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Clinical Significance of Tissue Levels of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases in Gastric Cancer*

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= 국문초록 =

위암 생검조직에서의 기질단백분해효소 및 기질단백분해효소억제제 발현의 임상적 의의

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심기남 · 정성애 · 주양희 · 유 권

목 적 : 본 연구는 암의 침윤과 전이에 필수적인 기질단백분해효소(matrix metalloproteinases, MMPs)와 억제인자(tissue inhibitor of metalloproteinase, TIMPs)의 발현정도를 위암의 생검조직에서 확인하고, 임상적, 병리학적 소견과의 상관관계를 분석하여 임상적 유용성을 평가하고자 하였다.

방 법 : 2003년 8월부터 2004년 7월까지 본원에서 위암으로 진단되어 근치적 치료를 받은 30명의 환자를 대상으로 하였다. 조직은 위내시경 검사시 정상 점막 및 암으로 진단한 병변에서 생검겸자를 이용하여 얻었다. 역전사중합효소연쇄반응(RT-PCR)을 이용하여, 정상 조직을 기준으로 암조직에서의 MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2의 발현정도를 반정량법으로 비교하였다.

결 과 : MT1-MMP의 발현정도는 위암의 분화도, 림프혈관계 침윤, 림프절 전이 및 암의 병기와 유의한 상관관계가 있었다($p<0.05$). 그 외의 MMP-2, MMP-9, TIMP-1, TIMP-2의 발현은 임상적 소견과 유의한 관계를 보이지 않았다.

결 론 : 반정량적 역전사중합효소연쇄반응을 이용하여 MT1-MMP의 발현 증가 유무를 확인하는 것은 위암의 공격적 특성을 예측하는데 유용할 것으로 생각되며, 이러한 검사는 수술 전에 얻어진 생검조직을 이용하여 가능하므로 암의 공격성에 대한 정보를 수술 전에 제공할 수 있을 것으로 사료된다.

중심 단어 : 위암 · 기질단백분해효소 · 역전사중합효소연쇄반응.

Introduction

Gastric cancer usually shows extensive local tumor invasion and early spread to metastatic sites. Metastasis of gastric cancer cells depends on some only partly understood factors ; angiogenesis, cellular attachment, proteolysis, migration through the barrier into secondary sites, and, of course, colonization and proliferation in the distant organs[1]. One important step in tumor invasion is

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the penetration of the basement membrane[2]. The basement membrane is a strong barrier to the movement of tumor cells. The invasion of the basement membrane proceeds through a series of discrete steps[3]. The matrix degradation in the basement membrane is closely related to activities of various subtypes of matrix metalloproteinase (MMPs) and the corresponding tissue inhibitors of matrix metalloproteinase (TIMPs). There are now more than 20 related enzymes, which are classified as secreted or soluble-type MMPs and membrane-type (MT)-MMPs. Among the MMPs, MMP-2 and MMP-9 have been the focus of attention in connection with cancer metastasis because of their ability to degrade type IV collagen, a major constituent of the vascular basement membrane[4-6]. MT-1 MMP is the first member of the MT-MMP family to be discovered since it is tethered to the plasma membrane[7][8]. MT1-MMP is distinguished from the soluble or secreted MMPs, such as MMP-2, MMP-9, by the presence of a hydrophobic transmembrane domain at the C-terminus. The expression of MT1-MMP has been thought to initiate multiple protein cascades on the cell surface[9-11].

MMP-2 (gelatinase A ; 72-kDa gelatinase ; type IV collagenase) is an important enzyme of the MMP family which is able to degrade collagen IV, a basic component of constitutive basement membranes[12]. Like other members of the MMP family, MMP-2 is secreted in a latent form which requires cleavage of N-terminal 80 amino acids to become active[13]. The activation and enzymatic activity of MMP-2 is regulated by TIMP-2[13]. The role of MMP-2 as essential for metastasizing tumor cells has been considered. In this context, evaluation of MMP-2 expression in lung, breast, and colon cancer appeared as a useful prognostic indicator[14-16].

