synthetic substances, e.g. cyanoacrylate, methylacrylate and polylactic acid. However, we selected natural substances-sodi-um alginate and bovine serum albumin as carriers, and 5-fluorouracil (5-FU) as model drug to prepare NP by emulsion solidification, and studied its distribution after oral administration.

## MATERIALS AND METHODS

### *Animal*

The Kunming rats weighing 20 g±2 g, were provided by the Experimental Animal Centre of Tongji Medical University.

### *Reagent*

Sodium alginate (chemical reagent), bovine serum albumin (Sigma), pentane dialdehyde (biochemical reagent, Merck), Na $^{125}$I (speific activity 7.78 TBq/L, Chinese Atomic Energy Institution), IV liquid nuclear emulsion (Physics Institute of Chinese Atomic Energy Research Institution).

### *Preparation of 125I bovine serum albumin (BSA)*

The BAS (10mg) was dissolved in 10mL distilled water and marked with $I^{125}$ according to the chloroamine-T method. After the reation was completed, the mixture was separated by column chromatography (10 mm×340 mm), using Sephadex G-50 as column material and $Na_2S_2O_3$ as eluant. Then the

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radio-chemical purity of eluate was determined by dichloroacetic acid method.

### Preparation of 125I sodium alginate-BAS NP

According to the literature, 1 mL-above $I^{125}$-BAS (1g/L, SpA 107 Bq) was added into sodium alginate solution to prepare sodium alginate- BSA NP.

### Determination of the diameter of nanoparticles

The NP size in the suspension was detected on TEM.

### Determination of the encapsulation efficiency

The standard curve of 5-FU was drawn first. One hundred and five mg of 5-FU was weighed precisely, dissolved in thin HCL solution (9-1000) and adjusted to given volume. This solution was diluted to its half concentration. After 3.0, 6.0, 9.0, 12.0 and 15.0 mL-diluted solution were taken into 100mL-volumetric flasks and adjusted to given volume respectively, their absorbance (*A*) values were detected on ultraviolet-visible spectrophotometer at 265 nm. The following regression equation was obtained: $C = 16.72A \pm 0.0416$ ($r = 0.9998$).

A total of 1.0 mL 5-FU NP suspension was added accurately to a test tube with 9 mL 6mol/HCl. The mixture was hydrolyzed for 20h at 100℃ water bath. The hydrolyzate was adjusted to given volume with distilled water to detect its A value as standard curve item, the hydrolyzate of bland NP suspe nsion served as control. The result showed that the control group had no obvious absorption at 265nm (A=0.01). By putting the A value into the regression equation we worked out the concentration of 5-FU in the hydrolyzate and got the encapsulation efficiency consequently.

### Determination of recovery rate

The same quantities of albumin, sodium alginate and pentane dialdehyde as "Prep aration of NP" items were mixed with 5-FU weighed precisely, the mixture was dissolved in distilled water. Its A value was detected according to the standard curve and the content of 5-FU was calculated.

### Determination of drug release

Appropriate quantity of 5-FU NP suspension was taken precisely, washed for three times with distilled water, and suspended in 200mL-thin HCL solution (9-1000). The drug release state of the suspension was measured at 37℃ by paddle method (100r/min). The samples were taken at the beginning, the 2nd, 4th, 8th, 12th, 24th, 48th and 72nd hour, and filtered through 0.2μm microporous membrane. The filter liquor was detected at 265nm to get its A value and cumulative rate of drug release.

### The distribution of 125I sodium alginate-BSA in rats

Fourty-five Kunming rats were divided into 15 groups (3 rats per group). After starvation for 16h, the rats were given the 5-FU NP orally $148 \times 10^8$Bq per rat. Right after its blood was taken from the eye at different time points (heparin was added to prevent coagulation), the rats were sacrificed by breaking its spine. The relevant tissues and organs were taken. When the water had been absorbed with filter paper after washing, they were weighed. The amounts of the NP in unit weight in different tissues were measured by GAMA Counter. In accordance with the counting result, we calculated the tissue blood ratio at different time points and used two-department model to process the data in order to get pertinent kinetic parameters.

### Radioautography

While carrying out the histological experiment, we chose the tissues of liver, spleen, lungs, and kidneys at some time points (the 4th, 8th and 12th hour), and frozen sections and paraffin wax sections about 6μm-thick were made. IV liquid emulsion was used for microradioautography.

## RESULTS

### Diameter of NP

On the 5-time enlarged photograph (diagram 1) of TEM, the average arithmetic mean diameter of 300 detected NPs was 166 nm±33 nm, 8% < 130 nm, 36% 130 nm - 150 nm, 42% 150 nm - 170 nm and 14% >180nm.

### Encapsulation efficiency

After two batches of 5-FU NPs were hydrolyzed in acid medium, their encapsulation efficiency was calculated (Table 1).

**Table 1 Encapsulation efficiency of 5-FU in NP**

| Weight of drug added (g) | *A* value | Weight of drug encapsulated (g) | Encapsulation efficiency (%) |
|---|---|---|---|
| 0.6162 | 0.679 | 0.208 | 33.8 |
| 0.6113 | 0.625 | 0.193 | 31.6 |
| 0.5917 | 0.676 | 0.195 | 32.9 |

### Rate of recovery

The rate of recovery of three batches of samples is shown in Table 2.

**Table 2 Rate of recovery of 5-FU**

| Weight of 5-FU added (g) | Weight of 5-FU determined (g) | Rate of recovery (%) |
|---|---|---|
| 0.1348 | 0.1326 | 98.4 |
| 0.1463 | 0.1449 | 99.1 |
| 0.1429 | 0.1395 | 97.6 |

### *Varitation of drug releasing ratio of 5-FU NP before and after storage*

The cumulative ratio of drug release within 72 h decreased from 84.8% before storage to 80.6% after storage under 40℃±1℃ for three months.

### *The pharmacokinetics of $^{125}$I sodium alginate-BAS NP in rats*

Thirty min after the rats administrated $^{125}$I sodium alginate-BSA NP orally, obvious radioactivity was shown in its blood. Eight hours after the administration, the radioactivity of their liver, spleen and lungs was 2.08 times, 2.32 times and 1.60 times, as much as that of blood, while 24 hours after the administration, they decreased to 1.18, 1.22 and 0.87 times respectively (Table 3).

The data in the parentheses show the ratio of radioactivity of the relative organ to that of blood. The data in Table 3 were put into computer, and processed according to the two-department model to calculate the relevant pharmacokinetical parameters. The results are shown in Table 4.

### *Radioautography*

The microradioautographical experiment indicated that the $^{125}$I sodium alginate-BSA NP were mainly distributed in the Kupffer cells of liver and liver parenchymal cells (Figure 2) after oral administration to rats. And there were Ag particles in the phagocytes of spleen (Figure 3) and also in the pulmonary cells (Figure 4).

**Table 3 Distribution of $^{125}$I sodium alginate-BSA NP in rats (*n* = 3, dosage $148\times10^8$ Bq per rat, po.)**

| Organ | Value of radioactive counting of 100mg tissue in 30s at different time points | | | | | |
|---|---|---|---|---|---|---|
| | 1h | 4h | 8h | 16h | 24h | 36h |
| Blood | 502±100 | 601±135 | 373±22 | 298±19 | 191±17 | 129±23 |
| Heart | 436±110(0.86) | 801±66(0.33) | 265±23(0.71) | 230±23(0.77) | 190±10(0.99) | 87±35(0.67) |
| Liver | 436±110(0.86) | 908±100(1.51) | 777±69(2.08) | 405±97(1.36) | 226±16(1.18) | 153±21(1.19) |
| Spleen | 663±140(1.32) | 1029±168(1.71) | 834±272(2.32) | 436±195(1.46) | 233±25(1.22) | 169±27(0.95) |
| Lungs | 717±314(1.42) | 818±172(1.36) | 597±121(1.60) | 443±31(1.49) | 166±4(0.87) | 119±27(0.92) |
| Kidneys | 526±139(1.05) | 185±39(0.14) | 401±71(1.08) | 387±105(1.30) | 133±13(0.69) | 123±14(0.95) |
| Brain | 105±31(0.21) | 112±49(0.19) | 111±47(0.30) | 96±27(0.32) | 50±5(0.26) | 39±11(0.30) |
| Stomach | 89654±7143 | 28674±3647 | 8362±1902 | 4641±1400 | 698±34 | 527±114 |
| Intestine | 765±137 | 5573±891 | 30±599 | 403±118 | 115±64 | 204±46 |
| Bowel | 1256±664 | 639±1023 | 30±599 | 3221±863 | 125±60 | 256±102 |

**Table 4 Pharmacolinetical parameters of $^{125}$I sodium alginate-BSA NP in rats**

| Parameter | Unit | Value |
|---|---|---|
| *A* | Bq | $3022\times10^2$ |
| α | 1/h | 0.07 |
| B | Bq | $3.03\times10^3$ |
| β | 1/h | -0.11 |
| Ka | 1/h | 1.46 |
| V/F | Bq | $1428\times10^3$ |
| $T_{1/2}\alpha$ | h | 9.42 |
| $T_{1/2}\beta$ | h | -6.07 |
| $T_{1/2}Ka$ | h | 0.47 |
| $K_{21}$ | 1/h | -0.11 |
| $K_{10}$ | 1/h | 0.07 |
| $K_{12}$ | 1/h | -0.00035 |
| AUC | Bq×h | 3.9×109 |
| CL(s) | Bq×h/Bq | $105\times10^3$ |
| *T*(peak) | h | 2.18 |
| Cmax | Bq | $2.45\times10^7$ |

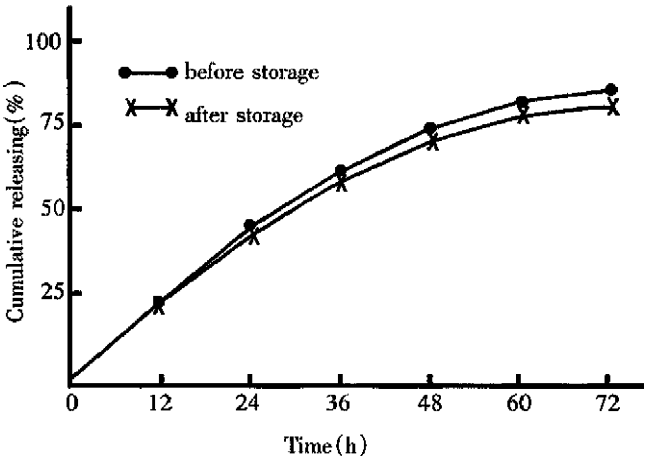


**Figure 1** Releasing of 5-FU from NP.

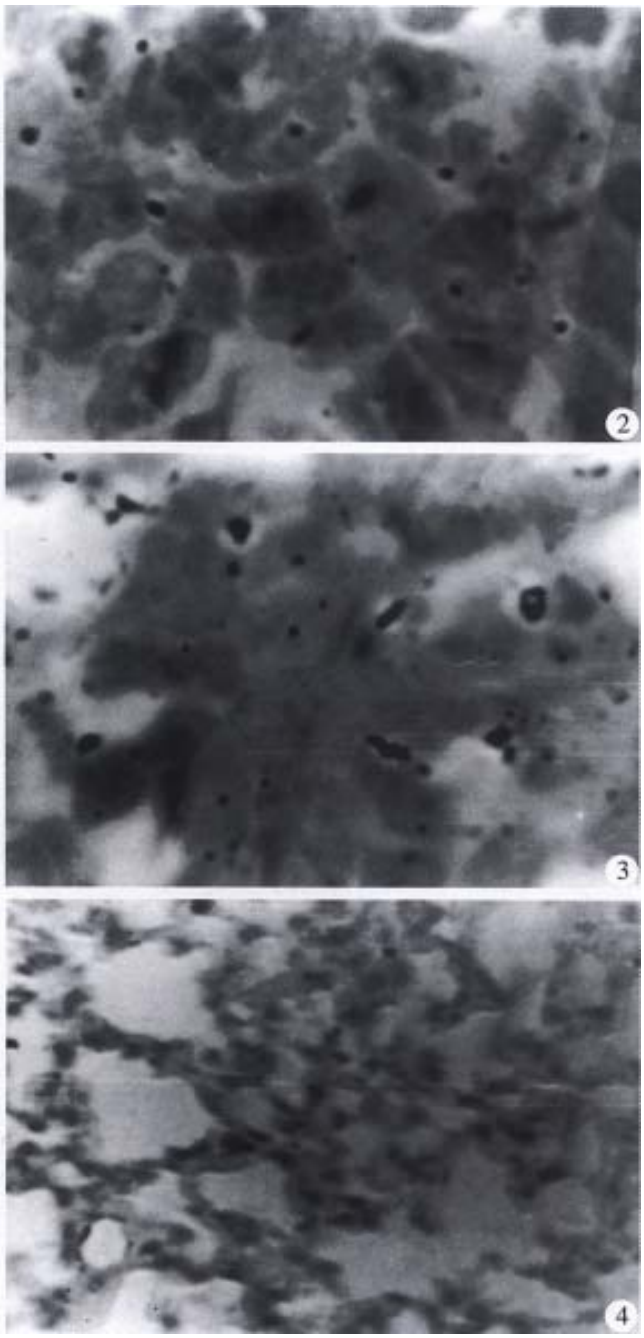

**Figure 2** NP in liver. ×1000
**Figure 3** NP in spleen. ×1000
**Figure 4** NP in lungs. ×1000

## DISCUSSION

Since the introduction of NP in the early 80s, scientists have done a lot of researches in its preparation, stability, distribution and targeting. It is suggested that the stability of NP is better than that of liposome and it can carry more drug than liposome.

At present, the materials, which are reported to prepare NP in literature abroad, are all man-made synthetic materials. While NP is mostly administrated in travenously, we chose natural polysaccharide and protein to compose the carrier, and observed its distribution and metabolism after oral administration to rats.

Thirty minutes after the rats administrated $^{125}I$ sodium alginate-BSA NP orally, the radioactive substances in their blood were detected.

The results indicate that NP is mainly distributed in the liver, spleen and lungs, quantitatively in heart and slightly in brain. This shows NP is difficult in passing through the blook-cerebral barrier. The experiments also display that the $^{125}I$ sodium alginate-BSA NP enters the body-circulation by gastrointestinal passage and is chiefly distributed in the tissues rich in phagocytes, such as in the liver and spleen.

In the study of its distribution, we discovered that there is still a large amount of radioactivity in the gastrointestinal passage 12 hours after the administration. This illustrates that the absorption of NP is not complete because NP exists in the suspension as cloudy polymer but not mono-dispersion, and the large diameter of the polymer makes it difficult for the NP to be absorbed by the gastrointestinal passage.

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*Combined Traditional and Modern Medicine*

# Plasma L-ENK, AVP, ANP and serum gastrin in patients with syndrome of Liver-Qi-stagnation *

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**Subject headings** Syndrome of Liver-Qi stagnation; leucine enkephalin/ blood; arginine vasopressin/ blood; atrial natural polypeptide/ blood; gastrin/blood

## Abstract

**AIM To investigate the pathophysiologic basis of syndrome of Liver-Qi stagnation and parameters for clinical differentiation.**

**METHODS Plasma L-ENK, AVP, ANP and serum gastrin were determined by RIA in 84 patients with neurasthenia, mastodynia, chronic gastritis, and chronic cholecystitis presenting the same syndrome of Liver-Qi stagnation in tr aditional Chinese medicine (TCM). Healthy subjects served as controls in comparison with patients having the same syndrome but with different diseases.**

**RESULTS Among the patients with Liver-Qi stagnation, the plasma L-ENK, ANP and gastrin levels were 38.83 ng/L ± 6.32 ng/L, 104.11 ng/L ± 29.01 ng/L and 32.20 ng/L ± 6.68ng/L, being significantly lower than those in the healthy controls ($P<0.01$, $t = 3.34, 6.17, 4.48$). The plasma AVP of the patient group (52.82ng/L ± 19.09ng/L) was significantly higher than that of the healthy contr ols ($P< 0.01$, $t = 5.79$). The above changes in patients having the same symptom complex but different diseases entities showed no significant differences, $P>0.05$.**

**CONCLUSION The syndrome of Liver-Qi stagnation is closely related to the emotional modulatory abnormality of the brain, with decrease of plasma L-ENK, ANP and gastrin, and increase of plasma AVP as the important pathophysiologic basis.**

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## INTRODUCTION

Liver-Qi stagnation syndrome is common in liver disease. The predominant clinical manifestations were characterized by emotional alterations and digestive disturbances. In order to explore the pathophysiological basis and indexes of differentiation, we investigated the alterations of neurohumoral parameters in association with modulating emotional and digestive function.

