# Expression and it´s significance of MMP–2 and TIMP–2 proteins in gallbladder carcinomas*

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**Abstract** **Objective** To study the relationships between expression of MMP-2 and TIMP-2 protein and clinical-pathological parameters in gallbladder carcinomas. **Methods** Carcinomas (n=45) and polypoid lesions of the gallbladder (n=15) were studied. Expression of MMP-2 and TIMP-2 protein was examined by immunohistochemical avidin-biotin-complex method and the image analysis. Clinical-pathological data in patients with carcinomas of the gallbladder such as histological type, grade of differentiation, level of infiltration, liver invasion and lymph node involvement, etc were recorded. **Results** The average level (1.123±0.108vs1.0301±0.054, P=0.002) of MMP-2 expression was significantly higher in carcinoma of the gallbladder than in polypoid lesions of the gallbladder. The significant difference was found between the expression of MMP-2 in early stage and advanced tumors. But there are no correlations between MMP-2 protein expression and histological type, differentiation degree, infiltration level, lymph node involvement or liver invasion. Though correlation was not observed between TIMP-2 expression and histological type or differentiation degree, the significant relationship was found between TIMP-2 expression and different Nevin stage, infiltration level, local lymph node involvement or liver invasion in patients with carcinoma of the gallbladder ($P<0.05$). **Conclusions** Expression of TIMP-2 protein could reflect more accurately biological character of gallbladder carcinomas when compared with MMP-2. If united examination, MMP-2 and TIMP-2 could help to differentiate malignant lesions from benign ones of the gallbladder and TIMP-2 might be a significant clinical indicator in the judgment of invasion or metastasis and the estimating of prognosis in patients with carcinoma of the gallbladder.

**Key words** gallbladder neoplasm; MMP-2; TIMP-2; immunohistochemistry; image analysis

Primary carcinoma of the gallbladder represents a very lethal malignant tumor because of its early metastasis, strong invasion and poor prognosis[1-4]. It is very important to estimate the malignant degree and invasion tendency in order to guide clinical diagnosis and treatment of gallbladder carcinoma. Breakage or degradation of ECM (extracellular matrix) and BM (basement membrane) is necessary in the process of tumor invasion [5-6]. Matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs), specially, MMP-2 and its tissue inhibitor (TIMP-2) take important roles in degradation of ECM and BM[7] and relating to tumor invasion [8-10]. So far, there are no reports on expression of MMP-2 and TIMP-2 in development process of gallbladder carcinoma. An effort is presently made to examine expression of MMP-2 and TIMP-2 proteins in carcinomas and polypoid lesions of the gallbladder, to study relationships between expression of MMP-2, TIMP-2 and clinical-pathological parameters in patients with carcinoma of gallbladder and to evaluate their clinical significance.

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## *MATERIALS AND METHODS*

### Samples

45 carcinomas and 15 polypoid lesions of the gallbladder underwent operational resection and confirmed histopathologically at Tongji hospital of Tongji University from 1995 to 2000 were studied. All samples were fixed in 10% formalin and embedded in paraffin. In 45 patients with carcinoma of gallbladder, there were 6 males and 39 females with a mean age of 61.9 years (rang 36~80 years). Of these, there were histologically 30 adenocarcinomas, 6 papillary carcinomas and 9 others (squamous, mucinous, undifferentiated and clear cell carcinoma); there were 24 G1 (well differentiation, 11 G2 (moderate differentiation) and 10 G3 (poor differentiation), according to the criteria established by World

Health Organization for histological type of tumors of the gallbladder and extrahepatic bile ducts[11]. Clinical-pathological data of each patient with carcinoma of gallbladder such as histological type, grade of differentiation, level of infiltration, liver invasion and lymph node involvement, etc were recorded. 15 polypoid lesions of the gallbladder were used as controls. Of them, there were 7 males, 8 females with a mean age of 45.5 years (range 27~74 years).

**Methods**

**Immunohistochemistry**

Immunohistochemical staining was performed on sections from formalin-fixed paraffin-embedded blocks by the avidin-biotin-complex method (SABC kit, BOSTER). Monoclonal MMP-2 antibody (Neomarker's) was used at a concentration of 1:100 and TIMP-2 antibody (Antibody Diagnostic) at 1:20. Goat serum, biotinylated secondary antibody (goat anti-mouse IgG) and DAB are all purchased from BOSTER. For negative control, the slides were treated with PBS in place of primary antibody.