The TIMP-1 transfected cells or carcinoma cells with abundant expression of TIMP-1 mRNA inhibit the MMPs' activity to invade the model of basement membranes in various human carcinoma cell lines[17-19]. Recent studies have reported an alternative function of TIMP-1, i.e., as a growth factor ; it is highly homologous with erythroid potentiating activity, which is an autocrine growth factor for the erythroid leukemia cell line K562[20][21]. Moreover, TIMP-1 also shares homology with a fibroblast elongation factor that is secreted from colon carcinoma cells and which stimulates tumor cell proliferation[22]. The TIMP-1 RNA levels were higher in primary colorectal carcinomas with distant metastasis than in those without metastasis[23], and the expression of TIMPs increased with the advance of the neoplastic process[24].

The expression and involvement of several MMPs and TIMPs in human gastric carcinoma have been determined in several studies. However, the studies showed relatively conflicting results about their contribution to the clinicopathological findings and prognosis of the patients with gastric cancer. In the present study, we examined the expression of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 mRNA in human gastric carcinoma tissues by an RT-PCR assay that enabled us to analyze small sample amounts, such as biopsy specimens, before surgery and the correlation between their expression and clinicopathological parameters.

Patients and Methods

1. Patients

Biopsy specimens from 30patients (18men and 12 women) with gastric carcinoma, from whom clinical and histopathological data concerning patients and carcinomas were available, were obtained during the diagnostic gastroscopic examination between August, 2003 and July, 2004. All patients underwent gastrectomy with curative intent. Mean age was 60.4±13.8 years with a range from 32 to 82 years. Four biopsy specimens of representative samples of the carcinoma and macroscopically normal mucosa respectively were frozen and stored at −70℃ until extraction. The study was approved by Human Research Review Committee and informed consent was obtained from all patients.

2. Method

1) RNA extraction

Total RNA was extracted from biopsy tissues using the easy-BLUE™ (intron biotechnology, Korea) total RNA extraction kit. Prepared fresh tissues were added to 800 μl easy-BLUE™ reagent and homogenized using a homogenizer or equivalent and vigorously vortexed at room temperature for 10 sec. 200 μl chloroform was

added and a vortex was applied. After centrifuging the solution at 12,000rpm (4℃) for 10 minutes, 400 μl of the upper fluid was transferred to an empty 1.5ml tube. 400 μl isopropanol (2-propanol) was added and we mixed it well by inverting the tube 2−3times. It was left for 10 minutes at room temperature. After centrifuging the solution at 12,000rpm (4℃) for 10 minutes, we removed the upper layer to obtain RNA pellet. 1ml 75% EtOH was added and the solution was mixed well by inverting the tube 2−3times. The mixture was centrifuged for 5 minutes at 12,000rpm (4℃). The upper layer was discarded and the remaining RNA pellet was dried. RNA was dissolved using 20−50 μl of DEPC treated distilled water for storage at −70℃. The amount and purity of extracted RNA was quantitated by spectrophotometry.

2) cDNA synthesis

cDNA was synthesized with 5 μg of total RNA and oligo dT primer. In a sterile RNase-free microcentrifuge tube, 0.5 μg of oligo dT primer and 5 μg RNA sample were added. The tube was hit at 70℃ for 5minutes, and cooled immediately on ice. The M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) (Promega, USA) 200 unit, rRNasin ribonuclease inhibitor (Promega, USA) 25 unit, 5×RT buffer, and dNTP were added to the tube. The tube was gently mixed, incubated for 60 minutes at 42℃, and hit for 5 minutes at 95℃. The cDNA was stored at −20℃.