## MATERIALS AND METHODS

### *Subjects*

Fifty-four patients with Liver-Qi stagnation syndrome selected from Xiangya Hospital and several general hospitals of Hunan Province from May 1995 to October 1996 were examined for plasma L-ENK, AVP and ANP. Seven were males and 47 females. Age ranged from 14-60 years, averaging 39±11.2 years. The diagnoses were mastod ynia (19), neurasthenia (17), chronic gastritis (10), and chronic cholecystitis (8). Another 30 patients selected from Xiangya Hospital through March 1997 to October 1997 were added, they were all females, aged 20-53 years, averaging 34.7±12.7 years. Mastodynia was diagnosed in eleven cases, neurasthenia in nine cases, chronic gastritis in seven, and chronic cholecystitis in three.

The healthy controls were selected from the blood donors and employees in our hospital, all of them were negative in physical examination, blood routine, liver function test and chest fluoroscopy to rule out organic disease of heart, liver, lung, kidney, and nervous system diseases. L-ENK, ANP and AVP were detected in 30 cases, whereas serum gastrin was examined in 33 cases.

### *Differentiation and diagnosis by TCM*

Based on previous criteria derived by our department[1], they were modified according to the clinical epidemiological survey as: hypochondrial, breast and lower abdominal pain; depression; restless and easily irritated; obstruction sensation at the pharynx; dysmenorrhea, amenorrhea, or irregular menstrual cycle; and the tense pulse. The patients presented with 3 or more of the above six items were considered to have Liver-Qi stagnation syndrome. (The disease entities selected including mastodynia, neurathenia, chronic gastritis, chronic cholecystitis

were diagnosed according to the textbook listed criteria and diagnosed by the departments of breast disease, neurology, general surgery, and digestive medicine).

### Laboratory methods

All the 4 parameters were determined by radioimmunoassay. The apparatus used was FJ-2008 P-type gamma immunocounter. Venous blood samples were collected during fasting in the morning and determined subsequently. Radioimmunoassay kits for L-ENK and AVP were provided by the Department of Neurobiology of the 2nd Military Medical University, and the ANP and gastrin immunoassay kits were provided by Northern Institute of Immunoreagents, Beijing. The standard curve for L-ENK was $r$ = 0.998, CV = 3.07%, RER 0.039; for ANP, $r$ = 0.998, CV = 7.15%, RER = 0.035; for AVP, $r$ = 0.998, CV = 3.15%, RER 0.039; and for gastrin, $r$ = 1.000, CV = 3.18%, RER 0.038; all fitted the quality control criteria.

### Statistical analysis

All data were expressed as $\bar{x}\pm s$. Student's $t$ test and $F$ tes $t$ were used for comparison between groups 2 and 3.

## RESULTS

Table 1 lists the results of measurement of plasma L-ENK, AVP, ANP and serum gastrin of the Liver-Qi stagnant patients group (LQSP) and of the normal controls and Table 2 shows the results of mesurements in patients with different diseas e entities.

**Table 1 The plasma L-ENK, AVP, ANP and serum gastrin levels in Liver Qi stagnation patients**

| Group | L-ENK | AVP | ANP | Gastrin |
|---|---|---|---|---|
| LQSP | 38.83±6.32(40)[b] | 52.82±19.09(30)[b] | 104.11±29.01(32)[b] | 32.20±6.68(30)[b] |
| Control | 45.19±9.58(30) | 29.88±10.35(30) | 149.50±28.89(30) | 47.02±15.64(30) |

In brackets are the number of cases; [b]$P$<0.01, *vs* control.

**Table 2 The plasma and serum neurohumeral parameters of patients with Liver Qi stagnantion syndrome of different disease entities**

| Group | L-ENK | AVP | ANP | Gastrin |
|---|---|---|---|---|
| Neurathenia | 40.72±17.18(14) | 42.95±1078(8) | 110.24±39.40(10) | 31.03±7.79(9) |
| Mastodynia | 36.40±21.12(10) | 61.31±23.57(13) | 104.73±34.43(13) | 33.49±7.08(11) |
| Gas-Chole* | 40.87±26.02(16) | 49.32±12.85(9) | 99.83±20.15(9) | 31.24±4.52(10) |
| $F$ value | 0.15 | 2.28 | 0.24 | 0.40 |
| $P$ | >0.05 | >0.05 | >0.05 | >0.05 |

Note: in the blackets are the number of cases; *Gas-Chole stands for chronic gastritis and chronic cholecystitis.

## DISCUSSION

In TCM, the function of liver is mainly dredging and storing of blood. The dredging function of the liver is extremely important in regulating the flow of Qi and blood in the body, the psychoemotion, digestion and absorption, water and fluid metabolism, menstruation and reproductive function. Psycho-emotional disturbance is the main cause of Liver-Qi stagnation, and in turn, psychoemotional change is the clinical feature of Liver-Qi stagnation, such as easily irritated and arousal of angry, depression and sighing, insomnia with nightmares, even suspicious and indifferent and grieving. RIA of the psychoemotional and digestive function related neurohumoral parameters in this series showed that among the Liver-Qi stagnantion patients, plasma L-ENK, ANP and serum gastrin were significant ly decreased and the plasma AVP increased as compared with those of the normal controls. However, between the different disease entities of various organ systems in modern Western medicine observed no significant difference. It strongly indicates that these alterations are the pathophysiological basis of Liver-Qi stagnation in common. These parameters are the indexes of syndrome, rather than the indexes of the disease entity. This study preliminarily explored the relationship between Liver-Qi stagnation syndrome and the modulatory neurohumeral factors associated with psychoemotional changes.

L-ENK is an active neuropeptide, its secretory neurons are distributed in thes triatal body thalamus, periaqua duct gray matter, and dorsal glial region of spinal cord, and are the modulators of emotional activity within the central nervo us system[2]. Gastrin is a gastrointestinal hormone secreted by G cells distributed in pylorus and upper duodenum.

Recent study reveals that some of the gastrointestinal peptide is also situated in CNS, the dual distribution of these peptides were called as brain enteric peptide[3]. Immunohistochemical studies demonstrated that gastrin and leucine enkephalin are also presented in the brain, stomach, intestine and pancreas[4]. Enkephalin is extensively distributed in the central nervous system and the digestive tract, and its secr etory cells coincided with gastrin secretory cells[5]. In this study, both plasma L-ENK and serum gastrin were sigificantly decreased in the patients with Liver-Qi stagnant syndrome, which might play an important role in the pathogenesis of psychoemotional modulation disorder and result in unstable emotional activity. Since the brain-enteric peptide secretion and release are closely related with the functional status of CNS and the vegetable nervous system, decreased brain-enteric peptide during Liver-Qi stagnation would certainly affect the gastric acid secretion, the intestinal, pancreatic juice, bile secretion and motility of the digestive tract, ultimately leading to digestive disturbance.

Vasopression, the antidiuretic hormone (ADH), is synthesized by the neurons of supraoptic nucleus of hypothalamus. Release of ADH is normally modulated by plasma osmotic pressure, blood volume and blood pressure; but pain, vomiting and emotional tension may promote ADH release, and antagonize the diuresis[4].

Atrial natriuretic polypeptide is mainly distributed in the brain, which is high in the hypothalamus and the diaphragmatic sellae region. Its secretion is influenced by physical, humoral and neural factors. Its functions include natiuresis, vasodilation, and decrease of blood pressiure[6]. It was reported that ANP significantly inhibited the release of AVP via hypothalamus neuropituitary axis, the antagonistic effect of ANP and renin-angiotensin not only existed peripherally, but also in the CNS, particularly in regulating the blood volume, electrolyte balance and maintenance of blood pressure[7]. Decrease of plasma ANP and elevation of AVP might be one of the pathophysiologic basis of the CNS regulatory dysfunction resulting in increased vascular tension, sodium and water retention, and hypertension in Liver-Qi stagnation syndrome. But the cause-effect relationship, the precise mechanism of the elevated AVP and decreased ANP, as well as the validity of the four neurohumeral parameters in this study as reference indexes in differentiation of the Liver-Qi stagnant syndrome await further clarification.

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***Review***

# Current medical therapy for ulcerative colitis

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**Subjects heading** colitis, ulcerative/drug therapy; inflammat ory bowel diseases/drug therapy; cyclosporin; glucocorticosteroids; sulphasalazine 5-aminosalicylic acids

## INTRODUCTION

In recent years, the advances in therapy of ulcerative colitis (UC) have been characterized mainly by the more extensive use of immunosuppression. Cyclosporin (CSA) may become a drug of choice to treat severe UC, but its long-term effect is insufficient. Topically, glucocorticosteroids (GCS) are hopeful in right ileocolonic UC, but no action for maintenance therapy[1-3]. The most significant development in recent years is the introduction of immunomodulatory treatments using cytokines and anticytokines. Immunomodulation therapy creates great expectations since early reset of the immunostat might be able to control inflammation in a long term. Current treatment strategies are anti-inflammatory and to modulate the immune response. Standard therapies with sulphasalazine (SAS) or 5-aminosalicylic acids ( 5-ASA, mesalazine or mesalamine), GCS and antibiotics yield a fair immediate success, but long-term response to these therapies is poor. The greatest advance has been the introduction of immunosuppressive strategies. The indexes like the clinical activity index (CAI) proposed by Rachmilewitz[1], although useful, have not received general acknowledgement.

Patients with an inflammatory bowel disease (IBD), such as UC or Crohn's disease, have recurrent symptoms with high morbidity. Mild disease requires only sympt omatic relief and dietary manipulation. Mild to moderate disease can be managed with 5-ASA, including olsalazine and mesalamine. Mesalamine enemas and suppositories are useful in treating proctosigmoiditis. Corticosteroids are beneficial in patients with more severe symptoms, but side effects limit their use, particularly for chronic therapy. Immunosuppressant therapy may be considered in patients with refractory disease that is not amenable to surgery. IBD in pregnant women can be managed with 5-ASA and corticosteroids[2]. Since longstanding IBD is associated with an increased risk of colon cancer, periodic colonoscopy is warranted.

Since lesions in UC are quite diffuse and uniform endoscopic indexes used are quite straightforward, clinical activity, endoscopic activity and histology show a reasonable correlation and it is useful to monitor disease activity also with flexible proctosigmoidoscopy. The persistence of active inflammatory lesions at histology in the presence of endoscopic remission predicts relapse. Bresci G *et al*[3] reported that the activity of the disease was evaluated by a Clinical Activity Index and an Endoscopic Index. Of 112 cases of UC observed, 95 showed no change in extent and were studied as examples of non-progressive UC, and in this group the extension of the disease was: pancolitis in 19%, left sided colitis in 39%, proctosigmoiditis in 17% and proctitis in 25%. A colectomy had to be performed in 5%. None of the enrolled cases developed a cancer during the follow up. The patients with ulcerative pancolitis or left-sided colitis were treated with 5-ASA-1.6g/d in a delayed-release formulation, while the patients with proctosigmoiditis or proctitis were treated with 5-ASA enem as 4g/d. The patients with more than one relapse/ year accounted for 39%. The proportion of patients with only one relapse/ year was 53%. The patients with steady remission for all the seven years of the trial were only 8%, but with a statistically significant difference between the groups with initial diagnosis of proctosigmoiditis or proctitis and the group with initial diagnosis of pancolitis or left-sided colitis (12% *vs* 5%). Among the patients with continuous remission, 37% showed colonic alterations, with an endoscopic score higher than 4 but a clinical score less than 6. Side effects were observed in 6% of patients but without treatment withdrawal. Non-progressive UC throughout the colon has a relatively good prognosis, which seems to be independent of the location of the disease, even if Bresci G *et al*[3] have found a statistically significant higher percentage of patients with steady remission among the patients with more distal diseases.

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## CURRENT TREATMENT OF ULCERATIVE COLITIS

Ulcerative colitis is a mucosal disease and therefore well suited for treatment in most instances with topically acting drugs at the level of the colonic mucosa. UC is controlled mainly using GCS and 5-ASA.

Ardizzone *et al*[4] had reviewed the role of corticosteroids, ASA and mesalazine ( 5-ASA, mesalamine), immunosuppressive agents and alternative nove l drugs for the treatment of distal UC. Short cycles of traditional, rectally administered corticosteroids ( methylprednisolone, betamethasone, and hydrocortisone) are effective for the treatment of mild to moderately active distal UC. In this context, their systemic administration is limited to patients who are refractory to either oral 5-ASA, topical mesalazine or topical corticosteroids. Of no value in maintaining remission, the long-term use of either systemic or topical corticosteroids may be hazardous. A new class of topically acting corticosteroids [budesonide, fluticasone, beclomethasone dipropionate, prednisolone -21- methasulphobenzoate, tixocortol (tixocortol pivalate)] represents a valid alternative for the treatment of active UC, and may be useful for refractory distal UC. Although there is controversy concerning dosage or duration of therapy, oral and topical mesalazine is effective in the treatment of mild to moderately active distal UC. Sulfasalazine and mesalazine remain the first-choice drugs for the maintenance therapy of distal UC. Evidence shows a trend to a higher remission rate with higher doses of oral mesalazine. Topical mesalazine (suppositories or enemas) is also effective in maintenance treatment. For patients with chronically active or corticosteroid-dependent disease, azathioprine and mercap topurine are effective in reducing either the need for corticosteroids or clinical relapses. Moreover, they are effective for long-term maintenance remission. CSA may be useful in inducing remission in patients with acutely severe disease that does not achieve remission with an intensive intravenous regimen. Existing data suggest that azathioprine and mercaptopurine may be effective in prolonging remission in these patients. The role of alternative drugs in the treatment of distal UC and its different forms are reviewed. In particular data are reported concerning the effectiveness of 5-lipoxygenase inhibitors, topical use of short chain fatty acids, nicotine, local anesthetics, bismuth subsalicylate enema, sucralfate, clonidine, free radical scavengers, heparin and hydroxychloroquine. The management of patients with acute severe UC requires careful in-hospital assessment of the patient and the coordinated treatment of a team of experienced gast roenterologists and surgeons. Complete understanding of the potential complications and their management, especially toxic megacolon, is essential.

The current medical arsenal advocates a standardized approach to management that includes continuous, high dose iv hydrocortisone, more aggressive use of topical steroids as well as feeding the patients and continuing (but not initiating) oral 5-ASA agents was reviewed[5]. For those patients whose disease proves refractory to iv steroids, iv. CSA (with an acute response rate of 82%) is an essential component in the medical management of these patients. Antibiotics should be used only when specifically indicated. Total parental nutrition has not been shown to be helpful in the acute setting. Air contrast barium enema and colonoscopy have been used to predict response but may be dangerous diagnostic modalities in these acutely ill patients and are not better than good clinical judgement. Marion *et al*[5] review and advocate long-term management of acute response using 6-mercaptopurine or azathioprine. The surgical experience and the postoperative complications of the ileal pouch anal anastomosis, which include acute pouchitis in 50% - 60%, chronic pouchitis in 5%-10% and recent reports of dysphasia among patients with chronic pouchitis, must be considered before colectomy is advised. Over 80% of patients with acute severe colitis can be spared colectomy using the current arsenal of medical therapies.

The inhibited release of 5-lipoxygenase products may account for some of the anti-inflammatory effects of ropivacaine seen in the treatment of UC[6]. Prompt diagnosis and exclusion of infection require a minimum of rigid sigmoido scopy, rectal mucosal biopsy and stool culture. Admission to hospital is mandatory for patients with features of severe disease, or who are in their first attack of UC and have bloody diarrhea, even if the criteria for severe disease are not met. Once admitted, plain abdominal X-ray, full blood count, and serum albumin and C reactive protein should be used to monitor the patients on alternate days; temperature and pulse rate should be recorded four times per day. Treatment should be instituted as soon as the diagnosis is made with an intravenous corticosteroid (hydrocortisone 100mg iv. four times daily or equivalent). Antibiotics may be included if infection cannot be confidently excluded. Free diet is allowed but attention should be given to nutritional, fluid and electrolyte status with intravenous replacement if necessary. Any evidence of colonic dilatation occurring despite maximal therapy should be regarded as an absolute indication for colectomy. The patient should be kept fully informed from an early stage about the

likely natural history of the condition and about the possible therapeutic options including surgery. CSA therapy should be reserved for patients who have a poor response to the first 3d-4d of corticosteroid therapy, particularly those with serum C reactive protein >45mg/L and who do not yet have absolute indications for colectomy. Most patients who have not convincingly responded within 10 days of starting medical therapy should undergo colectomy, although some responders who are febrile may reasonably continue for up to 14 days before a final decision. Approximately 30%-40% of patients with severe colitis will need colectomy within the first 6 months. With optimal management, mortality can be zero, but better medical therapies are urgently needed to reduce the colectomy rate[7].

