

• GASTRIC CANCER •

Relationship between matrix metalloproteinase-2 mRNA expression and clinicopathological and urokinase-type plasminogen activator system parameters and prognosis in human gastric cancer

Feng Ji, Yue-Liang Chen, En-Yun Jin, Wei-Lin Wang, Zi-Li Yang, You-Ming Li

Feng Ji, Yue-Liang Chen, En-Yun Jin, Wei-Lin Wang, Zi-Li Yang, You-Ming Li, Department of Gastroenterology, First Affiliated Hospital of Medical College, Zhejiang University, Hangzhou 310003, Zhejiang Province, China

Correspondence to: Dr. Feng Ji, Department of Gastroenterology, First Affiliated Hospital of Medical College, Zhejiang University, Qingchun Road 79, Hangzhou 310003, Zhejiang Province, China. jifeng1126@sina.com.cn

Telephone: +86-571-87236568

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Abstract

AIM: To investigate the relationship between matrix metalloproteinase-2 (MMP-2) mRNA expression and clinicopathologic and urokinase-type plasminogen activator (uPA) system parameter and prognosis in human gastric cancer.

METHODS: Expression of MMP-2 mRNA, uPA, and uPA-R mRNA in tumor tissues and ≥ 5 cm adjacent normal tissues from 67 cases of gastric cancer was studied using RT-PCR and Northern blot respectively. Survival analyses were done using the Kaplan-Meier method.

RESULTS: The expression rates of MMP-2 mRNA, uPA and uPA-R mRNA in tumor tissues (31%, 41%, and 51%, respectively) were significantly higher than those in ≥ 5 cm adjacent tissues (19%, 11%, and 9%; $\chi^2 = 4.59, 43.58,$ and 53.24 respectively, $P < 0.05, 0.0001,$ and $0.0001,$ respectively). Expression of MMP-2 mRNA was significantly correlated with lymph node metastasis (metastasis: 61.9%, no metastasis: 39.1%, $\chi^2 = 7.61, P < 0.05$), Lauren's classification of diffuse/mixed types: 54.2%, intestinal type: 26.3%, $\chi^2 = 4.25, P < 0.05$, expression of uPA and uPA-R mRNA (uPA+: 55.1%, uPA-: 22.2% and uPA-R+: 54.9%, uPA-R-: 18.8%, $\chi^2 = 5.72$ and 6.40 respectively, $P < 0.05$). Kaplan-Meier survival analysis of MMP-2 mRNA expression did not show significant difference in all 67 cases, but revealed an association of the expression of MMP-2 mRNA, uPA, and uPA-R mRNA with worse prognosis ($P = 0.0083, 0.0160,$ and $0.0094,$ respectively).

CONCLUSION: MMP-2 may play an important role in the development of invasion and metastasis of gastric cancer.

INTRODUCTION

Gastric cancer is one of the leading malignancies in many countries. In spite of radical surgeries, such as sub-total or total gastrectomy combined with other modalities, its cure rate is quite low because of local invasion and metastasis, which are known to be associated with increased activities of several proteolytic enzymes, including matrix metalloproteinase (MMP) and plasminogen activators. MMP-2 (72 ku type IV collagenase) is a member of the MMP gene family which degrades the macromolecules of connective tissues and ECM, such as collagen, proteoglycans, laminin and fibronectin. Thus MMP-2 is believed to play an important role in tumor invasion and metastasis^[1]. Our studies have shown that MMP-2 mRNA and protein are overexpressed in gastric cancer and related to the clinicopathologic parameters of cancer. In this context evaluation of MMP-2 expression in esophageal cancer, ovarian cancer, meningiomas and other tumors appears as a useful prognostic indicator^[2-7]. MMP-2 has been detected preferentially on cells from advanced gastric carcinoma by immunohistochemistry and an association of MMP-2 with poor prognosis of cancer patients has been suggested^[7].