3) Oligonucleotide primers

The primers used were 5'-AAG ATG ACC CAG ATC ATG TTT GAG-3' and 5'-AGG AGG AGC AAT GAT CTT GAT CTT-3' for β-actin, 5'-ACC TGG ATG CCG TCG TGG AC-3' and 5'-TGT GGC AGC ACCAGG GCA GC-3' for MMP-2[25], 5'-CCA TTT CGA CGA TGA CGA GTT G-3' and 5'-CTT GTC GCT GTC AAA GTT CGA G-3' for MMP-9[26], 5'-ATC TGT GAC GGG AAC TTT GAC-3' and 5'-ACC TTC AGC TTC TGG TTG TTG-3' for MT1-MMP[25], 5'-CTT CTG GCA TCC TGT TGT TGC T-3' and 5'-GGC TGT TCC AGG GAG CCA CGA-3' for TIMP-1[26], 5'-TGC AGC TGC TCC CCG GTG CAC-3' and 5'-TTA TGG GTC CTC GAT GTC GAG-3' for TIMP-2[25]. All primers were synthesized by TaKaRaKorea Biomedical Inc.

4) PCR amplification

The amplification reaction was carried out in the 20 μl of PCR mixture containing 4 μl of the synthesized cDNA solution, 4 μl of 5×polymerase reaction buffer, 200 μM of dNTP, 0.5 μM of each primer (sense and antisense) and 1unit Taq polymerase (Promega, USA). The PCR mixture was amplified using GeneAmp PCR System 9600 (PERKIN-ELMER Corp., USA). Amplified products (10 μl) were identified by electrophoresis of PCR on 1% agarose gel containing ethidium bromide and ultraviolet (UV) illumination. The housekeeping gene, β-actin was used as a control and for semiquantitative analysis of the MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2. A negative control, with H_2O instead of cDNA, was used. The levels of gene transcripts were quantified as the ratio of the intensity of the target gene to the intensity of β-actin.

5) Statistical analysis

The results were expressed as the mean±the standard deviation. The association between the clinicopathological variables and the expression of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 was analyzed using the Student's t-test and ANOVA test. The data were considered significant if the P value was <0.05. Statistical analyses were performed using the SPSS program (v. 11.0, SPSS Inc., USA).

Results

The mean age of the patients consisting of 18 men and 12 women was 60.4±13.8 years. The gross findings were 11 cases (38.7%) of EGC and 19 cases (61.3%) of AGC, and in terms of histological differentiation, there were 6 cases of well-differentiated type (20%), 7 cases of moderately-differentiated type (23.3%), and 17 cases of poorly-differentiated/signet ring cell type (56.7%). If we consider whether the lymphatic vessel was affected or not, there were 23 cases (76.7%) of groups with lymphatic invasion and 7 cases (23.3%) of groups without invasion. The blood vessel was not affected in 26 cases (86.7%) and was affected in 4 cases (13.3%). Regarding T stage, there were 12 cases (40%) of T1, 6 cases (20%) of T2, 12 cases (40%) of T3, and 0 case (0%) of T4, and

regarding N stage, there were 17 cases (56.7%) of N0, 8 cases (26.7%) of N1, 1 case (3.3%) of N2, and 4 cases (13.3%) of N3. Regarding M stage, there were 30 cases (100%) of M0 and 0 case (0%) of M1. If we consider TNM stage on the whole, there were 15 cases (50.0%), 5 cases (16.7%), 6 cases (20.0%), and 4 cases (13.3%) of stage I, II, III, IV, respectively (Table 1).

If we look at the histological differentiation of the tumors and the mRNA expression of the proteolytic enzymes (Fig. 1), the comparison between well-differentiated and moderately/poorly differentiated types, in the case of MT1-MMP, showed that the mRNA expression was significantly higher in the latter type ($p<0.005$), while in the rest cases of MMP-2, MMP-9, TIMP-1, and TIMP-2, there wasn't any significant difference between the two groups (Fig. 2).

If we consider the affection of the lymphatic vessel by the tumor and the mRNA expression of the proteolytic enzymes, the MT1-MMP mRNA expression was significantly higher when there was the affection of the lymphatic vessel ($p<0.05$). In the rest cases of MMP-2, MMP-9, TIMP-1, and TIMP-2, there was no significant difference between the two groups (Fig. 3).