Finnie *et al*[8] speculated that corticosteroids might cause beneficial stimulation of mucus synthesis, since this is a known action of carbenoxolone, a corticosteroid itself, and has also been proposed as a possible mechanism for the protective effect of smoking on UC. We have therefore compared the effects of corticosteroids including carbenoxolone, and nicotine on mucin synthesis, assessed by incorporation of N-[3H] acetylglucosamine into mucin by colonic epithelial biopsies in culture. In histologically normal biopsies from the left colon, hydrocortisone and prednisolone caused a very marked concentration-dependent increase in mucin synthesis, with maximal effect at 6 nmol/L ($P < 0.001$) and 1.5 nmol/L ($P<0.001$) respectively. The maximal effect of hydrocortisone was significantly greater than that of prednisolone ($P < 0.05$). Carbenoxolone, 0.17 mmol/ L, also increased mucin synthesis in the left colon [$P < 0.05$, $n = 15$ (three patients)]. In contrast, these corticosteroids caused only a small, non-significant increase in mucin synthesis in the histologically normal right colon; fludrocortisone, 2 nmol/L and 20 nmol/L, and aldosterone, 0.1 nmol/L - 10 nmol/L had no effect. Nicotine significantly increased mucin synthesis between 62.5 nmol/L and 6.25 nmol/L ($P < 0.05$ at all concentrations) in both the right and left colon. In biopsies from the relatively uninvolved right colon of patients with UC, corticosteroids and nicotine caused relatively smaller increases in mucin synthesis. The marked stimulation of mucin synthesis by corticosteroids suggests that this may account, at least in part, for their therapeutic effect in UC.

GCS act by binding to the GCS-R (glucocorticosteroid receptor). The activated receptor assembles to a dimmer that is transported in the nucleus of the cell where it binds to DNA and acts *via* enhanced or reduced transcription, reduced translation and breakdown of DNA. GCS have a very extensive anti-inflammatory and immunosuppressive action. Since the receptor is the same for all body cells, GCS in the circulation have many systemic effects. Many actions have been described for the aminosalicylates[9]. SAS and 5-ASA inhibit the production of cycloxygenase, thromboxane synthetase, platelet-activating-factor synthetase, and IL-1 by macrophages and can decrease immunoglobulin production by plasma cells. Both SAS and 5-ASA inhibit the production of reactive oxygen species and scavenge reactive oxygen metabolites. 5-ASA lacks the antibacterial effect of SAS.

## TREATMENT OF ACTIVE ULCERATIVE COLITIS

The two variables determining the therapeutic approach in UC are disease extent and disease severity (Table 1).

Effective medical treatment of UC is available. However, 20% - 40% of patients remains refractory and become steroid dependent or chronic active. Azathioprine and its metabolite 6-mercaptopurine have been found effective in this setting, although duration of treatment and doses are not entirely clear. Methotrexate has no definitive part in the treatment of refractory colitis. Iv. CSA induces remission in a considerable number of patients; follow-up treatment is, however, not defined. This approach may be useful for elective surgery. A number of other treatments have been proposed including chloroquine, interferons and anti-cytokines. None of these can currently be recommended for clinical practice. Anti-inflammatory cytokines such as IL-10 may be good candidates[5,10].

In the presence of proctitis or distal colitis, a topical approach should always be the first choice. To control active distal disease rectal 5-ASA is at least as effective as rectal GCS[11]. In patients with left-sided colitis, enemas are the best choice because of the retrograde spread up to the splenicflexure. GCS enemas have been used for a long time in the treatment of distal UC. Prednisolone (20 mg - 30 mg), hydrocortisone (100 mg-125 mg ), and betamethasone (5 mg) have all been shown to be effective. To minimize side effects, poorly absorbable GCS have been used for enema therapy including hydrocortisone foam and prednisolone metasulphobenzoate or molecules with increased first-pass metabolisation in the liver, e.g., betamethasone dipropionate and tixocortol pivalate. Budesonide enemas carry almost no systemic effects because of a very high first-pass effect. Doses of 2 mg are as effective as 20 mg - 30 mg of prednisone. Repeated therapy courses with budesonide enemas have been found safe without suppression of the HPA axis[5,12].

CSA has been proposed in the management of patients with acute UC in whom standard therapy failed and who were candidates for colectomy[13]. Seven academic hospitals contributed to this retrospective study which included 29 patients (median age: 33 years, 12 females and 17 males). The median duration of the disease was 4 years. For the responders, maintenance therapy included tapering dose of steroids ($n$ = 12), azathioprine ($n$ = 12), 5-ASA or salazopyrine ($n$ = 10), methotrexate ($n$ = 1) or oral CSA ($n$ = 11). The median duration of follow-up was 12 months (4 to 48 months). Among the 20 responders, 7 were subsequently referred for colectomy either selectively ($n$ = 3) or because of recurrence o f the disease ($n$ = 4). Among the 12 patients treated by azathioprine as a maintenance therapy, only 3 (25%) had to be referred for surgery. Among the 8 patients who did not receive azathioprine, 4 (50%) were subsequently subject to a colectomy (NS). In patients with acute refractory UC who received CSA, the short-term efficacy (avoidance of immediate colectomy) was obtained in 20 (69%) out of 29 patients. However, after a median follow-up of 12 months, only 13 (45%) patients were colectomy free.

Refractory distal colitis is a difficult medical problem and is defined as active distal inflammation unresponsive within 4 wk - 6 wk to a topical treat ment with 5-ASA or corticosteroids associated with oral salicylates or sulphasa lazine[6,7]. Although there is little controlled evidence, it is logical to increase the dose of the topically administered drug or to continue the drug for a longer time. A further step is to switch drugs. All clinicians have experi ence with patients in whom proctitis not responsive to 5-ASA responded to GCS enemas and *vice versa*. Another valuable approach seems to combine 5-ASA and GCS in one enema. Frequently, patients with refractory distal disease do not respond even to oral therapy with GCS and a rectal drip of GCS over several hours together with administration of antidiarrheals in hospital may be necessary or iv administration of high doses of GCS. Active disease extending beyond the rectum necessitates oral therapy. In mild-to-moderate disease oral SAS or 5-ASA formulations in high doses can be used or a combined approach of oral 5-ASA and topical 5-ASA. Many physicians prefer this approach because of the low incidence of side effects and the reluctance of the patients to take GCS[4-6].

Oral salicylates at doses of over 2g have been shown to be more effective than placebo to control mild-to-moderate attacks of UC[14]. There prob ably is a dose-response effect with doses up to 3.8g being increasingly more effective, but this was not demonstrated in all studies. 5-ASA was not more effective than SAS and was beneficial especially to the SAS-sensitive patient. Recent data[15] have shown that balsalazide is more effective and better tolerated than mesalamine in the treatment of active UC. Patients taking balsalazide not only experienced more asymptomatic days and achieved the first asymptomatic day more rapidly but with side effects. Differences were highly significant.

Improvement of symptoms of UC with 5-ASA may be slow and overall 5-ASA or sulphasalazine are certainly less effective to control active UC than GCS. There is a dose effect for oral GCS, 40 mg prednisolone daily being more effective than 20 mg; while 60 mg offers little extra benefit but is associated with a considerable increase in side effects[3,4].

Severe UC is defined using the criteria of Tnielove and Witts[16] as six or more bloody stools in a patient with fever, tachycardia, hypoalbuminemia and raised ESR. These patients will mostly be admitted to hospital to receive a continuous iv infusion of GCS. In severe left-sided or extensive disease GCS are mandatory and combined therapy with 5-ASA is probably not more efficaci ous than GCS alone. As soon as symptoms are controlled, tapering of the GCS can be started but proctoscopic monitoring of disease activity is valuable.

Recently parameters predictive of outcome of severe colitis under 3 days of intravenous glucocorticosteroids have been revised[17]. The need for colectomy was predicted in 85% of the patients on the basis of the presence of eight or more stools per 24 h or 4-5 stools per 24 h together with C-reactive protein >45mg/L. Based on these criteria one could make the decision to introduce intravenous CSA or to decide for colectomy. In patients who deteri orate or are admitted with toxic colonic dilatation, immediate colectomy has to be performed. If a pouch-anal anastomosis is constructed, a temporary diversion ileostomy is indicated. Many surgeons' three-step procedures, i.e . first colectomy with closure of a short rectal stump, subsequent construction of an ileoanal pouch with temporary ileostomy and finally closure of the stoma. The side effects of CSA are multiple (Table 2) and opportunistic infections by pneumocystis and cytomegalovirus may be life-threatening. These complications were encountered especially in elderly patients treated with long-term CSA and GCS. Anoth er serious side effect is epileptiform fits due to the CSA hydrophobic vehicle. Patients with lowered serum cholesterol or magnesium should not receive CSA.

**Table 1 Treatment of active ulcerative colitis**

| Severity | Extent | | |
|---|---|---|---|
| | Distal | Left-sided | Extensive |
| Mild | Topical GCS or 5-ASA | Topical GCS or 5-ASA +oral 5-ASA | Oral 5-ASA (+topical therapy) |
| Moderate/severe | Topical GCS or 5-ASA (+oral 5-ASA?) | Oral GCS | Oral or GCS iv |
| Refractory | Increase dose and duration | GCS iv+CSA | GCS iv+CSA |
| | Switch enemas | Surgery | Surgery |
| | Combine topical GCS and 5-ASA | | |
| | Oral GCS | | |
| | Others | | |

**Table 2 Adverse events reported with use of cyclosporin (iv+oral) in IBD[18]**

| Type of side effect | % | Type of side effect | % |
|---|---|---|---|
| Paresthesias | 26 | Headache | 5 |
| Miscellaneous | 13 | Infection | 3 |
| Hypertrichosis | 13 | Hepatotoxicity | 3 |
| Hypertension | 11 | Gingival hyperplasia | 2 |
| Tremor | 7 | Seizure | 1 |
| Nausea/vomiting | 6 | Anaphylaxis | 0.3 |
| Renal insufficiency | 6 | Side effects/patient | 0.94 |

**Table 3 Major side effects of glucocorticosteroids**

| | Short-term and long-term therapy | Long-term therapy |
|---|---|---|
| CNS | Pseudotumor cerebri | |
| | Psychosis | |
| Musculoskeletal | Myopathy | Osteoporosis |
| | Aseptic necrosis | |
| Ocular | Graucoma | Cataracts |
| Gastrointestinal | Ulcer-pancreatitis | |
| Cardiovascular | Hypertension | |
| | Fluid retention | |
| Endocrinological | | Permanent suppression of HPA-axis |
| | | Growth failure |
| Metabolic | Hyperglycemia | Fatty liver |
| | Hyperosmolar state | Hypokalemia |
| | Hyperlipidemia | |
| Skin | Acne, ecchymosis | Striae, atrophy, wound |
| | | Infection |
| | | Cushingoid fat |
| | | Distribution |

**Table 4 Immunosuppressives used in inflammatory bowel diseases**

| Drug | Mode of action | Mechanism of action |
|---|---|---|
| AZT/6-MP | Inhibition of ribonucleotide sysnthesis | Inhibition of proliferation of T-cell clones |
| Methotrexate | Folic acid inhibitor | Inhibition of T and B-cell |
| | | Function decrease of IL-1 and IL-6 |
| Cyclosporin (CsA | Inhibition of T-cell-receptor-stimulated | Inhibition of IL-2 production and |
| Tacolimua (FK 506) | Transcription of lymphokine genes | IL-2 receptors; inhibition of cytokines(TNFα,IFNγ) |
| Mycophenolate | Inhibition of guanosin nucleotide synthesis | |

**Table 5 Immunomodulation therapy in inflammatory bowel disease**

| | Cytokines | Anticytokines | Antisense nucleotides |
|---|---|---|---|
| Current studies | rhu IL-10, rhu IL-11 | TNF antibodies, inhibitors | ICAM-1 |
| Future studies | | IL-1 antibodies IL-1 ra | NFκB |
| | | IFN-γ antibodies | |
| | | IL-12 antibodies | |

Side effects of drug therapy in patients with refractory colitis result from cumulative toxicity of high-dose iv CSA and GCS. The exact role of CSA in the trea tment of severe colitis needs to be defined and will greatly depend on the long-term outcome of patients treated with this drug. The side effects associated with GCS therapy are important (Table 3). Short-term treatment carries mild side effects in the majority of patients but long-term therapy are sinetunes a ssociated with irreversible complications. In the past years, therefore, attempts have been made to develop GCS with high topical activity lacking the systemic activity of the drug and hence carrying fewer side effects.

Other approaches to therapy of UC are currently under investigation. Transdermal nicotine in doses of 15 mg - 25 mg daily added to conventional therapy was effective against placebo to control active disease[19]. The rationale to use this therapy is clear. Most UC patients are nonsmokers, and patients with a history of smoking usually acquire their disease within a few years after they have stopped smoking[20]. Among patients who continue to smoke, symptoms may improve, suggesting that smoking may have a beneficial effect. Another recent trial confirmed the moderate efficacy of nicotine in the treatment of UC. The use of nicotine as a treatment, however, remains highly controversial. The use of oral ridogrel, a thromboxane synthase inhibitor in UC is currently investigated. A study suggests that there is no benefit of adding azathioprine in patients with chronic stable colitis, whereas the drug is efficacious to maintain UC in remission. The evidence for the use of methotrexate in the treatment of chronic active UC is largely negative. It should be emphasized that UC is a curable disease. Colectomy with ileo-anal pouch anastomosis is a valuable treatment alternative in chronically active or intractable disease.

Rectal treatment with mesalazine enemas is the first-line therapy for distal UC. In order to improve the benefits of rectal therapy, a new 60 mL- 5-ASA rectal gel enema preparation has been developed using a device that excludes direct contact of the inert propellant gas with the active drug[21]. Twelve patients with active UC administered 4 g of the mesalazine rectal enema labelled with 100MBq technetium sulfur colloid (99mTc-SC). Anterior scans of the abdomen were acquired at intervals of 4 hours. Scans were analyzed to evaluate the extent of retrograde flow and homogeneity of distribution of the radiolabe lled enema in the rectum, sigmoid, descending and transverse colon. In addition, plasma levels of 5-ASA and Ac-5-ASA were measured for 6 hours. All patients retained the entire rectal gel throughout the course of the study without adverse events. In 11 (92%) out of 12 patients, the gel had spread homogeneously beyond the sigmoid colon and had reached the upper limit of disease in all cases. The maximum spread (splenic flexure) was observed in 6 (50%) out of 12 patients within the first 2 hours. The systemic absorption of mesalazine and its metabolite Ac-5-ASA were low. The new mesalazine enema represents an adequate alternative and a further technological improvement in the topical treatment of distal UC[20,21].

The choice between sulfasalazine and 5-aminosalicylate (5-ASA) drugs in the management of UC patients often depends on idiosyncrasies of drug tolerance and control of the disease in individual patients. Walker *et al*[22] sought to evaluate whether there were population differences in the effect of 5-ASA and SAS on the occurrence of clinically recognized adverse events. We also attempted to determine whether there were differences in the use of concomitant steroids and in the rates of hospitalization. A large computerized database drawn from general practices in the United Kingdom was reviewed. The 2894 patients who were diagnosed having UC were receiving ongoing medical therapy specific to UC. The period of data availability ran from early 1990 to late 1993. The average duration of observation was 2.1 years per patient. Patient histories were categorized into distinct periods according to the dose of 5-ASA and SAS, steroids, and immunosuppressants, and were further divided based on the UC activity. Within these categories, they examined the initiation and discontinuation of steroids, rate of new hospitalizations for UC, and clinical complaint of adverse events. The results show new clinical mentions of hepatic, pancreatic, renal, and hematological events other than anemia were similar among the 5-ASAs and were very infrequent generally. Hospitalizations for UC occurred with similar frequency (about 15 hospitalizations per 100 patients per year) among users of those drugs. Patients receiving SAS had lower rates of initiation of prednisolone than patients receiving 5-ASA, but SAS was used proportionately less often in patients who had been recently hospitalized, and it may be that SAS patients were somewhat less sick than patients using 5-ASA. The choice of drug did not affect discontinuation rates for prednisolone among established users. In the United Kingdom , during the period of this study, serious adverse reactions to drugs were not an important aspect of the management of patients with UC. Renal and pancreatic complications of SAS and 5-ASA therapy were extremely rare. SAS and 5-ASA drugs have similar steroid-sparing properties. Disease-specific

hospitalizations are approximately 100 times more common in UC patients than serious adverse drug effects. Considerations of drug efficacy should therefore dominate the choice between the rapeutic agents.