There are two types of plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator. In particular, the uPA and its specific cell surface receptor uPA-R, play important roles in tumor invasion and metastasis. Tissue levels of uPA, uPA-R are higher in several malignant tumors than those of the corresponding adjacent normal tissues, such as gastric cancer and esophageal squamous cell carcinoma^[8-10]. Many studies have shown that inhibition of uPA activity or uPA binding to uPA-R results in the suppression of tumor growth and reduces or abolishes formation of metastasis^[11].

The current study was designed to examine the possible

association between the expression of MMP-2 mRNA and clinicopathologic and uPA system parameters and prognosis in human gastric cancer.

MATERIALS AND METHODS

Samples

The samples of tumor tissue and ≥ 5 cm adjacent normal tissues from 67 gastric cancer patients were obtained from the Surgery Department of our hospital and People's Hospital of Yunhe County, Zhejiang. Gastric tissues from 10 benign ulcer patients after partial gastrectomy were used as controls. The histological diagnosis was made by the pathologists of these two hospitals. Patients' clinical features are shown in Table 1. The mean age was 62.2 years (SD 9.3, range 23-78 years), the male/female ratio was 1.79 (43/24). All samples were quenched in liquid nitrogen immediately after operation and then stored at -70 °C for study.

Table 1 Relationship between MMP-2 mRNA expression and clinicopathologic and uPA system parameters

Variable	Case number	Expression of MMP-2 mRNA, n (%)	χ^2	P
Depth of invasion				
Early cancer	11	3 (27.3)	1.91	>0.05
Advanced cancer	56	28 (50.0)		
Differentiation			0.65	>0.05
Well-moderate	16	6 (43.8)		
Poor	51	25 (47.1)		
Lymph node metastasis			7.61	<0.01
Positive	21	13 (61.9)		
Negative	46	18 (39.1)		
Liver metastasis			3.01	>0.05
Positive	8	6 (75.0)		
Negative	59	25 (42.4)		
TNM stage			3.49	>0.05
I+II	13	3 (23.1)		
III+IV	54	28 (51.9)		
Lauren's classification			4.25	<0.05
Diffuse/mixed types	48	26 (54.2)		
Intestinal type	19	5 (26.3)		
uPAmRNA			5.72	<0.05
Positive	49	27 (55.1)		
Negative	18	4 (22.2)		
uPA-RmRNA			6.40	<0.05
Positive	51	28 (54.9)		
Negative	16	3 (18.8)		

RT-PCR analysis of MMP-2 mRNA

dNTP, RNAsin and MMLV reverse transcriptase and Taq DNA were provided by Stratagene, La Jolla, CA, USA. MMP-2 primer pair was synthesized by Shanghai Cell Research Institute, Chinese Academy of Sciences and its sense and anti-sense were 5'-ACAAAGAGTGGCAGTG-CAA-3' and 5'-CACGAGCAAAGGCATCATCC-3' respectively. The expected size of MMP-2 product was 302 bp. Total RNA was extracted from frozen tissues by cesium chloride purifying method. A total amount of 20 μ L reaction solution contained 5 μ g RNA sample tissue, 1 mmol/L dNTP, 10 U RNAsin, 100 mmol/L Tris-HCl pH 8.4, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 200 mg/mL

BSA, 100 pole random six-polyoligonucleotide and 100 U MMLV reverse transcriptase. The reverse transcriptional condition was at 37 °C for 1 h, and at 95 °C for 5 min. Twenty microliters of cDNA reverse transcriptase product was put in PCR reaction solution containing 100 mmol/L MgCl₂, 200 mg/mL BSA, 30 pmol sense and anti-sense primers, and then 2 U Taq DNA polymerase was added in the solution. The PCR amplification conditions were: denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min for 35 cycles. Ten microliters of DNA amplified product was subjected to electrophoresis in 4% agarose gel, stained with ethidium bromide and observed under ultraviolet light. The photographs of PCR results were used to measure the level of optical density (*A*) of MMP-2 cDNA bands by densitometry (Backman CD 2000).