When we consider the affection of the blood vessel by the tumor and the mRNA expression of the proteolytic enzymes, the MT1-MMP mRNA expression had a tendency to be associated with invasion of the blood vessel. But there was no statistical significance ($p=0.054$). In the

Table 1. Patients' characteristics and clinicopathological parameters

Variables	N (%)
Gross findings	
EGC	11 (38.7)
AGC	19 (61.3)
Differentiation	
Well	6 (20.0)
Moderately	7 (23.3)
Poorly/signet ring cell	17 (56.7)
Lymphatic invasion	
−/+	23 (76.7)/7 (23.3)
Venous invasion	
−/+	26 (86.7)/4 (13.3)
Depth of invasion	
T1	12 (40.0)
T2	6 (20.0)
T3	12 (40)
T4	0 (0)
Nodal status	
N0	17 (56.7)
N1	8 (26.7)
N2	1 (3.3)
N3	4 (13.3)
Distant metastasis	
M0	30 (100)
M1	0 (0)
Stage	
I	15 (50.0)
II	5 (16.7)
III	6 (20.0)
IV	4 (13.3)

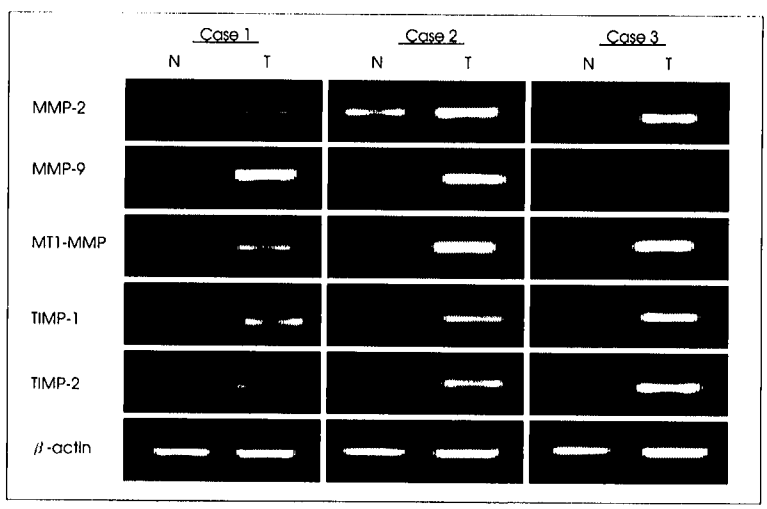

Fig. 1. RT-PCR analysis of mRNA expression for proteolytic enzymes in normal and tumor tissues. N : normal tissue, T : tumor tissue.

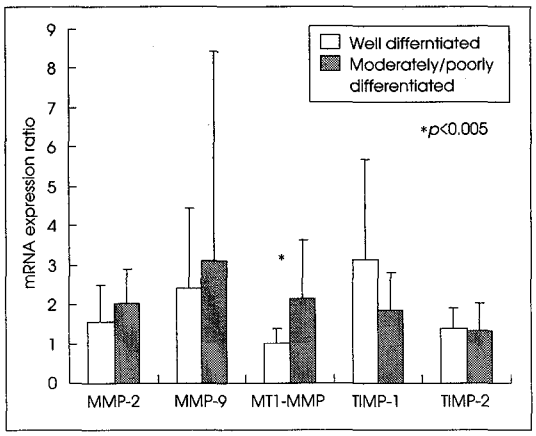

Fig. 2. Relationship between the differentiation of tumor and mRNA expression of proteolytic enzymes.

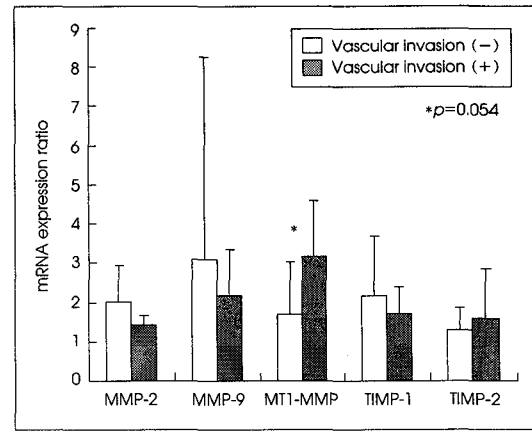

Fig. 4. Relationship between vascular invasion and mRNA ex-pression of proteolytic enzymes.