The efficacy and safety of 5-ASA suspension enema were compared with oral SAS in patients with active mild to moderate distal UC[23]. Thirty-seven patients were randomly assigned to treatment with either rectal mesalamine, 4 g at night ($n$ = 19) or oral SAS, 1 g four times a day ($n$ = 18) in a 6 week, double blind, double-dummy, parallel-group, multicenter study. A physician-rated Disease Activity Index (DAI), which included symptom evaluations and sigmoidoscopic findings, assessed efficacy, by physician-rated Clinical Global Improvement (CGI) scores, and by Patient Global Improvement (PGI) scores. Adverse event reports, clinical laboratory tests, and physical examination assessed safety. Mean DAI scores indicated significant improvement from baseline in both treatment groups. CGI scores indicated that 94% of the 5-ASA patients were either "very much improved" or "much improved" at wk 6 *vs* 77% of the SAS patients. PGI ratings showed more improvement in the 5-ASA treatment group than in the SAS group at week 2 ($P$ = 0.02) and at 4 week ($P$ = 0.04). Adverse events, primarily headache and nausea, occurred significantly more frequently ($P$ = 0.02) in the SAS than in the 5-ASA group (83% *vs* 42%). Three patients were withdrawn from SAS treatment because of adverse events. Rectally administered 5-ASA is as effective as oral SAS in treatment of active distal UC but is associated with fewer and milder adverse events. Patients treated with 5-ASA reported improvement earlier than those treated with SAS.

Since transdermal nicotine is of value in the treatment of active UC but is often associated with side effects, an alternative in the form of topical therapy with nicotine enemas has been developed. In an open study[24], 22 patients with active colitis, all non-smokers, were asked to take a 100mL enema containing 6mg of nicotine every night for 4 weeks. Pre-trial treatment using mesalazine ($n$ = 16), oral prednisolone (8), cyclosporin (1) and azathioprine (1) was kept constant for the month prior to assessment and during the study period. Symptoms, with stool frequency, were recorded on a diary card and an endoscopy was performed with rectal biopsy at the beginning of the study and after 4 weeks. Seventeen of the 22 patients completed 1 month of treatment. Mean duration of relapse was 29 weeks. Sixteen of 17 improved their St Mark's score. Urgency and stool frequency improved in 12 patients, sigmoidoscopic and histological scores in 10. Three patients had a full remission of symptoms with normal sigmoidoscopy. Six of 10 patients with a partial response continued with the enemas for the second month, and five showed further improvement with full remission in two. The enema appeared effective when added to conventional treatment and produced few side effects. Topical nicotine therapy for UC may have a place in future management, but case control studies are needed[25]. Immunosup pressives used in IBD (Table 4), and immunomodulation therapy in IBD are directed toward suppressing the action of proinflammatory cytokines for enhancing the effects of antiinfl ammatory mediators (Table 5).

## MAINTENANCE THERAPY IN ULCERATIVE COLITIS

Aminosalicylates are used as standard treatment for maintaining remission in UC[26]. As yet, there is no other existing alternative with proven efficacy. In the light of the hypothesis that the intestinal environment may contribute to the pathophysiology of UC, a trial was conducted to test the effects of probioti c treatment with an oral preparation of non-pathogenic *E. coli*. A total of 120 patients with inactive UC were included in a double blind, double-dummy study comparing mesalazine 500mg three times daily. An oral preparation of viable *E. coli* strain Nissle (Serotype 06:K5:H1) for 12 weeks was studied with regard to their efficacy in preventing a relapse of the disease. Study objectives were to assess the equivalence of the clinical activity index (CAI) under the two treatment modalities and to compare relapse rates, relapse free times and global assessment. The start and end scores of the CAI demonstrated no significant difference between the two treatment groups. Relapse rates were 11.3% under mesalazine and 16.0% under *E. coli* Nissle 1917 (NS). Life table analysis showed a relapse-free time of 103 d±4 d for mesalazine and 106 d±5 d for *E. coli* Nissle 1917 (N.S.). Global assessment was similar for both groups. Tolerability to the treatment was excellent. No serious adverse events were reported. From the results of this preliminary study, probiotic treatment appears to offer another option for maintenance therapy of UC. Additional support is provided for the hypothesis of a pathophysiological role for the intestinal environment in UC.

Distal UC can be maintained with a topical approach. Regimens such as alternate-day enema administration has been shown to be effective, while for maintenance, the use of suppositories may be feasible as far as compliance is concerned, and the long-term use of enemas is difficult. The benefit of

maintenance treatment with oral 5-ASA of extensive UC is well established. SAS reduces the relapse rate by fourfold and all 5-ASA formulations have comparable efficacy with fewer side effects. The rate of GI side effects is especially decreased with 5-ASA. The optimal 5-ASA dose may be 2 g. A dose effect was not demonstrated for most 5-ASA formulations and SAS, except for olsalazine[27].

## PROSPECTS

There is overwhelming evidence that genetic factors play a role in the predisposition to develop the chronic IBD[28-30]. The genetic analysis of complex diseases, such as UC, is difficult. The presence of disease heterogeneity, the relative low frequency in the population, the degree to which first-degree rel atives are affected (approximately 10%), the presence of genes with minor genetic effects, and ethnic differences are some of the difficulties encountered in identifying disease susceptibility loci. Two major approaches to identify these genes are being followed at present. The first, family-based, consists of studying linkage analysis in sibling pairs and parental transmission in genome-wide screening using microsatellite markers. These studies are appropriate and helpful for finding genes of major or moderate effects but may be difficult in identifying genes with minor effects; and can be considered in the future in genome-wide screens with technologic advances. The second approach is based on conventional epidemiological designs, population-based studies, using candidate genes in the framework of a biologic hypothesis. Recent data using both approaches in both Crohn's disease and UC are reviewed. The results of genome-wide linkage studies have not reached consensus, but suggest that these diseases are different and polygenic in nature. We have started our studies with the hypothesis that an abnormal immune disbalance contributes to the biologic basis of the disease. Therefore, polymorphisms in genes encoding proinflammatory and regulatory cytokines were studied. Preliminary data of these association studies suggest the importance of several genes with small effects in determining the severity and prognosis of these diseases. If the promised breakthrough of immunomodulation therapy is achieved in IBD, one may anticipate quite dramatic changes in the treatment of IBD. GCS still are the mainstay of therapy of UC within 5-10 years.

This review[29] focuses on current developments in the major categories of the therapy used in the management of IBD. Conventional corticosteroids, although a mainstay of the acute treatment of IBD for many years, have many drawbacks, including a variety of side effects,particularly with chronic use. Budesonide appears to be relatively safe and at least moderately effective in inducing remission in active distal UC. Aminosalicylates, both oral and topical, have been proved useful in managing mild to moderate active UC, as well as in maintaining remission. Data from recent trials suggest that higher doses of mesalamine are generally more efficacious than lower doses. In addition, a combination of oral and rectal formulations is successful, but is not so when single route is used.The immunomodulatory agents azathioprine, 6-mercaptopurine, and methotrexate have been shown to be effective in the treatment of IBD and are now widely accepted as valuable parts of the therapeutic armamentarium. CSA, although effective, is associated with much toxicity, and patients must be monitored closely in centers experienced with this agent. Clinical trials of IL-10, IL-11, and anti-TNF-α have also shown promise. Antibiotics have been used empirically for many years in the treatment of IBD. Larger clinical trials are warranted to explore the potential efficacy of antibiotic therapy. The acemannan, heparin, and transdermal nicotine have also shown variable degrees of promise as possible therapies for IBD. Despite the variety of agents available for the treatment of IBD, none is ideal or universally accepted. Ongoing research into the well-established therapeutic agents, as well as novel drugs with more precise targets, may contribute to the design of a more optimal regimen for IBDin the not too distant future.

Both UC and Crohn's disease are considered to be the result of an unrestrained inflammatory reaction, but an explanation for the aetiopathogenesis has still not emerged[30]. Until the predisposing and trigger factors are clearly defined, therapeutic and preventive strategies for these disorders must, therefore, rely on interrupting or inhibiting the immunopathogenic mechanisms involved. Current therapies, such as glucocorticoids and 5-ASA, inhibit raised concentrations of interdependent, soluble mediators of inflammation, which may amplify one another or have parallel effects. Future medical options for treatment of UC aim at removing perpetuating antigens, blocking entry of inflammatory cells by manipulating adhesion molecules, targeting soluble mediators of inflamm ation by blocking proinflammatory molecules or by preserving endogenous suppressive molecules, or correcting genetic defects. It remains, however, to be determi ned whether targeting multi-inflammatory actions or a single key pivotal process is the better therapeutic strategy and whether subgroups of UC with different clinical courses will require different treatment approaches[24,31].

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# Species differentiation and identification in the genus of *Helicobacter*

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**Subject Headings** *Helicobacter*; genus; species; biological features; biochemical tests; identification; differentiation

As early as nineteen century, incidental presence of spiral organisms was noted in the stomachs of dogs[1], rats and cats[2]. In the early years of this century, spiral organisms were also found in the gastric contents of patients with ulcerative carcinoma[3]. During the ensuing 30 years, there were scattered reports of these organisms being found in the stomach of patients with benign peptic ulcers. Doenges[4] showed a prevalence of 43% of spiral organisms in a comprehensive autopsy study in 242 human stomach specimens. However, he did not associate the presence of the spiral organism with various gastric diseases.

Controversy existed over the possible role of these spiral organisms in human gastric disease. It was suggested that the bacteria observed in gastric biopsies might represent bacterial contaminants introduced from mouth. This hypothesis gained support with the publication of an extensive histologic study of gastric biopsies from 1000 subjects by Palmer[5]. After the publication of the report the interest in gastric bacteria waned.

Interest in the role of gastric bacteria in the pathogenesis of peptic ulcer disease was rekindled when Steer and Colin Jones[6] reported the presence of bacteria deep in the mucus layer of gastric mucosa in patients with gastric ulceration. It was suggested that the bacteria might cause a reduction in gastric mucosal resistance via predisposal to ulceration. Attempts to culture this bact erium yielded growth of *Pseudomonas aeruginosa*. Retrospectively, careful examination of the figures in this publication[6] suggests that the organism seen on the mucosa is a spiral bacterium, a morphological form not associated with *P. aeruginosa.* It is now assumed that the culture of *P. aeruginosa* by these authors represents a contaminant cultured from the endoscope. With the discovery of *Helicobacter pylori* by Warren and Marshall[7], it has been shown that *H. pylori* is associated with gastroduodenal disease[8,9].

The spiral organism was first named Campylobacter pyloridis in 1984[10]. However, the rules of Latin grammar changed the name to *Campylobacter pylori*[11]. Ribosomal ribonucleic acid sequences showed that the bacterium did not belong to the *Campylobacter* genus[12-14]. In 1989, Goodw in *et al*[15] proposed a new genus called *Helicobacter* on the bases of 5 major taxonomic features: ultrastructure and morphology, cellular fatty acid profiles, menaquinones, growth characteristics and enzyme capabilities. *C. pylori* was, therefore, transferred to the new genus and renamed as *Helicobacter pylori*. The major features[15,29] of *Helicobacter* genus consist of ① Helical, curved or straight unbranched morphology. ② Gram negative. ③ Endospores are not produced. ④ Rapid, darting motility by means of multiple sheathed flagella that are unipolar or bipolar and lateral, with terminal bulbs. ⑤ Optimal growth at 37℃; growth at 30℃ but not at 25℃; variable growth at 42℃. ⑥ Microaerophilic, variable growth in air enriched with 100mL/L -$CO_2$ and anaerobically. ⑦ External glycocalyx produced in broth cultures. ⑧ Susceptible to penicillin, ampicillin, amoxicillin, erythromycin, gentamicin, kanamycin, rifampin and tetracycline. Resistance to nalidixic acid, cephalothin, metronidazole and polymysin. ⑨ G+C content of chromosomal DNA of 200mol/L-440 mol/L.

It has been a decade since the genus of *Helicobacter* was created. This genus expands rapidly from at first only two species,*viz. H. pylori* and *Helicobacter mustelae*, to 20 species[15-35] and one associated species[36] with a wide variety of sources isolated from either human beings and/or different animals. The characteristic details of the *Helicobacter* genus, which might be useful in the differentiation and identification of different *Helicobacter* species in microbiological laboratory, are listed in Table 1, 2, 3 a nd 4. The genus of *Helicobacter* will surely continue to enlarge as more data of *Helicobacter* features are available and more animal hosts are investigated. Molecular methods, such as PCR, will provide the most accurate tests in diff erentiation and identification in future with the publi-cation of the genomic library of *H. pylori*[37].

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**Table 1 Locations, key morphological features and growth characteristics of *Helicobacter* species colonizing either humans and/or animals**

| Charastistic | *H. pylori* | *H. canis* | *H. cinaedi* | *H.felis* | *H. fennelliae* | *H. pullorum* | *H. westmeadii* |
|---|---|---|---|---|---|---|---|
| Host | Human | Dog, human | Human | Cat,dog, human | Human | Poultry, human | Human |
| Location | Stomach | Intestine | Blood, rectum | Stomach | Intestine | Intestine | Blood |
| Cell size (μm) | 0.5×3.0-5.0 | 4.0 | 0.3-0.5×1.5-5.0 | 0.4×5-7.5 | 0.3-0.5×1.5-5.0 | 3×4 | 0.5×1.5-2.0 |
| Flagella | | | | | | | |
| Number | 4-8 | 2 | 1-2 | 14-20 | 1-2 | 1 | 1 |
| Distribution | Polar | Biopolar | Polar | Biopolar | Polar | Monopolar | Monopolar |
| Sheath | + | + | + | + | + | - | + |
| Periplasmic fibers | - | - | - | + | - | - | |
| Growth at: | | | | | | | |
| 25℃ | - | - | - | - | - | - | - |
| 37℃ | + | + | + | + | + | + | + |
| 42℃ | - | + | - | + | - | + | - |
| Growth on: | | | | | | | |
| 10g/Lglycine | - | | + | - | + | | |
| 15g/L NaCl | - | | - | - | - | | |
| Tolerance to: | | | | | | | |
| 10g/L bile | - | + | Vary | - | - | + | |
| Safrain 'O' | - | - | - | - | + | - | |
| Methyl orange | - | - | + | - | Vary | + | |
| Growth under: | | | | | | | |
| Aerobic conditions | - | - | - | - | - | - | - |
| Microaerobic conditions | + | + | + | + | + | + | Weak+ |
| Anaerobic | Weak+ | - | - | + | - | - | + |
| Susceptibility to: | | | | | | | |
| Nalidixic acid | R | S | S | R | S | S | S |
| Cephalothin | S | S | I | S | S | R | R |
| Cefoperazone | S | S | S | S | S | R | |
| Metronidazole | S | S | | S | | | |

**Table 2 Locations, key morphological features and growth characteristics of *Helicobacter* species colonizing animals**

| Charastistic | *H. acinonyx* | *H. bilis* | *H. bizzozeronii* | *H. cholecystus* | *H. hepaticus* | *H. nuridarum* | *H. mustelae* | *H. nemestrinae* | *H. pametensis* | *H. rodentium* | *H. salomonis* | *H. trogontum* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Host | Cheetah | Mice | Dog | Hamster | Mice | Rat, mice | Ferret | Macaque | Bird, swine | Mice | Dog | Rat |
| Location | Stomach | Bile,live, intestine | Stomach | Gallbladder | live, intestine | Intestine | Stomach | Stomach | Intestine | Intestine | Stomach | Intestine |
| Cell size (μm) | 0.3×1.5 -2.0 | 0.5×4.0 -5.0 | 0.3×5 -10 | 0.5-0.6× 3.0-5.0 | 0.2-0.3× 1.5-5.0 | 0.5×3.5 -5.0 | 0.5-2.5× 5.0 | 0.2×2.0 -5.0 | 0.4-1.5 | 0.3×1.5 -5.0 | 0.8-1.2× 5.0-7.0 | 0.6-0.7× 4.0-6.0 |
| Flagella | | | | | | | | | | | | |
| Number | 2-5 | 3-14 | 10-20 | 1 | 2 | 10-14 | 4-8 | 4-8 | 2 | 2 | 10-23 | 3-7 |
| Distribution | Monopolar | Biopolar | Biopolar | Polar | Biopolar | Biopolar | Peritrich ous | Polar | Biopolar | Biopolar | Biopolar | Biopolar |
| Sheath | + | + | + | + | + | + | + | + | + | - | + | + |
| Periplasmic fibers | - | + | - | - | - | + | - | - | - | - | - | + |
| Growth at: | | | | | | | | | | | | |
| 25℃ | - | - | - | - | - | - | - | - | - | - | - | - |
| 37℃ | + | + | + | + | + | + | + | + | + | + | + | + |
| 42℃ | - | + | + | + | - | - | + | + | + | + | - | + |
| Growth on: | | | | | | | | | | | | |
| 10g/Lglycine | - | + | - | + | + | - | - | - | + | + | | - |
| 15g/L NaCl | - | - | | - | + | - | - | - | - | + | | - |
| Toleranceto: | | | | | | | | | | | | |
| 10g/L bile | | + | - | + | | - | - | - | | | - | |
| Safrain 'O' | | | | | | - | - | - | | | | |
| Methyl orange | | | | | | - | - | - | | | | |
| Growth under: | | | | | | | | | | | | |
| Aerobic conditions | | | | - | - | - | - | - | - | - | | - |
| Microaerobic conditions | + | + | + | + | + | + | + | + | + | + | + | + |
| Anaerobic | | | | + | + | - | + | Weak+ | Weak+ | + | | - |
| Susceptibility to: | | | | | | | | | | | | |
| Nalidixic acid | R | R | R | I | R | R | S | R | S | R | R | R |
| Cephalothin | S | R | S | R | R | R | R | S | S | R | S | R |
| Cefoperazone | | | S | | | S | R | S | | | S | |
| Metronidazole | S | S | S | | S | | S | S | | | S | S |