**Quantified analysis of stained intensity**[12]

Stained intensity was quantified with software of analysis system of clinical-pathological image (version 2, for windows 95/OSR). These methods were as follows: (1) estimating percentage of positive tumor cells, no positive cells, regarded as 0%. (2) examining average gray value of positive cells of individual slide with image analysis system to be used as antigen concentration. (3) calculating antigen content: Content=gray value × percentage of positive cells. Control samples were examined with same methods.

**Data alternating and statistical analysis**

Because some samples were negative the examined data need to be alternated. Regarding examined data as X, alternated data as Y. Alternating methods were as follows:

$$Y=ex \qquad e=2.71828$$

All the statistical analyses were performed using SPSS 10.0 for windows. $P<0.05$ or $F<0.05$ was considered to be of statistical significance

## *RESULTS*

**Expression of MMP–2 and TIMP–2 proteins in carcinomas and polypoid lesions of the gallbladder**

Expression of MMP-2 and TIMP-2 proteins was observed in tumor cells and epithelial cells of benign lesions, tumor stromal tissues, muscularis of gallbladder and vas wall, some endangium were stained. The proteins of MMP-2 and TIMP-2 were mainly expressed in cytoplasm of positive cells. Although the expression pattern of MMP-2 and TIMP-2 proteins in gallbladder carcinomas was identical to that in polypoid lesions of the gallbladder, expressed value of MMP-2 in carcinomas was significantly higher than that in polypoid lesions. But there were no difference in TIMP-2 protein expression between two groups (Table1, Figure 1, 2).

**Table 1** Expression of MMP-2 and TIMP-2 proteins in carcinomas and polypoid lesions of the gallbladder

| | n | Expression value (mean±s) | |
|---|---|---|---|
| | | MMP-2 | TIMP-2 |
| GBC | 45 | 1.1231±0.108* | 1.077±0.090 |
| PLG | 15 | 1.0301±0.054 | 1.104±0.072 |

* $P=0.002$ GBC: gallbladder carcinoma
PLG: polypoid lesions of the gallbladder

**Relationships between expression of MMP–2 and TIMP–2 proteins and clinical–pathological parameters in patients with carcinoma of the gallbladder**

Relationship between expression of MMP-2 and TIMP-2 proteins and clinical-pathological parameters in patients with carcinoma of the gallbladder was shown in table 2 ( Figure 3~8). There were no relationships between expressions of MMP-2 or TIMP-2 proteins and their histological type or differentiated degree (F test, $P>0.05$). Based on Nevin stage criteria, our files included early stage (S1, S2. n=11) and advanced stage tumors (S3~S5, n=34). Expression of MMP-2 protein was only correlated with Nevin stage ($P<0.05$), while expression of TIMP-2 proteins was positively correlated with Nevin stage and infiltration level ($P<0.05$), but was reverse correlated with lymph node metastasis and liver invasion ($P<0.05$).

## *DISCUSSION*

One of the typical characteristics of malignant tumor is its invasion and metastasis. Its ability of invasion and metastasis is mainly responsible for their lethality. So it is necessary to understand the molecular and cellular mechanism of tumor dissemination so as to develop novel therapies based on this knowledge. Tumor invasion is considered to be a dynamic, complex, and multi-step process[13], but the essential step is degradation of

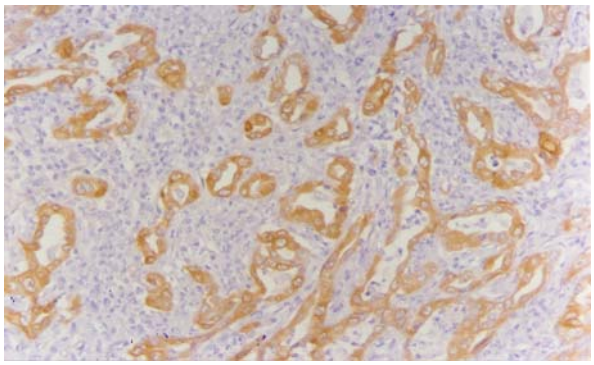

Fig.1 Expression of MMP-2 proteins in adenocarcinomas cells of the gallbladder(×100)