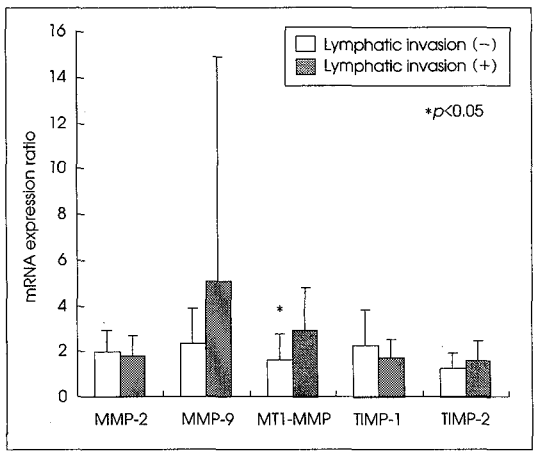

Fig. 3. Relationship between lymphatic invasion and mRNA ex-pression of proteolytic enzymes.

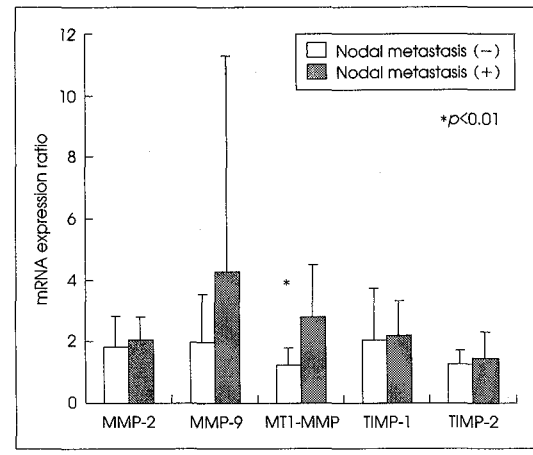

Fig. 5. Relationship between nodal metastasis and mRNA ex-pression of proteolytic enzymes.

rest cases of MMP-2, MMP-9, TIMP-1, and TIMP-2, no significant difference was between the two groups (Fig. 4).

Regarding the relation between the presence or absence of invasion to the lymph node of the tumor and the mRNA expression of the proteolytic enzymes, the MT1-MMP mRNA expression was significantly higher when there was invasion ($p<0.01$). In the rest cases of MMP-2, MMP-9, TIMP-1, and TIMP-2, there was no significant difference between the two groups (Fig. 5).

If we consider the TNM stage of the tumors and the mRNA expression of the proteolytic enzymes, the MT1-MMP mRNA expression significantly increased according to the stages ($p<0.001$). In the rest cases of MMP-2, MMP-9, TIMP-1, and TIMP-2, there wasn't

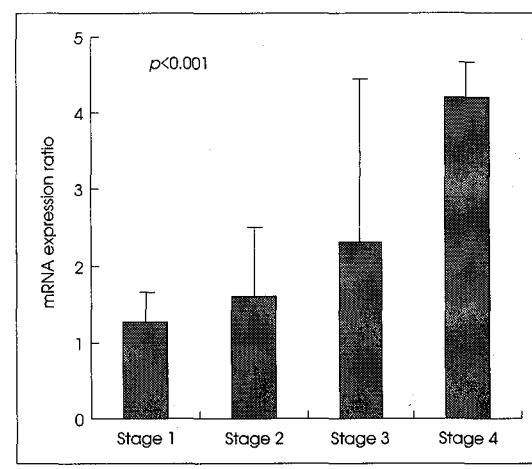

Fig. 6. Relationship between the tumor stage and MT1-MMP mRNA expression of proteolytic enzymes.

any significant difference among the stages of gastric cancer (Fig. 6).