**Table 3 Key and differential biochemical characteristics of *Helicobacter* species colonizing either humans and/or animals**

| Characteristic | *H. pylori* | *H. canis* | *H. cinaedi* | *H. felis* | *H. fennelliae* | *H. pullorum* | *H. westmeadii* |
|---|---|---|---|---|---|---|---|
| Catalase activity | + | - | + | + | + | + | + |
| Urease activity | + | - | - | + | - | - | - |
| Oxidase activity | + | + | + | + | + | + | + |
| Alkaline phosphatase activity | + | + | - | + | + | - | + |
| γ-Glutamyl transpeptidase activity | + | | - | + | - | | |
| $H_2S$ production | - | | - | - | - | | - |
| Indoxyl acetate hydrolysis | - | + | - | - | + | - | - |
| Hippurate hydrolysis | - | - | - | - | - | - | + |
| Nitrate reduction | - | - | + | + | - | + | + |
| $C_4$ esterase | + | | + | | + | | + |
| $C_8$ esterase lipase | + | | + | | + | | + |
| Leucine arylamidase | + | | - | + | - | | + |
| Acid phosphatase | + | | + | | + | | + |
| Naphthol-AS-B1-phosphohydrolase | + | | + | | + | | + |
| DNase activity | + | | | + | | | |
| G+C content (mol%) | 35-37 | 48 | 37-38 | 43 | 37-38 | 34-35 | |

**Table 4 Key and differential biochemical characteristics of *Helicobacter* species colonizing animalsCharacteristic**

| | *H. acinonyx* | *H. bilis* | *H. bizzozeronii* | *H. cholecystus* | *H. hepaticus* | *H. muridarum* | *H. mustelae* | *H. nemestrinae* | *H. pametensis* | *H. rodentium* | *H. salomonis* | *H. trogontum* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Catalase activity | + | + | + | + | + | + | + | + | + | + | + | + |
| Urease activity | + | + | + | - | + | + | + | + | - | - | + | + |
| Oxidase activity | + | + | + | + | | + | + | + | + | Weak+ | + | + |
| Alkaline phosphatase activity | + | | + | + | | + | + | + | + | - | + | - |
| γ-Glutamyl transpeptidase activity | + | | + | - | | + | + | | - | - | + | + |
| H2Sproduction | | + | | - | + | - | - | - | - | | | - |
| Indoxyl acetate hydrolysis | - | - | + | - | + | + | + | - | - | - | + | |
| Hippurate hydrolysis | - | - | - | - | - | - | - | - | - | - | - | - |
| Nitrate reduction | - | + | + | + | + | - | + | - | + | + | + | + |
| DNase activity | | | + | | | | | - | | | + | |
| G+C content (mol%) | 30 | | | | | 35 | 36 | 24 | 38 | | | |

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***Brief Reports***

# Transient expression and antigenic characterization of HBsAg of HBV nt551 A to G mutant *

FANG De-Xing[1], LI Fa-Qing[1], TAN Wei-Guo[1], CHEN Hua-Biao[1], JIN Hui-Ying[1], LI Su-Qin[1], LIN Hou-Ji[2] and ZHOU Zhen-Xian[2]

**Subject headings** hepatitis B virus;HbsAg;point mutation;antibodies.monoclonal

## INTRODUCTION

From the late 80s, there have been increasing number of reports on hepatitis B (HB) patients with atypical HBV serological markers, some of them even lack any HBV immunological markers. Analysis of the HBV in those patients demonstrated mutants. Mutations could be found within the C, S, P and X genes[1]. The most important S gene mutants are those which affect the antigenicity of HBsAg α determinant (HBsAg amino acid residue 124 to 147). There are several reports on HBV S gene mutants affecting amino acid position 126, 144 and 145 of HBsAg[1-3]. We have described another HBV mutant with a point mutation at nt551 (A to G) of HBV genome, leading to a substitution of Met to Val at amino acid re sidue 133 of HBsAg[4]. In this study, we investigated the antigenicity of the mutant HBsAg by different mAb.

## MATERIALS AND METHODS

The recombinant bacteriophage pM13T was constructed and the mutant HBsAg coding region was sequenced (GenBank accession number AF052576)[4]. Construction of the mutant HBsAg expression plasmid pSHBsT and transfection of COS7 cell followed the reference[1]. The reactivity of the expressed HBsAg protein to mAb was detected by using a solid RIA kit (Beijing Atomic Energy Institute, China).

## RESULTS

The wild HBsAg[1] and mutant HBsAg were expressed under the regulation of SV40 early promoter in COS7 cell in a transient fashion. A mAb against HBsAg *d* determinant (anti-*d*), S4 (Shanghai Institute of Biological Products, China), was used for the quantitation of the expressed HBsAg proteins. After a series of dilution and detection, both HBsAg preparations were adjusted to a concentration of 2.1 μg/L.

Three different mAb against HBsAg-α determinant (anti-α), A6, A11 and S17, from different manufacturers were selected to characterize the binding activity of the expressed HBsAgs. Under the condition of the same concentration of HBsAg proteins determined by anti-*d*, the reactivity of the mutant HBsAg to three anti-α mAb was weaker than that of the wild HBsAg, as shown in Table 1. The result implied that the Met to Val s ubstitution at amino acid position 133 of HBsAg resulted in the alteration of the antigenicity.

**Table 1 Detection of the reactivity of the expressed HBsAg to anti-α mAb by radioimmunoassay***

| Anti-a mAb | pSHBs(133Met) | pSHBsT(133Val) |
|---|---|---|
| A6 | 1118(5.82) | 774(3.93) |
| A11 | 932(4.80) | 744(3.76) |
| S17 | 945(4.87) | 630(3.14) |

*Counter per minute (cpm), the number in the parentheses is P / N-value. According to the solid RIA kit producer's instructions, P / N = (sample cmp-background) / (negative control cpm-background). Untrans fected cells were used as negative control, average cpm was 240. Blank polystyrene beads were used as background, average cpm 58. P / N ≥ 2.10 is cons id ered to be positive reactivity. The more the P / N value, the stronger the r eactivity.

## DISCUSSION

Since Carman *et al*[3] described the HBV immune escape mutant in 1990, many researchers have reported that HBV DNA mutations are related to immune escape, but most of which are limited to the detection by PCR amplification and direct nucleotide sequencing. So far, only the mutant HBsAg with substitution of Ile to Ser at aa126[1], HBsAg with substitution of Asp to Ala at aa144[2], and HBsAg with substitution of Gly to Arg at aa145[3] were

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characterized in detail. Because the mutation of A to G at nt551 affect the HBsAg α determinant, it was likely that the mutation could cause immune escape[4]. This study showed that the mutation resulted in decreased reactivity of the HBsAg to anti-α monoclonal antibodies, confirming the hypothesis that the HBV is a new immune escape mutant.

The finding of HBV immune escape mutant has caused attention from scientists all over the world. Some experts recommended that it is worth considering to add mutant immunogen (HBsAg) into the future hepatitis B vaccine. But it is very important to know what mutants are immune escape ones and prevalent ones. A mutation specific PCR (msPCR) method for detecting the mutation at nt551 of HBV genome was established and the investigation and survey of the mutant among child and adult patients is in progress.

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# Experimental study of cholagogic cream for refractory jaundice

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**Subject headings** jaundice; cholagogic cream bile duct stenosis; cholestatic hepatitis

## INTRODUCTION

"The refractory jaundice" in this paper implies the benign icteric disease which was repeatedly treated with either western or traditional Chinese medicine but gave no evidence of improvement, mainly including primary sclerosing cholangitis, intrahepatic sand-like calculi, bile duct stenosis and cholestatic hepatitis etc[1,2]. Such diseases are difficult to treat by both traditional C hinese and wes tern medicine. In this study, the cholagogic and anti-inflammatory effects of cholagogic cream, and the combined effects with trepibutone on refractory jaundice were observed and evaluated by experimental pathologic and biochemical examination.

## MATERIALS AND METHODS

### *Ingredients of cholagogic cream*

Dandelion herb 20 g, curcuma root 15 g, Fructus Aurantii 10 g, Sichuan chinaberry 10 g, oriental wormwood 20g, lysimachia 20g, gentian root 10g, Chicken's gizzard-skin-15 g, root bark of the tree peony 15 g, red peony root 15 g, red sage root 20 g, burreed tuber 15 g, zedoary 15 g, rhubarb 10 g, mirabilite 10 g, Herba Lycopi 15 g, earthworm 15 g.

### *Animal experiment*

Thirty-two healthy adult hybrid dogs of either sex (mean weight, 18.1 kg) were selected. The animals were anesthetized intravenously with 2% pentobarbital sodium (30 mg/ kg). Epigastric median incision was performed to reveal the common bile duct and hepatic porta. One percent formaldehyde solution was evenly and carefully infiltrated into the extrahepatic bile duct wall with a small needle. The volume depended on appearance of white on the bile duct wall. A mushroom like catheter (a catheter with a mushroom tip) was placed into the gallbladder, fixed on the abdominal wall, and the abdomen was closed after gastrostomy. The appearance of jaundice after one month indicated the success in model preparation.[2] The 32 dogs were randomly divided into control group, cholagogic cream group, trepibutone group, and cholagogic cream plus trepibutone group, 8 for each.

### *Route of drug administration*

The medicine was administered through the gastrostomy tube, followed by 1/2 hour observation to make sure that no vomiting occurred.

The administration regimens (1 week) were:

Cholagogic cream plus trepibutone group: cholagogic cream 0.3 g/kg, twice a day; and trepibutone 0.75g/kg,three times a day.

Cholagogic cream group: cholagogic cream 0.3 g/kg, twice a day.

Trepibutone group: trepibutone 0.75 mg/kg, Three times a day.

Control group: normal saline 100 mL, twice a day.

### *Observation methods*

The bile flow from the cholecystostomy tube in 24 hours was recorded at 08:00 every day, and 5 mL bile was taken for viscosity measurement. The remaining bile was trasfused bach to duodenum via the gastrostomy tube. The bile in the gallbladder was also collected regularly before and after operation for viscosity measur ement.

Changes of the serum total bilirubin, direct bilirubin and glutamic-pyruvic transminase were routinely detected.

The preparation and observation of the scanning electron microscopic sections were carried out in the Center for Computation and Test, Nankai University, Ti anjin. Hitachi-650 scanning electron microscope was used, the maximal resolving power was 60A. The paraffin-embedded sections by routine HE staining were obse rved under optical microscope.

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## RESULTS

After therapy, the bile flow showed no significant change in the control group, but dramatically increased in trepibutone group and cholagogic cream group, especially in cholagogic cream plus trepibutone group.

The bile viscosity displayed no significant change in the control group, but dropped significantly in trepibutone group and cholagogic cream group, especially in cholagogic cream plus trepibutone group.

Serum total bilirubin and direct bilirubin were apparently decreased in all the groups except the control group.

Glutamic-pyruvic transaminase (GPT) was significantly decreased in all the groups, especially in cholagogic cream plus trepibutone group, except the control group.

Seven days later, the sections were observed under optical microscope. In the control group, inflammatory cell infiltration occurred in the bile duct wall, especially in the mucous submucous layers. In trepibutone group, inflammatory cell infiltration reduced, but the mucosal exfoliation was obvious. In cholagogic cream group, the inflammatory cells were decreased, and the endoscopic appearance of the gallbladder mucosa returned to normal. In cholagogic cream plus trepibutone group, the inflammatory cells were markedly decreased, and many new mucous membranes were observed.

The mucous membrane of the bile duct was observed under scanning electron microscope for 7 days. It was found that the mucosal membrane was swollen, and the microvilli were exfoliated from the surface of the mucosa in the control group. The edema was markedly alleviated, and the microvilli were quite rare in trepibutone group. A part of the microvilli appeared, and the edema was markedly reduced in cholagogic cream group. The edema was completely resolved and many neoformative microvilli were seen in cholagogic cream plus trepibutone group.

## DISCUSSION

Sclerosing cholangitis, representative of the typical refractory jaundice clinically, served as the icterus model. This experiment found that cholagogic cream possesses a strong cholagogic effect (soothing the liver and normalizing the function of the gallbladder, eliminating blood stasis and removing obstruction in the meridians, and relieving jaundice), which results mainly from its ingredients such as dandelion herb, lysimachia, oriental wormwood, curcuma root, gentian root and rhubarb. The mechanism might be explained in two aspects. Cholagogic cream, on one hand, can promote the bile secretion from liver cells and bile capillaries, resulting in marked increase of bile flow, and sharp decrease of bile viscosity and serum total bilirubin; and on the other hand, it can also resolve mucosal edema in the bile duct and relax the sphincter, which can be explained by the observation under the scanning electron microscope and change of the serum direct bilirubin. Therefore, it is extremely beneficial to patients with refractory jaundice.

This experiment also proves that cholagogic cream has a strong anti-inflammatory effect (clearing away heat and toxic materials or expelling toxin by cooling, and promoting Qi flow and blood circulation). It could increase the phagocytos is of inflammatory cells by regulating the immunologic function, leading to the elimination of the inflammations in the liver cells and the bile duct wall, which can be proved by the sharp decrease of serum GPT level and the rapid resolution of the inflammation in bile duct wall observed under the optical microscope. The clinical experiences indicated that many refractory jaundices were mutually affected with other hepatic diseases, such as liver dysfunction, hepatic interstitial cells, the bile duct stenosis, edema and inflammation. So, we believe that the cholagogic cream possesses a unique advantage of overall regulation, which is absent in western medicine.

Besides, the combination of cholagogic cream and trepibutone can enhance the effects, the mechanism, beyond profound discussion here, might be complex, but it is certain that the combination of the traditional Chinese and western medicine for refractory jaundice is undoubtedly practicable.

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# Application of BRV-R mAbs to detection of corresponding receptors

ZI Zi-Qiang, XU Yan, SUN Wen-Min and REN Zhong-Yuan

**Subject headings** retavirus; antibodies, monoclonal; IgG

## INTRODUCTION

Rotavirus is a major pathogen of acute gastroenteritis in human infants and young animals. It has been the second cause of infant death especially in developing countries. The infections sometimes occur in adults too. We have used the BRV-R mAb to study the BRV and the BRV-R on MA104 cell surface in three aspects: ① the conjugation test of the BRV-R mAb and rabbit anti-BRV IgG; ② the a nti-infectious action of the BRV-R mAb; and ③ the immuno-spot test of the BRV-R mAb.

## MATERIALS AND METHODS

### *Conjugation test of BRV-R mAb and anti-BRV IgG*

**Preparation of rabbit anti-BRV IgG** Virosomes were extracted from the BRV (NCDV strain) suspension concentrated by PEG using ultracentrifugation and observed under electron microscope. Using the virus ($10^7$/ mL), we prepared the rabbit anti-BRV immune serum. Antibody titre was determined by the complement fixation test. The rabbit anti-BRV IgG was extracted by salting-out method and DE52 chromatographic analysis. Antibody protein was 7.2 g/L measured by Lowry method and was preserved at -20℃.

**Preparation of ascities BRV-R mAb** The concentional procedure adopted in our lab was used.

**Conjugation test of BRV-R mAb and rabbit anti-HBV IgG** ELISA was employed for the detection. The main procedures were: The 96-pore polythylene plate (America Costor) coated with rabbit anti-BRV IGG (130 mg/ L) was incubated for 24 h at 4℃, washed and enclosed. Its horizontal rows were added with doubling diluted a scitic type BRV-RmAb. The first spot of each row was added with diluted fluid as blank control, and the $SP_{2/0}$ ascities was used as a negative control. After that, the plate was incubated, washed and added with goat anti-mouse IgG-HRP. It was then put in substrate fluid for color reaction Bio-RAD (America) 2550 type enzyme linked immune measurer was used to detect A (the OD value, $\lambda = 492$). The titre of BRV-R mAb was determined and the IgG of BRV-R mAb was extracted by DE52.