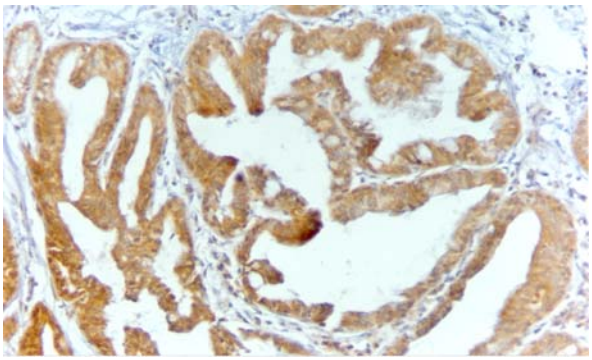

Fig.2 Expression TIMP-2 proteins in adenocarcinomas cells of the gallbladder(×100)

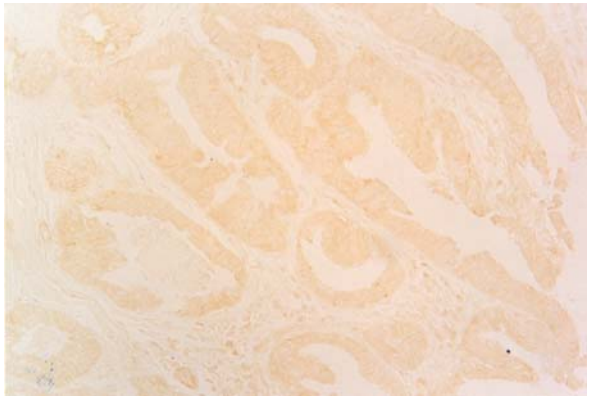

Fig.3 Expression of MMP-2 proteins in adenocarcinomas cells of the gallbladder(×100)

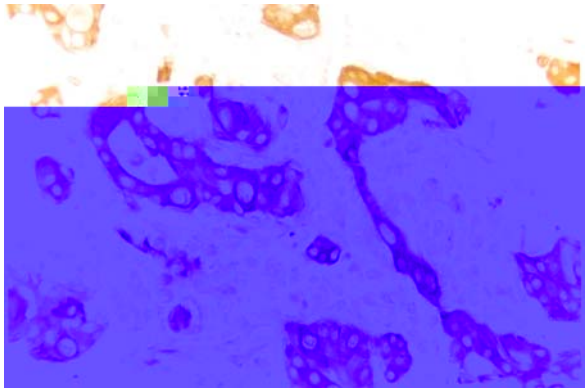

Fig.4 Expression of MMP-2 proteins in adenocarcinomas of the gallbladder with Nevin IV-stage

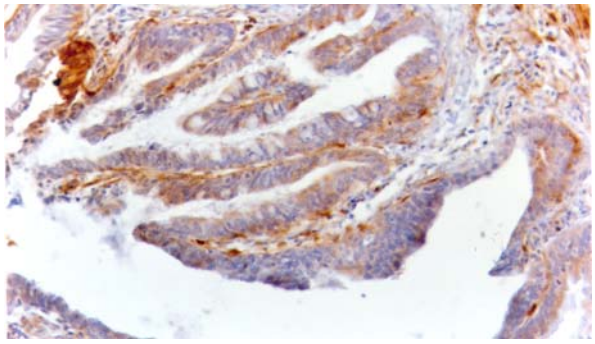

Fig.5 Expression of MMP-2 proteins in adenocarcinomas of the gallbladder with LN(-)(Expression level 1.101,×200)

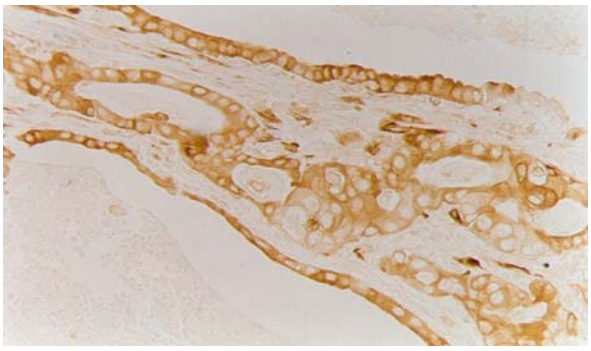

Fig.6 Expression of MMP-2 proteins in adenocarcinomas of the gallbladder with LN(+)(Expression level 1.270, ×200)