Northern blot analysis of uPA and uPA-R mRNA

The probes of uPA and uPA-R cDNA encoding the PGEM recombinant plasmid of human uPA cDNA 387 bp Acc I-BglII and uPAR cDNA 585 bp BamHI respectively were provided by Shanghai Sangon Biotechnology and β -actin DNA probe (1.0 kb) was provided by Huamei Biotechnology. All probes used in Northern blot analysis were labeled with α -³²P-dCTP in Shanghai Sangon Biotechnology. Total RNA was isolated from frozen tumor tissue and ≥ 5 cm adjacent normal tissues using a guanidine isothiocyanate method^[12]. Twenty-five micrograms of total RNA was fractionated in 0.8% agarose gel containing 1% formaldehyde at 30 V overnight, transferred to a nylon membrane and fixed by UV cross-linking. The filter was hybridized at 80 °C for 2 h with a radioactive labeled probe in ExpressHyb solution (Clontech), washed with 2 \times SSC/0.05% SDS for 40 min at room temperature and then with 0.1 \times SSC/0.1% SDS for 40 min at 50 °C. The probe was removed from the blot by incubating with 0.5% SDS in H₂O at 90 °C for 10 min. The filter was then re-equilibrated in the ExpressHyb solution and re-probed with a new sequence. The hybridization signals were quantitated by scanning the autoradiograms with a laser densitometer (Computing Densitometer Model QTM970), and normalized to the quantity of mRNA and the specific activity of different probes.

Statistical analysis

The significance of differences in expression rates and *A* values among groups was determined by χ^2 test and Student's *t*-test respectively. Kaplan-Meier analysis was used to evaluate group oriented life-table curves which were confirmed by log-rank statistics. *P*<0.05 was considered statistically significant. All statistical analyses were performed using SPSS software.

RESULTS

Expression of MMP-2, uPA, and uPA-R mRNA in tumor and adjacent tissues

In 67 cases of gastric cancer, the positive rate of MMP-2 mRNA expression was significantly higher in tumor tissues (expressed in 31 cases) than in ≥ 5 cm adjacent normal ones (expressed in 19) ($\chi^2 = 4.59$, *P*<0.05, Figure 1). The *A*

values of MMP-2 cDNA in 10 normal controls were very low and considered to be negative. The average A value of MMP-2 cDNA was 4.05 times higher in tumor tissues than in ≥ 5 cm adjacent normal tissues ($t = 3.437$, $P < 0.01$). The positive rates of uPA and uPA-R mRNA expression were significantly higher in tumor tissues (expressed in 49 and 52 cases respectively) than in ≥ 5 cm adjacent normal (expressed in 11 and 9 respectively), ($\chi^2 = 43.58$ and 53.24 respectively, $P < 0.001$). The average IOD value of uPA and uPA-R was 5.1 and 5.5 times higher in tumor tissues than in ≥ 5 cm adjacent normal ones ($t = 3.841$ and 4.026 , $P < 0.001$, Figure 2).

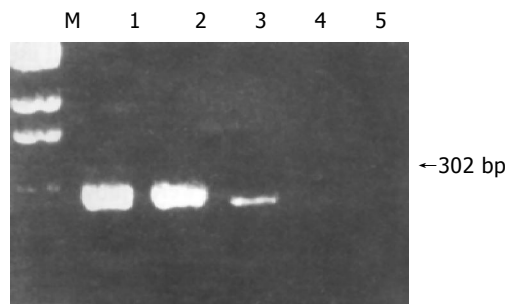


Figure 1 Expression of MMP-2 mRNA in gastric cancerous tissues and tumor adjacent tissues. Lane M: DNA marker; lanes 1 and 2: gastric cancerous tissues where MMP-2 mRNA is overexpressed; lanes 3 and 4: tumor adjacent tissues; lane 5: normal control.

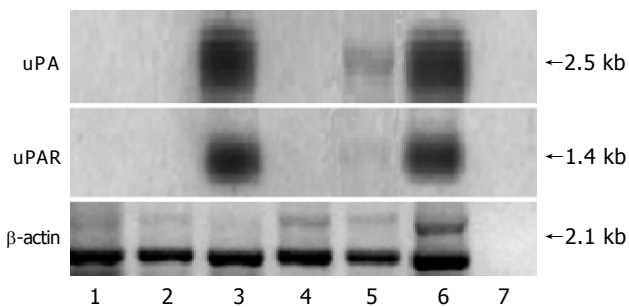


Figure 2 Expression of uPA and uPA-R mRNA in gastric cancerous tissues and tumor adjacent tissues. Lanes 1 and 4: normal controls; lanes 2 and 5: tumor adjacent tissues; lanes 3 and 6: gastric cancerous tissues where uPA and uPA-R mRNA are overexpressed; lanes 7: empty control.