### *Anti-infectious action of BRV-R mAb*

MA104 strain culture and BRV (NCDV strain) suspension preparation were completed using our own procedure. We set up five control groups: ① the normal MA104 strain; ② BRV; ③ rabbit anti-BRV IgG; ④ BRV-R mAb; ⑤ fluid combined with the rabbit anti-BRV IgG and BRV. Test groups: ① MA104 strain tube was first incubated with BRV-R mAb for 30min at 37℃ and then added with BRV (NCDV strain) suspension. It was supplemented with maintenance media after 30min at 37℃; ② The rabbit anti-BRV IgG and BRV-R mAb were quantitatively mixed, incubated for 30min at 37℃, and then put in the MA104 strain tube. After another 30min at 37℃, it was added to BRV (NCDV strain) suspension. Maintenance media was added in the tube after the third 30min at 37℃. The cells of the control and test groups were cultured at 37℃ and CPE was observed.

### *Immuno-spot test of BRV-R*

**Preparation for BRV-R** After routinely cultured, amplified, digested, collected and washed with PBS, the MA104 strain cells were suspended in the 0.01 mol/ L PB (pH 7.0), swollen thoroughly at 4℃ and mechanically splitted. Supernatant was collected after centrifugation 1000×*g* for 5 min and discarded after immersing cell membrane-*via*-centrifugation 20 0000×*g* for 1 h. Add 3g/L sodium deoxycholate-Tris chlorhydric acid and incubate for 40min at 4℃. After centrifugation 100000×*g* for 45min, supernatant was taken, which is BRV-R, and preserved at -20℃.

**Immuno-spot test** After drying at room temperature, BRV-R was dropped on the pyroxylin membrane (British product) with a total dose of 30μm, and then put in the 10% ovi albumin-0.1 mol/ L glucine-Tris chlorhydric acid buffer solution for 1h at 37℃ for enclosing. After washing, it was placed into the BRV-R mAb preparation for 1h at 37℃,

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rewashed and added with the goat anti-mouse IgG-HRP conjugate for 1h at 37℃. Substrate solution was added for color reaction and observed with naked eye.

## RESULTS

### *The conjugation test of BRV-R mAb and rabbit anti-BRV IgG*

The positive result was judged if the OD value of the sample was 0.1 higher than that of the negative control determined by ELISA. The conjugation titre of the ascitic type BRV-R mAb and rabbit anti-BRV IgG was 1:5120 and when the BRV-R mAb was extracted by DE52 chromatographic analysis it was 1: 2560.

### *Anti-infectious action of BRV-R mAb*

The BRV was inoculated into the MA104 strain cells. The CPE of each group was observed at different time points every day. The result is shown in Table 1.

### *Immuno-spot test*

The result indicated that the BRV-RmAb can combine with the BRV-R, and colored brown, while the control groups showed no color.

**Table 1 The anti-infectious action of the BRV-R mAb**

| Groups | CPE at different time point (h) | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Control group 1 | - | - | - | - |
| Control group 2 | + | ++ | +++ | ++++ |
| Control group 3 | - | - | - | - |
| Control group 4 | - | - | - | - |
| Control group 5 | - | - | - | - |
| Test group 1 | - | - | - | +/- |
| Test group 2 | + | ++ | +++ | ++++ |

## DISCUSSION

The conjugation reaction was produced by the rabbit anti-BRV IgG and BRV-R mAb when detecting the plate coated with rabbit anti-BRV IgG using ELISA. The cytoprotection test indicated that the BRV-R mAb was able to prevent the corresponding sensitive cell strain MA104 from being infected by the BRV. We inferred that the BRV-R mAb and BRV shared the correlative antigen determinants, which were related to the BRV-R on the cell surface. The immuno-spot test demonstrated that the antigenicity of the BRV-R on the bovine enteric mucosal cell surface was correlated with that of the BRV-R on the MA104 strain cell surface. To a certain extent, it has laid a basis for purifying the BRV-R on the MA104 strain cells by affinity chromatography, studing its property, and searching the correlative receptors on different tissues and cell surface *in vivo* utilizing labelled BRV-R mAb.

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# A successful case of combined liver and kidney transplantation for autosomal dominant polycystic liver and kidney disease *

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**Subject headings** liver; transplantation; kidney transplantation; kidney disease; liver disease

With advances in transplantation, multiorgan transplantation has become a treatment of choice for end-stage organ failure which can not be reversed with other modalities. In 1984, the first case of combined liver and kidney transplantation was introduced by Witts at Innsbruck University Hospital in a 30-year-old man with HBsAg positive cirrhosis. He survived more than 9 years[1]. Since then, an increasing number of such combined transplantation has been performed. But in Asia, this technique has not been put into clinical practice yet. We report such a case below.

## CASE REPORT

### *Physical examination*

A 52-year-old woman was admitted to our hospital with fatigue, jaundice and severe abdominal pain. She suffered from congenital polycystic liver and kidney disease at 40 years of age. Two weeks before her admission, she underwent laporatory in a local hospital for unbearable abdominal pain. She was found to have anemia and jaundice in physical examination. She had an enormous liver extending to pelvic cavity with an enlarged spleen. Biochemical tests showed hemoglobin 103 g/ L, WBC $3.2\times10^9$/ L, platelet $7.4\times10^9$/ L, serum total bilirubin 42.7 μmol/L, albumin 30 g/L, creatine 62 μmol/L, BUN-8.5 μmol/L and prothrombin time 18 seconds (control 12 seconds). The renogram indicated that her renal function had been slightly damaged. Two weeks after her admission, she received combined liver and kidney transplantation.

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### *Procurement of donor organs*

The donated liver and kidney were harvested using the rapid multiple organs harvesting technique[2]. Both organs were flushed *in situ* and then cold preserved in the Winsconson University solution. Lymphocyte cross-matching was negative and the panel reactive antibody (PRA) was 8%.

### *Operative and postoperative course*

The liver was transplanted orthotopically using venovenous bypass during anhep atic phase. Following implantation of the liver, the kidney was placed intrapert ioneally into the right iliac fossa after 12.5h of cold ischemia. Bile was produced promptly, indicating immediate graft function. The kidney assumed normal color and consistency after revascularization and produced copious amounts of urine. The total operative blood loss was 3500mL, and 2800mL blood products were infused. Immunosuppressive regimen including cyclosporine A, 2mg/kg per day intravenously, and methylpredisolone (starting with 1 g per day and tapering to 10 mg daily) was given.

### *Results*

The early postopertive course was uneventful. The patient was discharged two months after combined liver and kidney transplantation. Six months after transplantation, however, she developed jaundice though her general condition was good. Following the jaundice period, liver function began to show signs of deterioration. The biliary sludge was diagnosed. She was reoperated due to biliary obstruction. The biliary sludge which was 2.0 cm × 0.5 cm in size located in the middle portion of common bile duct. The patient was soon recovered from the second operation and discharged on the 20th day with nearly normal liverfunction. She is doing well and is in good health.

## DISCUSSION

The high success rate achieved with single organ transplantation has stimulated the assumption of double organ transplantation for patients with complex multiorgan failure. The development of new immunosuppressive agents and improved surgical ap-

proaches as well as sophisticated postoperative management make assumption become clinical practice.

The main concern in combined liver and kidney transplantation is the selection of candidates. According to Margreiter[3], the indications for combined liver-kidney transplantation are divided into three categories: ① Disease affecting both organs, such as autosomal dominant polycystic disease. ② Renald isorders with liver involvement or liver disorders with renal involvement, inclu ding primary type I hyperoxaluria, type I glycogen storage disease, cholesterol acyltransferase deficiency, etc. ③ Separate diseases of both organs. This category consisting of patients on hemodialysis with liver disease and patients with end-stage liver disease and renal function impairment other than hepatorenal syndrome is the main indication for combined liver and kidney transplantation.

Autosomal dominant polycystic kidney disease is often present in newborns, but dose not become clinically evident until adulthood. It is often associated with liver cysts which may reach enormous size (in our case, it weighs 5.0kg) and can therefore be extremely disabling considering both the rigors of renal ischemia during transplantation and nephrotoxic effects of cyclosporine A, and some necessitating antibiotics may worsen the previously impaired renal function and lead to postoperative renal failure. We treated the patient with combined liver-kidney transplantation.

The surgical technique of combined liver-kidney transplantation was exactly the same as for transplantation of the liver or the kidney alone. For the reason that liver is more subject to cold ischemia damage than kidney, the liver is gener ally implanted prior to the kidney. In case a venovenous bypass should be needed, the axilla and thigh, preferentially on the left side, must be prepared. Because of the poor coagulation status of the recipient, heparin may not be required for prevention of clotting.

A protective role of the liver allografts in the survival of other solid organ transplants has been noted, but the exact mechanism of this phenomenon has not been elucidated. The liver has the potential to protect the other organs immunologically. The efficacy of simultaneous liver-kidney transplantation in the prevention of hyperacute renal rejection in patients with reformed anti-HLA lymphocytotoxic antibodies is also demonstrated in clinical practice. However, these observations have been challanged by reports documenting hyperacute rejection of kid ney or/and liver allografts when the roles of ABO matching and donor/recipient crossmatching have been violated[4-5]. Although our patient did not experience any rejection and showed some protection of the transplanted kidney by the liver graft, we believe that careful selection of suitable donor with HLA typing crossmatching and PRA test are essential to success in combined liver-kidney transplantation.

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# Resection of gastric carcinoma with preservation of pancreas and clearance of lymph nodes along splenic artery: theory, technique and results

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**Subject headings** stomach neoplasms / surgery; lymph nodes; panc reas / blood supply; splenic artery

## INTRODUCTION

Cancers of whole gastric stomach, cardia, and gastric corpus often metastasize to splenic hylus and lymph nodes along the splenic artery[1]. There is ample statistical evidence to support the routine clearance of the lymph nodes along the splenic artery in cases of cancers in whole stomach, gastric corpus, cardia, and in certain invasive cancers in gastric antrum. Beginning from the mid 1940s, many surgeons adopted the procedure of resection of the spleen together with pancreatic body and tail in order to clear the lymph nodes of splenic hylus and along the splenic artery (pancreatic resection procedure). However, combined resection procedure carries the disadvantage of increased operative complication and mortality, and reduction of pancreatic function, especially in insulin output, hence increasing the incidence or aggravating diabetes mellitus. Based on the data and rationale of all those mentioned above, we began in 1968 our research on the operative procedure for gastric cancer, with preservation of pancreatic parenchyma with clearance of lymph nodes along the splenic artery (pancreatic preservation procedure)[2].

## MATERIALS AND METHODS

### *Theoretical grounds of pancreatic preservation*

The lymph flow from the stomach does not enter into the pancreas. By injecting 2ml of methylene blue into the gastric cardia or corpus subserous space during operation, we observed the direction of gastric lymph flow in 54 cases. The direction of cardia lymph flow could be judged from the flow direction during operation and from anatomic examination of lymph nodes specimens. ① The direction of the lymph flow from the gastric cardia is ascending toward mediastinal lymph nodes along the esophageal wall. ② Lymph from the lesser curvature flows toward the left gastric artery and then into lymph nodes along the celiac artery. ③ Lymph flowing along the short gastric arteries is running away from the greater curvature, and drains into lymph nodes of splenic hylus, then out along the splenic artery and finally to lymph nodes along the celiac artery. ④ Lymph from the posterior gastric wall along the post-gastric artery flows to retroperitoneal space and then into the lymph nodes along the splenic artery at the upper border of pancreas. ⑤ Lymph flowing along esophagocardiac branch of left subdiaphragmatic artery comes from the left side of gastric cardia, and drains to the para-aortic lymph nodes. The direction of lymph flow from the upper gastric corpus, besides being the same as that from cardia, may enter into lymph nodes of the splenic hylus and along the splenic artery via the left gastroepiploic artery and its lymph nodes, and finally accumulate into lymph nodes along the celiac artery. Not a single case was found with lymphatic flow entering into pancreatic parenchyma.

It is infrequent for cancers from gastric cardia, gastric corpus, and whole stomach to invade pancreas itself. During the period from 1968 to 1986, in our hospital 439 cases of cancers in gastric cardia, gastric corpus, and whole stomach could be resected, of which 25 (5.7%) cases were found invading into the pancreas. From examination of the resected specimens of pancreatic body and tail in 22 cases, we found that direct invasion of gastric cancer to pancreatic parenchyma and capsule occurred in only 6 cases, with no metastasis to lymph nodes. This result is in agreement with the conclusion of the research on gastric lymphatic flow.

No necrosis of pancreatic body and tail occurred after resection of splenic artery and vein. The pancreatic body and tail is vascularized by pancreatic transverse artery, splenic artery, great pancreatic artery and pancreatic tail artery. The pancreatic transverse artery is the left branch of pancreatic dorsal artery. The splenic artery arises from the celiac artery and gives off 2-10 arterioles supplying the pancreas, one of which is called greater pancreatic artery, located between the middle and distal thirds of the pancreas. The right branch anastomose with branches from pancreatic transverse artery and the splenic artery and a left branch anastomoses

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with artery of the pancreatic tail. The artery of the pancreatic tail arises from the distant 1/3 of splenic artery or from the left gastroepiploic artery. The pancreas has an abundant venous anastomosis, with the veins mainly accompanying synonymous arteries. There are 3-13 veins from the pancreatic body and tails (average 7). They drain into the splenic vein, more often into the upper mesenteric vein and upper part of the inferior mesenteric vein or left gastroepiploic vein. There is usually a vein which accompanies the inferior pancreatic artery and drains into the upper mesenteric vein, someti mes into the inferior mesenteric vein or the splenic vein[3]. Since about 40% of the pancreatic dorsal artery arises from the first part of the splenic artery, it is enough to resect the splenic artery at the distal end of the pancreatic dorsal artery. The pancreatic body and tail may be supplied by the left branch of the dorsal pancreatic artery, i.e., the transverse pancreatic artery. After resection of splenic vein, the pancreatic body and tail can be drained by transverse pancreatic vein and collateral vein with surrounding tissues several days after operation, therefore necrosis of pancreatic body and tail will not occur.

### *Procedure for preservation of pancreatic parenchyma with resection of the splenic artery and vein and pancreatic capsule with surrounding lymphatic and neural connective tissues*

We first resected the omentum with concomitant freeing of the transverse mesocolon to the lower border of pancreas. After clearance of the capsule in front of the pancreas from the lower pancreatic border, the spleno-renal ligment was then resected up to gastric fundus and the left side of the cardia. After lifting up the spleen, we freed the pancreatic body and tail along the retroperitoneal space, and cleared the lymph nodes along the common hepatic artery, celaic artery and the root of left gastric artery together with the attached fatty and connective tissues. After the roots of the left gastric artery and the splenic artery were exposed, these arteries were separated and ligated. If the pancreatic dorsal artery arised from the first part of the splenic artery, one should ligate the splenic artery to the left of the dorsal artery, or when it arised from other arteries at the root of the splenic artery. After the operator changed his position to the left side of the patient, the assistant lifted the spleen and pancreatic body and tail out of the incision and turned them to the right side. After incising the splenic vein sheath, the splenic vein was exposed and resected at the left of the entrance of the inferior mesenteric vein. With slight traction of the severed ends of the splenic artery and vein, the vascular branches which arose from the splenic artery and entered into the pancreatic parenchyma, the most important of which were the great pancreatic artery and artery of the pancreatic tail, should be freed and ligated. Concomitantly, we resected several venous tributaries arising from the pancreatic parenchyma and draining into the splenic vein. In the end, we freed completely the pancreatic body and tail, the splenic artery and vein, the pancreatic capsule and surrounding lymphatic, fatty, nerve and connective tissues.

By adopting the procedure described above, we found the operation expedient and with less possibility of injuring the pancreatic dorsal artery and inferior mesenteric vein. The lymph nodes and connective tissue were cleared in front of distal splenic vessels and at the periphery from the branch of the distal splenic artery and vein to the root of splenic vessels and finally the pancreatic capusle and splenic artery and vein surrounding lymphatic, fatty, neural and connective tissues were freed from the pancreatic body and tail. The other operative procedure was the same as the radical operation for gastric cancer.

## RESULTS

### *Comparison of lymph node metastasis between two different operation groups*

From 1968 to 1992, we performed radical resection of gastric carcinoma by two different techniques, on 216 cases with preservation of pancreas and clearance of lymph nodes along the trunk of splenic artery and 30 cases with resection of pancreas. The metastasis rates of lymph nodes in splenic hylus and those along the trunk of splenic artery were 20.8% (45/216) and 25% (54/216) in the first group, and 20% (6/ 30) and 23.3% (7/ 30) in the second group. There was no sig nificant difference statistically ($P>0.05$).