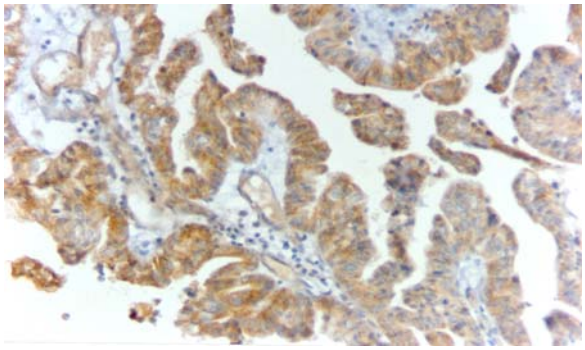

Fig.7 Expression of TIMP-2 proteins in adenocarcinomas of the gallbladder with Ln(-)(Expression level 1.88×200)

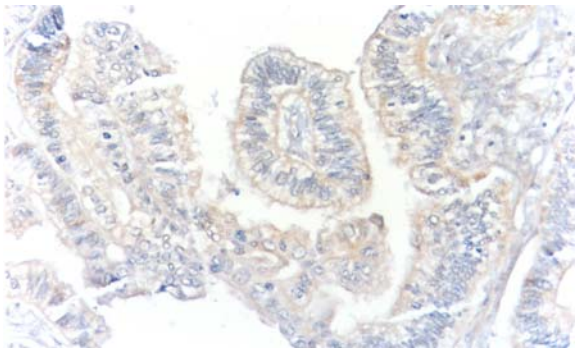

Fig.8 Expression of TIMP-2 proteins in adenocarcinomas of the gallbladder with LN(+)(Expression level 1.122,×200).

Table 2 Relationships between expression of MMP-2 and TIMP-2 proteins and clinical-pathological parameters in patients with carcinoma of the gallbladder.

| | n | Expression value (mean±s) | |
|---|---|---|---|
| | | MMP-2 | TIMP-2 |
| histological type | | | |
| adenocarcinoma | 30 | 1.140±0.113 | 1.077±0.089 |
| papillary carcinoma | 6 | 1.101±0.106 | 1.117±0.094 |
| others | 9 | 1.082±0.086 | 1.042±0.084 |
| differentiated degree | | | |
| well | 24 | 1.117±0.099 | 1.089±0.095 |
| moderate | 11 | 1.041±0.131 | 1.062±0.083 |
| poor | 10 | 1.120±0.110 | 1.064±0.086 |
| Nevin stage | | | |
| S1. S2 | 11 | 1.063±0.077* | 1.168±0.067* |
| S3~S5 | 34 | 1.143±0.110 | 1.048±0.075 |
| Infiltration level | | | |
| muscular | 13 | 1.088±0.099 | 1.170±0.062* |
| serosal | 32 | 1.137±0.109 | 1.039±0.069 |
| Lymph node | | | |
| LN(+) | 29 | 1.131±0.109 | 1.039±0.076* |
| LN(-) | 16 | 1.120±1.107 | 1.147±0.083 |
| Liver invasion | | | |
| (+) | 21 | 1.136±0.107 | 1.048±0.074* |
| (-) | 24 | 1.112±0.110 | 1.103±0.095 |

*$P<0.05$

extracellular matrix (ECM) and basement membrane (BM)[13-14]. It was reported that MMPs (matrix metalloproteinases) is important for degradation of ECM. MMPs hydrolyze specifically type Ⅳ, Ⅴ, Ⅶ, Ⅹ collagens and fibronectin, elastin, et al, which are all important component of ECM and BM, and are closely associated with invasiveness and metastasis of tumor[15-20]. TIMPs (tissue inhibitors of metalloproteinases), as the specific inhibitors of MMPs, have such ability to form tight binding, non-covalent inhibitory complexes with multiple members of MMP family that they inhibit MMPs activity of ECM degradation and have anti-metastasis function[21-23].

There have been many reports about correlation between expression of MMP-2 and TIMP-2 and tumor development[23-26]. However, no reports concerning relationships between content of MMP-2 and TIMP-2 and clinical-pathological parameters have been found in carcinoma of gallbladder. In an immunohistochemical study in 27-pancreatic cancers done by Bramhall[27], a strong correlation was found between over-expression of MMP-2 and the aggressive phenotype of pancreatic carcinoma. Another study of 177-breast cancer consisting of mainly of invasive ductal carcinoma showed that the activation rate of pro-MMP-2 is significantly higher in node-positive carcinomas than in node-negative cancers or benign neoplasms. Patients with positive staining for MMP-2 were significantly associated with shortened survival and 3.6 fold increase in the risk of death. MMP-2 was an independent prognostic indicator in multivariate analysis [28]. Ara et al[5] have observed the expression of high level of TIMP-2 in early stage and the inverse correlation with the corresponding MMP-2 in cases of neuroblastoma, and this may represent a mechanism by which tumor cells and stromal tissues control the proteolysis and remodeling of ECM that occurs during invasion and advancement of stages. Also, the correlation of TIMP-2 over-expression with better chance of survival seemed to be identical with the ability of TIMP-2 to inhibit MMP-2's activities and tumor invasion. But recently some studies suggested that expression level of TIMP-2 related to poor prognosis. Ree[29] showed that TIMP-2 mRNA level correlated with the development of distant metastases. It was reported in another study that TIMP-1 and TIMP-2 mRNA levels were positively correlated with lymph node metastasis, reduced 5-year survival and