Relationship between MMP-2 mRNA expression and clinicopathologic and uPA system parameters (Table 1)

Although the expression rates of MMP-2 mRNA were lower in cases with early cancer, MMP-2 mRNA expression in tumor tissues correlated neither with depth of invasion, differentiation and liver metastasis nor with TNM stage. A significant correlation was seen, however, between lymph node metastasis, Lauren's classification and positive expression of uPA and uPA-R mRNA ($P < 0.05$).

Association between MMP-2 mRNA expression and prognosis of patients

Kaplan-Meier survival analysis of MMP-2 mRNA detection did not show significant differences in 31 positive cases with

a mean survival time (MST) of 30.41 mo and in 36 negative cases (MST: 46.51 months, $P = 0.1321$, Figure 3). Among the 48 cases of Lauren's diffuse/mixed types, MST of 26 positive cases (25.05 mo) was significantly lower than that of 22 negative cases (51.77 mo, $P = 0.0083$). Among the 49 positive cases of uPA mRNA, MST of 27 positive cases (29.79 mo) was significantly lower than that of 22 negative cases (49.07 mo, $P = 0.0160$). Among the 51 positive cases of uPA-R mRNA, MST of 28 positive cases (25.82 mo) was significantly lower than that of 23 negative cases (52.11 mo, $P = 0.0094$).

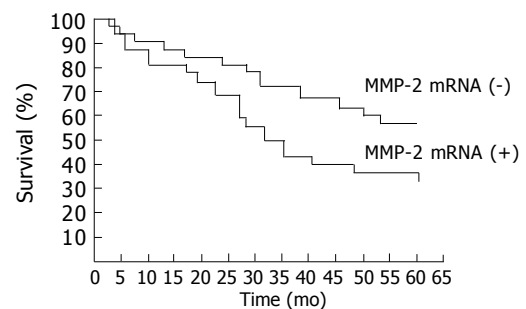


Figure 3 No significant differences between positive cases and negative cases in Kaplan-Meier survival analysis of MMP-2 mRNA.

DISCUSSION

Studies have shown that MMP-2 is mainly expressed in membrane of tumor cells while it cannot be detected in normal gastric mucosal cells and that MMP-2 positive cells are more numerous in poorly differentiated and advanced gastric cancers than in well differentiated and early gastric cancers^[13-15]. Our previous study also revealed that MMP-2 mRNA and protein overexpressions are more significant in poorly differentiated and advanced gastric cancer cells than in well differentiated and early gastric cancer cells. In the current study, MMP-2 mRNA could not be detected in 10 cases of normal controls, demonstrating that in normal gastric tissues, MMP-2 is rarely expressed or the mRNA level is too low to be detected. Schwartz *et al*^[16], reported that MMP-2 mRNA is expressed in invasive SK-GT1, SK-GT5 and SK-GT6 cell lines but not in noninvasive SK-GT2 and SK-GT4 cell lines. In ultrastructural study, MMP-2 mRNA is expressed markedly in cancer cells with rich false feet and rapid movement in culture, but insignificantly expressed in cancer cells with few false feet from unmetastatic and uninvasive gastric cancerous tissues, indicating that MMP-2 secretion is correlated with the invasion and metastasis of gastric cancer^[17,18]. Studies have shown that downregulation of MMPs or reduction of MMP-2 expression results in inhibition of tumor growth and reduces or abolishes formation of metastasis^[19,20]. In our study, the rates of positive MMP-2 mRNA expression were low in early gastric cancer with no liver metastasis and stages I and II gastric cancer, but there was no significant difference between MMP-2 mRNA expression and the degree of invasion, differentiation, liver metastasis and TNM stage of gastric cancer. However, the