### *Comparison of postoperative complication, mortality and survival rates*

In the first operation group (preservation of pancreas), the postoperative complications occurred in 9 (4.2%), diabetes mellitus in 2 (0.9%) and death in 2 (0.9%); and in the second operation group in 12 (40%) diabetes mellitus in 3 (10%) and death in 1 (3.3%). The 5-year survival rates were 57% and 36% and 10-year survival rates were 47% and 30%, respectively. There was significant statis tical difference ($P<0.05$) between the two in the incidence of complication, but no marked difference in the mortality rate.

## DISCUSSION

The severe organic deficiency caused by expanded resection of gastric cancer has been of great concern

in recent years. It is a subject of dispute among surgeons that after clearance of lymph nodes along the splenic artery, whether one should adopt the procedure of pancreatic body and tail preservation or resection. After 20 years' research on this subject, we think that pancreatic preservation conforms better to the rationale of radical gastric cancer resection. The reasons are: ① The results of our research on lymphatic flow of gastric cardia and corpus indicated that the direction of the lymphatic flow from those area is along the upper border of the pancreas and the splenic artery, draining to relevant lymph nodes, but not into pancreatic parenchyma. Maruyama[4] found no case with gastric lymphatic flow entering into pancreatic parenchyma with preoperative gastroscopic photography after injection of opaque medium into inferior posterior wall of gastric cardia and upper part of gastric corpus and photography of specimens 1 to 5 days later and by injecting dye into gastric subserous space during operation. ② By serial sections of autopsy material of gastric cancer and pancreatic body and tail resected together with gastric cancer, Maruyama[5] found that only a small part of gastric cancer lesion invaded pancreas directly from the serous surface without any intrapancreatic lymphatic metastasis. We got the same conclusion from pathologic examination of resected pancreatic body and tail. ③ Maruyama[5] adopted the procedure of pancreatic preservation with clearance of lymph nodes along the splenic artery in 76 cases after 1976, with an operative mortality of 1.1%. Various common complications occurred in 25% cases, and pancreatic complication in 6.5%, but not a single case of diabetes mellitus occurred postoperatively. During the same period, Maruyama adopted pancreatic resection procedure in 58 cases and common complications occurred in 59.2% and pancreatic complications in 25%, and postoperative diabetes mellitus in 9.2%, and the operative mortality was 2.6%. In our group of 216 cases, postoperative complication occurred in 4.2%, the mortality rate being 0.9%, and the postoperative diabetes mellitus was found in 0.9%. In 30 cases with resection of pancreas, the postoperative complication occurred in 40%, and the postoperative mortality being 3.3%, postoperative diabetes mellitus in 10%. The incidence of postoperative complications and diabetes mellitus of pancreatic preservation adopted by our group and Maruyama were lower than those applying procedures with pancreatic resection ($P$<0.05-0.001). The postoperative survival rates after pancreatic preservation operation by our group and Kinoshita, Maruyama[6] were higher than those applying pancreatic resection procedure ④. The lymph nodes of splenic hylus and along the splenic artery can be completely removed by pancreatic preservation procedure with resection of spleen, splenic artery and vein, pancreatic capsule, and surrounding lymphatic tissues to gether with the fatty, nerve and connective tissues. Maruyama[5] first cleared the lymph nodes along the splenic artery with preservation of pancreatic parenchyma, then resected the pancreas and the specimens were examined histologically. The result showed that on lymph nodes were left. In summary, the procedure with pancreatic preservation has advantages of easy performance, complete clearance of lymph nodes along the splenic artery, and low incidence of common postoperative complications and diabetes mellitus, and the 5-year survival rates being higher than that with pancreatic resection.

The indications for pancreatic preservation procedure are cancers from gastric cardia, corpus, whole stomach and certain cases with cancer from gastric antrum requiring clearance of lymph nodes along the splenic artery. Patients with preoperative diabetes mellitus or diabetic tendency are indicated absolutely for this procedure. If, however, the cancerous lesion or metastatic lymph nodes have invaded the pancreas parenchyma and Borrmann 4 type carcinoma, the combined resection procedure should be adopted.

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# Comparison of serum Zn, Cu and Se contents between healthy people and patients in high, middle and low incidence areas of gastric cancer of Fujian Province *

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**Subject headings** stomach neoplasms/etiology; stomach neoplasms/mortality; trace elements/blood; copper/blood; selenium/blood; zinc/blood

## INTRODUCTION

To find out the difference of Zn, Cu and Se contents in the sera between healthy group in high gastric cancer incidence area and in low incidence area, and the difference among healthy, gastric cancer or other tumor groups, we collected 453 serum samples from healthy, gastric cancer or other tumor groups in high gastric cancer incidence areas of Changle and Putian, middle incidence area of Shaxian, and low incidence area of Fuan between 1992 and 1995, and measured and compared the serum contents of Zn, Cu and Se. The results are presented as follows.

## MATERIALS AND METHODS

### *Sampling objects*

According to the gastric cancer mortality from the data of resident retrospective survey on death causes in 1986-1988, we selected high gastric cancer incidence area Changle with a gastric cancer mortality of $92.26/10^5$ and Putian with a mortality of $58.61/10^5$, middle incidence area Shaxian with a mortality of $18.86/10^5$ and low incidence area Fuan with a mortality of $7.76/10^5$. Samples were collected in terms of sex and age proportion from healthy check-up people in the three areas, and from patients with gastric cancer and other tumors diagnosed by hospitals. Table 1 shows the number of samples collected.

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**Table 1 Sample number of different groups among the three areas**

| Area of incidence | County | Healthy (group I) | Gastric cancer (group II) | Other tumor (group III) |
|---|---|---|---|---|
| High | Changle | 100 | 41 | 3 |
| | Putian | 52 | 39 | 14 |
| Middle | Shaxian | 95 | 14 | 15 |
| Low | Fuan | 49 | 15 | 16 |
| Total | | 296 | 109 | 48 |

### *Sampling method*

All appliances and recipients were washed carefully and disinfected to avoid contamination during sampling. Blood was collected from veins. Sera were separated by centrifugation of samples and transferred into plastic tubes, sealed up, frozen, then stored at low temperature.

### *Sample analysis and measurement*

**Cu and Zn** The samples were diluted and contents of Cu and Zn were measured by flame spectroscopy in Pekin-Elmer 5000 atomic absorption spectr ophotometer.

**Se** The samples were digested and content of Se was determined by hydride generating method in Pekin-Elmer 5000 atomic absortion spectrophotometer.

### *Quality control*

To assure the accuracy of analysis, we adopted the strict quality-control measures, i. e. analyzed each batch of samples while analyzing national stair standard referential material GBW-09 cattle serum.

### *Data analysis and statistics*

SYSTAM software was used to manage and analyze the data of this survey.

## RESULTS AND DISCUSSION

### *Difference of serum microelement contents of healthy group in high, middle and low gastric cancer incidence areas*

Results of serum Zn, Cu and Se in healthy people of different areas were analyzed (Table 2).

**Table 2 Serum contents of trace elements in healthy group in high, middle and low gastric cancer incidence areas ($\bar{x}\pm s$)**

| | *n* | Zn(mg/L) | Cu(mg/L) | Se(μg/L) | Cu/Zn |
|---|---|---|---|---|---|
| High (I) | 152 | 0.886±0.015 | 0.911±0.015 | 84.82±2.18 | 1.065±0.025 |
| Middle (II) | 95 | 0.885±0.015 | 0.942±0.019 | 84.57±1.23 | 1.099±0.035 |
| Low (III) | 152 | 1.002±0.019 | 0.867±0.032 | 76.87±3.22 | 0.867±0.033 |
| Analysis of variance | | $F$ = 9.326 | $F$ = 2.479 | $F$ = 2.297 | $F$ = 9.939 |
| | | $P$ = 0.00012 | $P$ = 0.086 | $P$ = 0.102 | $P$ = 0.000066 |
| Newmen-Keul test in | I:III | $q$ = 5.782[a] | | | $q$ = 6.827[a] |
| comparison of different | I:II | $q$ = 0.0303 | | | $q$ = 0.955 |
| areas | II:III | $q$ = 5.456[a] | | | $q$ = 6.105[a] |

[a]$P$<0.01.

**Table 3 Contents of Zn, Cu and Se in sera of healthy, gastric cancer and other tumor groups ($\bar{x}\pm s$)**

| | *n* | Zn(mg/L) | Cu(mg/L) | Se(μg/L) | Cu/Zn |
|---|---|---|---|---|---|
| Healthy (I) | 294 | 0.905±0.010 | 0.914±0.011 | 83.22±1.32 | 1.044±0.018 |
| Gastric cancer (II) | 109 | 0.843±0.019 | 1.045±0.023 | 73.58±1.68 | 1.308±0.035 |
| Other tumor (III) | 48 | 0.858±0.028 | 1.127±0.040 | 75.29±2.33 | 1.404±0.078 |
| Analysis of variance | | $F$ = 5.137 | $F$ = 29.168 | $F$ = 9.278 | $F$ = 35.220 |
| | | $P$ = 0.006 | $P$<0.00001 | $P$ = 0.000073 | $P$<0.000001 |
| Newmen-Keul test in | I:III | $q$ = 4.305[a] | $q$ = 7.660[b] | $q$ = 5.852[b] | $q$ = 9.423[b] |
| comparison of different | I:II | $q$ = 2.362[b] | $q$ = 8.949[b] | $q$ = 3.426[a] | $q$ = 9.113[b] |
| groups | II:III | $q$ = 0.673 | $q$ = 3.094 | $q$ = 0.664 | $q$ = 2.673 |

The serum Zn content of healthy group had no difference as compared with that in high incidence area and that in middle incidence area, but was obviously lower than in low incidence area. The ratio of Cu/Zn in high incidence was incon siderably different from that in middle incidence area, but significantly higher than in low incidence area, the difference being statistically significant. However, no obvious difference was found in serum Cu and Se contents among these areas.

The correlation between contents of Cu and Se in healthy human serums and gastric cancer mortality among different areas was insignificant, but in high incidence area the content of Zn was much lower than in low incidence area and the ratio of Cu/Zn was higher. The result indicated that contents of microelement had a certain relationship with gastric cancer incidence although we could not conclude the contents affected gastric cancer incidence. Because of limited inves tigation scope, the conclusion was uncertain in a way, nevertheless it provides a clue to further study the causes of gastric cancer.

### *Comparision of microelement contents in human serum between healthy and gastric cancer groups*

Zn, Cu and Se in the samples collected from healthy, gastric cancer and other tumor groups in the three areas were measured and the ratio of Cu/Zn was calculated. The results of statistics and analysis are presented in Table 3.

According to Table 3, the serum levels of Zn, Cu and Se and the ratio of Cu/Zn are significantly different statistically between the healthy group and gastric cancer group, however the difference between the gastric cancer group and other tumor group was not significant. Newmen-Keuls test was used in compar ison of all groups.

### *Zn content*

The Zn content in the serum of gastric cancer and ot her tumor patients was lower than in healthy group, and the difference being statistically significant. However this difference did not exist between gastric cancer group and other tumor groups. Zn was considered one of the necessary compositions of many enzymes in human body, involved in the synthesis of DNA and RNA polymeric enzymes, took part in the nucleic acid metabolism and immunosurveillance prolection, affecting the process of cancerization directly or indirectly. Epid emiological studies also indicated that content of Zn in serum of tumor patients was lower than in healthy persons. The results of this study showed that the serum content of Zn was closely related to gastric cancer. Though it was uncertain that there existed a cause and effect relationship. Zn was proved to play an

important role in physiological and biochemical process, disease production and c ancerization.

### *Cu content*

The Cu content in serums in gastric cancer and other tumor patients was obviously higher than in healthy group, the difference being statis tically significant. Though the relationship between Cu and cancer are controversial and the mechanism remained ambiguous, most clinical and experimental studies showed that a large variety of cancers are connected with considerably higher Cu content in serum and enhanced activity of cuprein in plasma. Our results were similar to theirs.

### *Ratio of Cu/Zn*

It was reported that contents of Cu and Zn in human serum existed proportionally and affected each other. The determination of ratio of Cu/Zn was helpful for diagnosing many diseases, observing their transformations, preventing recrudescence, and reflected the nutritive status of Zn in human body more effectively than Zn content in serum. It was said that if the ratio was above 2, it would lead to cancerization. The ratio of Cu/Zn in healthy group was markedly lower than in gastric cancer and other tumor groups, with statistically significant difference. In many gastric cancer cases, the ratio exceeded 2, indicating its implication in observing, diagnosing and distinguishing gastric cancer cases. It also provided references on etiology.

### *Se content*

Researches on relationship between Se and cancer were popular. Though they did not come to an agreement in etiology, many epidemiolo gical reports supported that Se content in serum decreased in cancer cases, especially those who suffered from tumors of alimentary canal. Measurement of Se con tent in serum is of some value in diagnosing and distinguishing the kinds of cancers. Se was one of the necessary composites of glutathione peroxidase (GSH-Px), thus can prevent lipid peroxidation from producing free radicals. Most clinical and experimental studies showed that the activity of GSH-Px in consumptive chronic and cancer patients decreased obviously. Our results indicated the content of Se in serum of gastric cancer and other tumor patients was much lower than in healthy population, with statistically significant difference. Most researches supported such a hypothesis.

## CONCLUSION

A great deal of investigations have demonstrated that contents of Zn, Cu and Se are connected with tumor. Our study indicated that in healthy population, Zn con tents in serum and ratio of Cu/Zn had significant differences between high incidence area and low incidence area while contents of serum Se and Cu were si milar, and Zn and Cu in sera of gastric cancer patients were found much higher than in healthy population by determining contents of Zn, Cu and Se in 453 serum samples collected from healthy, gastric cancer and other tumor population in high, middle and low incidence areas. Such results were identical to those presented in most epidemiological surveys. The result is of reference value for diagnosing and differentiating tumors, and has provided fundamental data for further investi gation on etiology of tumors.

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# *nm*23 expression in gastric carcinoma and its relationship with lymphoproliferation

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**Subject headings** stomach neoplasms; nm23 protein; gene expression; lymphoproliferation; lymphocytes

## INTRODUCTION

Tumor spread is a complex biological process closely related to tumor growth, which is regulated by many genes within the cell. Recent studies have revealed that nm23 is intimately related to tumor metastasis in its biochemical nature, structure and function and its regulating role of the gene itself[1]. In this study, gene product nm23 expression was performed in 97 cases of gastric cancer and observations were made on its relationship to hyperplasia of lymphatic tissue.

## MATERIALS AND METHOD

Specimens were collected from 97 cases of gastric cancer treated by radical surgery together with 482 enlarged regional lymph nodes (including 214 with reactive hyperplasia and 268 showing cancer metastases). Specimens were fixed in 100mL/L-formalin solution and embedded in paraffin wax. Sections of 4μm in thickness were made and routinely stained with HE stain. In accordance with the literature[2], gastric carcinoma was divided into: stage T1, where cancer tissue invades the mucosa or submucosa; stage T2, with invasion of muscular layer; stage T3, with invasion of serosa; stage T4, with invasion of tissue outside the serosa or of adjacent organs.

### *Observation of lymphocytes surrounding the cancer*

Lymphocytes in the advancing aspect of cancerous invasion were observed but excl uding lymphocytes in between cancer nests and the submucosal lymphocytic reaction.

Observations were made separately for each type and each stage of gastric ca rcinoma.

### *Observation of lymph node metastasis*

Changes were observed in lymph node metastasis, which were into 4 stages[3]: Stage 1, structure of lymph nodes is undamaged. Peripheral sinuses or elsewhere show invasion by solitary or multiple cancer cells which may be scattered or form cancerous foci comprised of 3 - 5 cells each; Stage 2, metastatic cancer cells comprise < 1/3 of surface area of section of lymph gland and usually with intact lymph follicles, dilated lymph sinuses filled with cancer cells and an intact lymph node capsule; stage 3, metastatic cancer cells comprise > 2/3 of cross sectional area with intact lymph node capsule; and stage 4, the lymph node and its capsule are both invaded by metastatic cancer cells, or there is invasion of surrounding fibrofatty tissue, muscle fibres, glands etc. with li ttle residual lymphatic tissue.

### *Antibody and staining methods*

One section was randomly selected from the sections made from the 4 pieces of tissue obtained from around the cancer and tested for expression of *nm*23 gene product using the streptomyces antibiotin peroxidase linkage method (S-P). DAB was used coloration, and haematoxylin for background staining.

## RESULT

Expression of nm23 of gastric carcinoma and results of examination for lymphocytes around the cancer are shown in Table 1.

### *Relationship between expression of nm23 and reactive hyperplasia in lymph nodes*

In each type of gastric cancer showing enhanced expression of nm23, reactive hyperplasia of regional lymph nodes was active, whereas this was diminished in those cases showing negative or weak expression of nm23. High expressivity of nm23 shows positive correlation with the amount of reactive hyperplasia of lymph nodes in the drainage area of the cancer, and the latter was related somewhat with the histological type of tumor. In papillary adenocarcima and tubular adenocarcinoma there was a greater amount of reactive hyperplasia, while the hyperplasia was low in adenocarcinoma, with low grade differentiation, mucinous adenocarcinoma and signet ring cell carcinoma.