Duke's classification in primary colorectal carcinomas. A study on stomach cancer showed that TIMP-1 and TIMP-2 were identified in 41% and 57% of tumors, respectively. Normal gastric mucosa was negative. No correlation was observed between the presence of TIMP-2 and tumor stage, histological type, lymph node status or survival[30].

Primary carcinoma of the gallbladder represents a lethal malignant tumor because of its early metastasis, poor prognosis and great difficult in management[1-4]. Focusing on the key step of ECM degradation in metastasis and discussing the expression of MMP-2 and TIMP-2 and the relationship between the expression level and clinical-pathological parameters would be important for early diagnosis, judgment of invasion or metastasis and prognosis in patients with carcinoma of the gallbladder. The present study examined the expression of MMP-2 and TIMP-2 in primary carcinomas of the gallbladder by immunohistochemical and image analysis methods so as to evaluate accurately invasive potential of tumor cell. Results showed that several type tissues expressed MMP-2 and TIMP-2 protein, such as tumor cells, muscularis of gallbladder and vas wall, stromal cells and epithelial cells of benign lesions, indicating that MMP-2 and TIMP-2 come from epithelial cells (normal or transformed) and other stromal cells. The staining pattern of tumor cells and epithelial cells of benign lesions was cytoplasmic type, identical to previously immunohistochemstry study[31-32]. Although expression of MMP-2 and TIMP-2 could be observed in gallbladder carcinomas and polypoid lesions of the gallbladder, the MMP-2 expression level in tumor was significantly higher than in benign lesion. There wee no difference in TIMP-2 level between two groups, indicating that relatively high expression of MMP-2 was the basis of tumor invasion and metastasis.

With regard to correlations between the expressions of MMP-2 or TIMP-2 and clinical-pathological parameters in patients with carcinoma of the gallbladder, the present study showed that expression of MMP-2 related to Nevin stage. Expression value in advanced stage was obviously higher than in early stage ($1.143 \pm 0.110$ vs $1.063 \pm 0.077$, $P<0.05$), but has no relationship with lymph node status and infiltration level. These seemed in contradiction with above reports. The reason was, on one hand, difference of study methods and antibodies[14], on the other hand, double effects of TIMP-2. TIMP-2 can not only inhibit MMP-2 activities but also take part in activation of MMP-2 on the cell surface. If the relation between MMP-2 and TIMP-2 was not considered only examining expression of MMP-2, invasive characteristics would not be reflected completely. In addition, there were no significant difference in expression value of TIMP-2 between groups of different histological type and grade of differentiation. Expression of TIMP-2 in early stage was obviously higher than in advanced stage, and correlated with infiltration level, local lymph node metastasis and liver invasion. All these suggested that there were relationships between TIMP-2 expression and clinical parameters standing for prognosis in patients with carcinomas of the gallbladder. TIMP-2 may be one of indicators judging Nevin stage, invasion and lymph node metastasis.

In view of the important role of TIMPs in tumor invasion and metastasis, inhibition of MMPs activity has been investigated as a method of preventing or decreasing tumor spread and MMP inhibitors are being tried in tumor therapy. Several pharmaceutical companies are currently developing low-molecular-weight MMP inhibitors for clinical use. One of the first to be tested in patients was batimastat (BB-94, developed by British Biotech together with Oxford University), a potent broad-spectrum inhibitor of MMP-1, -2, -3, and -9. Batimastat has been reported to have anti-metastasis effects in several animal models[33]. Marimastat (British Biotech) is a second-generation synthetic MMP inhibitor, structurally similar to batimastat, is water-soluble and can be given orally. The results of phrase 3 clinical trials are awaited. MMP inhibitors have been proved to possess inhibitory function in tumor spread and development and offered an available method for treating primary gallbladder carcinoma.

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