rates significantly increased in lymph node metastasis and diffuse/mixed types. This phenomenon implies that gastric cancer cells with more malignant and metastatic potential may secrete much more MMP-2 protein. Sier *et al.*^[21], reported that MMP-2 expression is significantly enhanced in gastric cancerous tissues compared with that in adjacent normal mucosal tissues. In our previous studies, adjacent normal mucosal tissues had lower MMP-2 mRNA positive expression and lower MMP-2 cDNA *A* than gastric cancerous tissues. This study also showed that MMP-2 mRNA expression and MMP-2 *A* were higher in gastric cancerous tissues than in ≥ 5 cm adjacent tissues. Although the levels of MMP-2 cDNA signals in tumor-adjacent tissues were lower than in tumor tissues, MMP-2 mRNA was overexpressed in tumor-adjacent tissues, suggesting that both gastric cancer cells and adjacent mesenchymal cells, including fibrocytes, endothelium cells, macrophages, and lymphocytes have the ability to secrete MMP-2. There may be information exchange between cancer cells and these mesenchymal cells through the dissolvable intercellular substances and membrane cement factors, and such information exchange may regulate the production of MMP-2. This may be very important in elucidating the mechanism of invasion and metastasis of cancer cells^[22].

The role of MMP-2 protein and mRNA evaluation in the prognostic judgment of gastric cancer is still controversial. Mori *et al.*^[4], concluded that the expression of MT1-MMP may influence prognosis via tumor invasion of gastric wall and lymph node metastasis, and activation of MMP-2 may be clinically relevant to the progression of gastric carcinoma tumors. Caenazzo *et al.*^[15], suggested that the ratio of MT1-MMP and MMP-2 mRNA of gastric carcinoma tissue is a new preoperative molecular level prognostic factor for gastric carcinoma. Allgayer *et al.*^[5], showed that Kaplan-Meier survival analysis of immunohistochemical MMP-2 detection does not show significant differences in disease-free and overall survival of curatively resected patients. In diffuse types, however, a significant association of MMP-2 with disease-free and overall survival is revealed in curatively resected patients. These results demonstrate that there is an association of MMP-2 with prognosis of cancer patients. For diffuse gastric cancers, MMP-2 is a significant prognostic parameter; however, it is of no independent impact. In our study, the average survival period of 31 MMP-2 mRNA positive patients was shorter than that of negative ones, but the difference did not show statistical significance. In diffuse/mixed type, the average survival period was significantly shorter than negative ones, which was similar to the immunohistochemical results of Allgayer's^[5].

The uPA/uPA-R system not only takes part in tumor formation, but also plays an important role in tumor invasion and metastasis^[23-26]. In this study, there was a significant difference in uPA and uPA-R mRNA expression between gastric cancer and adjacent tissues, and the IOD was significantly higher in cancer tissues. Some studies reported that the uPA and uPA-R protein activity in tumor center, tumor border and adjacent normal tissue determined by ELISA and immunohistochemistry decreases gradually, which is similar to the results of our study^[23]. These studies show that either the protein or the mRNA level of uPA

and/or uPA-R tends to decline following the decline of malignant degree of tumors. uPA-R as the membrane-bound center of the uPA system is able to concentrate on uPA activity by focusing on receptor-bound uPA and enzymes in the proteolytic cascade like MMP-2 may be activated, resulting in stroma protein hydrolysis and tumor cell invasion and metastasis^[26-28]. Thus, MMP-2 and uPA/uPA-R system co-operate during tumor invasion and metastasis. Allgayer *et al.*^[5], reported a significant correlation between MMP-2 immunohistochemical detection and prognosis in cancers with uPA-R but not uPA overexpressed. While in our study, MMP-2 mRNA level in uPA and uPA-R positive patients increased significantly. In 49 uPA and 51 uPA-R mRNA positive patients, the survival time of MMP-2 positive patients was significantly shorter than that of negative ones, suggesting that MMP-2 can be designated as a prognostic factor when MMP-2 activated enzymes such as uPA/uPA-R are overexpressed^[15,29]. In conclusion, MMP-2 mRNA expression may play important roles in gastric cancer formation, invasion, and metastasis.

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