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**Table 1 Results of positive expression of nm23, hyperplasia of lymphatic tissue and metastasis in various types of gastric carcinoma**

| Type of cancer | Number of cases | Positive expression of nm23 cases (%) | Enlarged lymph nodes (nodes) | Reactive hyperplasia of lymph nodes (nodes) | Cancer metastasis of lymph nodes (nodes) | | | | Lymphocytes around cancer (cases) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | I | II | III | IV | - | + | ++ | +++ |
| Papillary adenocarcinoma | 8 | 7(87.5) | 34 | 26 | 2 | 4 | 2 | 0 | 1(12.5) | 3(37.5) | 3(37.5) | 1(12.5) |
| Tubular adenocarcinoma | 17 | 14(82.4) | 105 | 38 | 4 | 28 | 29 | 6 | 0(0.0) | 3(17.6) | 8(47.1) | 6(35.3) |
| Poorly differentiated adenocarcinoma | 37 | 30(81.1) | 163 | 97 | 2 | 16 | 29 | 19 | 4(10.8) | 7(45.9) | 11(29.7) | 5(13.5) |
| Mucinous adenocarcinoma | 23 | 16(69.6) | 89 | 35 | 1 | 14 | 26 | 13 | 3(13.0) | 12(52.2) | 6(26.1) | 2(8.7) |
| Signet ring cell carcinoma | 12 | 9(75.0) | 91 | 18 | 2 | 24 | 32 | 15 | 1(12.5) | 6(50.0) | 5(41.7) | 0(0.0) |
| Total | 97 | 76(78.4) | 482 | 214 | 1 | 86 | 118 | 53 | 9(9.3) | 41(42.3) | 33(34.0) | 14(14.4) |

**Table 2 Relationship between stage of gastric cancer and expression of nm23 and hyperplasia of lymphatic tissue**

| Group | No. of cases | Positive nm23 expression No. (%) | Lymphocytes around cancer cases (%) | Reactive hyperplasia of lymph nodes (nodes) | Metastatic lymph nodes (nodes) |
|---|---|---|---|---|---|
| T 1 | 6 | 6(100.0) | 6(100.0) | 48 | 0 |
| T 2 | 15 | 14(93.3) | 14(93.3) | 52 | 4 |
| T 3 | 27 | 24(85.2) | 25(92.6) | 63 | 19 |
| T 4 | 49 | 33(67.3) | 43(97.8) | 51 | 245 |
| Total | 97 | 76(78.4) | 88(90.7) | 214 | 268 |

### *Relationship between expression of nm23 and lymph node metastasis*

In all types of gastric cancer when positive expression of nm23 protein was enhanced there was generally no spread to the lymph nodes in the drainage area of the cancer. When expression was negative, there was usually metastasis to regional nodes. There was a negative correlation between high expressivity of nm23 and the number and degree of regional lymph node involvement (Table 2).

### *Relationship between expression of nm23 and histological type and depth of infiltration in gastric carcinoma*

There was some relationship between expression of nm23 protein and the histological type of gastric cancer and the depth of invasion. A high positive rate was seen in papillary adenocarcinoma, tubular adenocarcinoma and poorly differentiated adenocarcinoma as compared with mucinous adenocarcinoma and signet ring cell carcinoma, but the difference was not marked ($P>0.05$). Positive rate of nm23 expression decreased as depth of invasion increased. Stages T1 and T2 show marked difference as compared with Stage T4 ($P<0.01$).

## DISCUSSION

The appearance of large numbers of lymphocytes around a cancer is the morphological expression of the body's immunological reaction to the tumor. Tumors can indirectly inhibit the antineoplastic cellular immunity of the host by means of lymphocytes. The degree of inhibition shows a parallel relationship with the degree of malignancy of the tumor[4]. A considerable portion of these lymphocytes are immunoresponsive and having lethal activity on tumor cells. They directly prevent tumor growth by releasing lymphokines or through the lethal action of cytotoxins[5,6]. Our results are basically the same with those reported in literature. The degree of lymphocytic infiltration around a cancer is related to the stage of the tumor. The covatation degree in the early stage of adenocarcinoma is more serious, and the lymph nodes with reactive hyperplasia are higher in number than those in the late stage. The degree of lymphatic tissue hyperplasia was not significantly related to the age and sex of the patient.

Gene *nm*23 is a type identified through the CDNA archives for low grade metas tasing melanoma cell line K-1735 of mice using different hybridization technics. In this gene, the levels of mRNA and the encoded protein are markedly lowered in many experimental tumors of high matastatic phenotype, hence it is considered as a matastasis-inhibiting gene. Human nm23 gene has two subtypes: nm23-H1 and nm23-H2[7,8], located in human chromosome number 17 in its long arm in the vicinity of the centromere, its encoded product being a 17kD protein composed of 152 aminoacids[9]. The relationship between human nm23 genetic protein and nu-

cleoside diphosphate kinase (N DP K) expression universally present inside cells and tumor spread and prognosis, is still contraversial in the literature[10,11]. In this group of 97 cases, positive expression is seen in 78.4% which is intimately related to the degree of lymphatic tissue hyperplasia. Lymphocytic infiltration was found around the cancer in 88 cases accounting for 90.7% of the total. Marked surrounding infiltration was seen with positive expression of nm23 in 79% of 14 such cases, while in 9 cases with absence of lymphocytic infiltration, nm23 expression was found in 32.5%, difference being significant between the two groups ($P$ <0.01).

Lymph nodes in the area of drainage of the cancer were presented with a stage of reactive hyperplasia and a stage of metastasis, each showing corresponding characterisitic changes in histological structure, and difference in quantity and in degree[12]. In this study, the degree of reactive hyperplasia of the regional lymph nodes and lymph node metastasis is related closely to nm23 gene expression. In this group, of the 482 enlarged lymph nodes, 21 had reactive hyperplasia and 268 had metastasis. When reactive hyperplasia is large in number and severe in degree, the positive expression rate of nm23 is increased, if opposite, the rate decreased. When the number of metastatic lymph nodes is large and the degree of involvement is severe, the positive expression of nm23 is reduced, and increased if opposite. This shows that level of exptession of nm23 is intima tely related to enlargement of lymph nodes in the drainage area. This means specifically, that expressivity of nm23 is in direct ratio to the amount and degree of reactive hyperplasia in lymph nodes, but inverse ratio to the number and degree of lymph node involvement in metastasis. It is likely that nm23 gene inhibits the metastatic action of the tumor after cell malignancy transformation. Such close relationship of nm23 with inhibition of tumor spread and the reduction of lymphatic tissue hyperplasia in gastric carcinoma awaits further investigations. Expression of nm23 gene which helps understand hyperplasia and metastasis of lymphatic tissue, and evaluate the depth of invasion of gastric carcinoma provides a useful method in radiotherapy and chemotherapy.

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# Clinical significance of Fas antigen expression in gastric carcinoma *

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**Subject headings** stomach neoplasms/pathology; Fas antigen; lymphatic metastasis; prognosis; immunohistochemistry

## INTRODUCTION

In order to determine whether Fas antigen plays a role in the gastric carcinogenic sequence, an immunohistochemical study of Fas antigen expression in gastric carcinoma and its relation to clinical status, pathomorphological parameters and prognosis was carried out and reported below.

## MATERIALS AND METHODS

### *Histological specimens*

Fifty-nine cases of surgically resected gastric carcinomas (male 37, female 22; mean age 55.6 years) were selected from the files of the Department of Pathol ogy of our hospital. All blocks were fixed in 10% formalin and embedded in paraffin. Serial sections were cut from each block in 4μm, HE stained and confirmed pathologically. All patients underwent curative resection, and followed up for 2.7 to 52 months.

### *Immunohistochemical methods*

Immunohistochemical staining for Fas antigen was performed using SP technique. S lides were deparaffinized and then were hydrated and detected with immunotist chencal kit according to the mannal of the mountecturer. The sections were then counterstained with hematoxylin. With each batch of test samples, a positive control consisting of a tissue section from liver was evaluated. A negative control was prepared for each sample using an irrelevant antibody of the same isotype as the primary antibody. The immunostaining of Fas antigen was visually classified into negative and positive groups.

### *Statistics*

Correlations between Fas antigen expression and clinicopathologic parameters were examined using Chi-square test. Survival data was analyzed by a log-rank test. $P<0.05$ was considered to be statistically significant.

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## RESULTS

### *Expression of Fas antigen in gastric carcinoma*

Twenty-seven (45.8%) of the 50 gastric carcinomas showed immunoreactivity for Fas antigen in gastric carcinoma cells. The Fas antigen immunoreactivity appeared brown or dark brown, which was mainly located in the cytoplasm (Figure 1), a few specimens simultaneously expressed Fas antigen on the cell membrane of tumor cells. Some of the mature lymphocytes infiltrating in the stroma of gastric carcinoma had Fas antigen expression with a strong staining intensity.

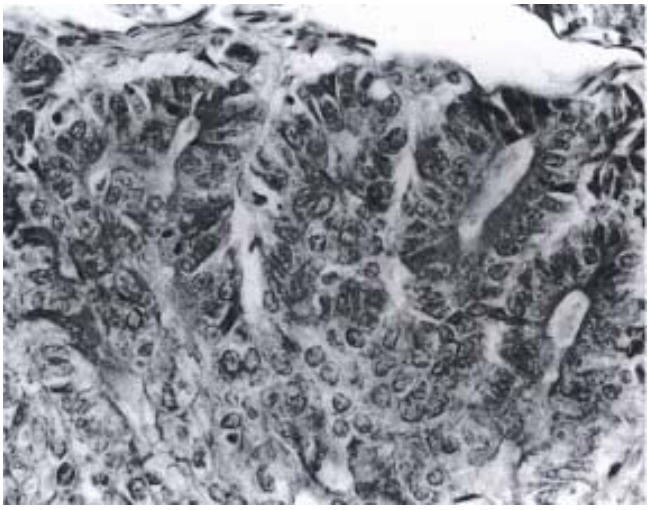

**Figure 1** Immunoreactivity of Fas antigen detected in the cytoplasm of gastric carcinoma cells. SP×200

### *Correlation between Fas antigen expression and clinico-pathological parameters of gastric carcinomas*

Fas antigen expression was related to clinical pathological staging of gastric carcinoma. The rate of Fas antigen expression was not correlated with patient age, sex, tumor size, grades of differentiation and depth of invasion ($P>0.05$ ). The immunoreactivity of Fas antigen was significantly associated with lymph node status and clinical stages of gastric carcinoma. Sixteen (61.5%) of 26 gastric carcinomas without lymph node metastasis were immunoreactive

versus 11 (33.3%) of 33 cases with lymph node metastasis ($P<0.05$). Twenty-one (58.3%) of 36 gastric carcinomas in clinical stages I and II were immunoreactive versus 6 (26.1%) of 23 gastric carcinomas in clinical stages III and IV ($P<0.05$).

### *Relationship between Fas antigen expression and prognosis*

The survival rate of patients with Fas antigen expression was compared with that of those without Fas antigen expression. Patients with Fas antigen expression in gastric carcinomas showed a significantly longer survival period as compared with those without Fas antigen expression ($P<0.05$).

## DISCUSSION

Fas antigen is a type I transmembrane protein, its molecular weight is 45 000, and it belongs to the tumor necrosis factor/ nerve growth factor receptor family[1]. Fas antigen as a receptor exists in the body and can induce a poptosis in target cells. In recent studies, Fas antigen expression has been ide ntified in various human organs, e. g. , heart, liver, lung, kidney, and ovary[2,3]. But, little is known about Fas antigen expression and its relationship with the biological behavior and prognosis of human gastric carcinoma.

In this study, we found that Fas antigen also expressed in gastric carcinoma tissues. Since Fas antigen is a transmembrane protein, it should appear both on the surface and in the cytoplasm of gastric carcinoma cells. But, in our study, most of the specimens expressed Fas antigen only in the cytoplasm of tumor cells, a few specimens expressed Fas antigen both on the surface and in the cytoplasm of tumor cells. There are several possible explanations for this. First, under pathological conditions, normal Fas antigen expression may be down-regulated, but expressi on of soluble Fas antigen is up regulated[4]. Second, Fas antigen may be affected by mutation on its DNA, or certain abnormalities may occur in the maturation process of this protein. Third, the structure of Fas antigen, which originally expressed on the surface, may be destroyed through a certain mechanism, e . g. , its binding site on the membrane undergoes proteolysis, and only cytoplasmic Fas antigen expression remains. However, the mechanism remains to be elucidated in future in vitro studies.

Our findings concerning the relationship between Fas antigen expression and the pathological characteristics of gastric carcinoma showed that Fas antigen expression could relate to lymph node status and clinical stages. The rate of Fas antigen expression was significantly higher in gastric carcinomas without lymph node metastasis than in those with lymph node metastasis, and in clinical stages I and II than in clinical stages III and IV gastric carcinomas. This indicated that aberrant Fas antigen expression may be involved in lymph node metastasis of gastric carcinoma. In addition, the survival period of patients with Fas antigen expression was longer than those without Fas antigen expression. The results demonstrated that Fas antigen expression may be of some value in predicting prognosis in patients with gastric carcinoma.

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# Distribution of nitric oxide synthase in stomach wall in rats

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**Subject headings** stomach/ physiology; nitric oxide synthase/analysis

## INTRODUCTION

It has been shown that neuronal nicotinamide adenene dinucleotide phosphate-diaphorase (NADPH-d) may correspond to the neuronal nitric oxide synthase (NOS), and may be used as a marker for NOS in the central and peripheral nervous system. Thus, NADPH-d histochemistry provides us with a mean to specifically identify neurons producing nitric oxide (NO)[1,2].

Recent pharmacological and physiological-studies demonstrated that NO is a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves in the mammalian gastrointestinal tract. It may play a very important role in then euronal regulation of gut. NOS activity is present in neurons and fibers of the major enteric nerve layer in intestine[3]. However, there have been far fewer studies of NOS activity in stomach wall. If NO is a transmitter of NANC in hibitory nerves, it should be present in neurons innervating the muscularis. What proportion of nerves produce NO? What is the pattern of innervation of these neurons? To answer these questions, we examined the distribution and morphological feature of NOS positive neurons in the stomach wall with improved whole mount preparation technique.

## MATERIALS AND METHODS

Adult male Wistar rats weighing 210g-250g were provided by the Center of Laboratory Animals of our university. The experimental rats were fasted overnight prior to the experiment, and anaesthetized with sodium pentobarbitone (50 mg·kg$^{1}$, ip). Stomach was excised and rinsed with PBS and dipped in 40 mL/L paraformaldehyde for 4 h, then stored in 200 g/L sucr ose in PBS for 48 h at 4℃. Thereafter, whole mount preparations were made, and histochemistry staining of NADPH-d and control test were performed as previously reported[4].

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### *Statistical analysis*

All results were expressed as the $\bar{x}\pm s$, and data were analyzed by S tudent's $t$ test. $P$ values <0.05 were considered statistically signifi cant.

## RESULTS

Our study showed that NOS was widely distributed in the gastric wall, and most of them were located in the myenteric plexus, and distributed in submucosal plexus, gastric mucosal epithelium and gastric gland. In the myenteric plexus, the cytoplasm of the NOS positive neurons completely labelled except for the nucleus. The cell body shape was basically similar, most of them shaped round, oval or fusiform while their density, size and staining intensity varied greatly in the different parts of stomach. The density was (62 ± 38) cells/mm$^2$, (43 ± 32) cells/mm$^2$ and (32 ± 28) cells/mm$^2$ respectively in the antrum, body and fundus ($P$<0.01). Tow subtypes of NOS positive neurons could be distinguished on the basis of size, staining intensity and number of progresses. In fundus, about 75% neurons were large, and dark-stained. Neurons of the second subtype were sligh tly smaller, with only one or two processes and were mainly located in the antrum (approximately 65%). In the body of stomach, the character of NOS positive neu rons was an intermediate state from fundus to antrum.

## DISCUSSION

The results of our experiment provide the first morphological evidence for the presence of NOS positive neurons in the stomach myenteric plexus. Nerve bundles also contained a large number of reactive fibers. Many bead-like structures strung together by NOS positive varicosities in nerve fibers, some were closely adherent to the outer walls of blood vessels and smooth muscle fibers. This finding has provided morphological evidence of NO involved in the modulation of motility and blood circulation of gastrointestinal tract. The significant difference of the distribution of NOS positive neurons among the myenteric plexus in different parts of the stomach may be related to the physiological function of the stomach.

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