Discussion

Invasion and metastasis are the most insidious and life-threatening aspects of cancer. A critical proteolytic event occurring early in the metastatic cascade appears to be the degradation of the basement membranes. Recent studies have suggested a major role for MMP-2 and MMP-9 in the digestion of basement membrane type IV collagen, as an important mechanism for vessel invasion and metastasis[4-6]. Although soluble MMPs have been shown to be produced as a zymogen (proMMP-2) in cells, the fate of MMPs and TIMPs after their secretion from producing cells or the spatial regulation of their molecular activation is not fully understood. However, it has been shown that the activation of MMPs is strictly controlled by plasma membrane-associated events[9-11] Briefly, of the TIMPs, TIMP-2 preferentially binds first to the MT1-MMP localized on the cell surface. The TIMP-2/MT-1 MMP complex subsequently functions as a receptor, allowing proMMP-2 to form a ternary complex. ProMMP-2 in the complex can then be activated by the adjacent TIMP-2 free MT1-MMP, and the active MMP-2 in turn activates MMP-9[9)10)26-29)]. Koyama showed that the cell surface expression of MMP-2, MMP-9, MT1-MMP, and TIMP-2 increased during the development of invasion and/or metastasis of gastric carcinoma[30)].

Because of its ability to degrade the basement membrane, MMP-2 has been postulated as a potential marker of tumor progression and prognosis. In several studies, MMP-2 staining by immunohistochemistry correlated significantly with depth of tumor infiltration (T-stage), lymph node metastasis (N-stage), and distant metastasis (M-stage)[4)5)31)]. Bando et al[32)] reported that MMP-2 immunoreactivity was associated with advanced-stage gastric cancer and that it contributed to tumor progression, invasion, and metastasis. On the other hand, Allgayer et al[33)]. have demonstrated a correlation between distant metastasis (M-stage) and MMP-2 status using immunohistochemistry, but not between lymph node metastasis and MMP-2 immunoreactivity in gastric cancer. The latter feature was also noticed by Kabashima, who also demonstrated no correlation between MMP-2 expression and lymph node metastasis[34)]. Some *in vitro* and *in vivo* experiments showed that matrix metalloproteinase levels were related to the invading and metastatic potential of colorectal cancer. Sier et al. demonstrated that the higher tissue levels of total and the pro-forms of MMP-2 and MMP-9, as well as the active form of MMP-2 indicated a poor prognosis in patients with gastric carcinoma[5)].

It has been reported that the matrix-degrading activity of MMP-9 is nearly 25 times higher than that of MMP-2, and that MMP-9 is more important for the metastatic potential of carcinoma than MMP-2[35)36)]. It was proved that MMP-9 expression by immunohistochemistry was significantly correlated with lymphatic permeation and lymph node metastasis in intramucosal gastric cancers[37)]. But another study showed that MMP-9 mRNA expression did not have significant correlation with the prognosis of the patients with hepatocellular carcinoma. In this study, the level of MMP-2 and MMP-9 mRNA expression was not correlated with clinicopathologic parameters of gastric cancer.

MT1-MMP, the first member of a more recently established group of MMPs containing a membrane-spanning sequence, has been shown to have an important role in MMP-2 activation in cell membranes and its overexpression seems to have a significant effect on tumor growth. Expression of MT-1 MMP mRNA has a tendency to be associated with a lower degree of differentiation in hepatocellular carcinoma and has a strong statistical association with a poor outcome of patients[37)]. A similar tendency was also observed in pancreatic adenocarcinomas, but the association did not reach statistical significance[37)]. And Caenazzo et al[38)]. showed that the augmented MT1-MMP mRNA expression resulted in the poor prognosis of gastric cancer. In this study, increased expression of MT1-MMP was significantly correlated with lower degree of differentiation, lymphatic invasion, venous invasion, and tumor stage of gastric cancer. To this date, there are at least four mechanisms by which MT-1 MMP can enhance tumor progression ; it can activate MMP-2 on tumor cell membranes[7)8)] ; it is a very effective degradative enzyme by itself, having substrates such as fibronectin, tenascin, nidogen, aggregan, and perlecan[39)] ; MT-1 MMP is a

very potent regulator for neovascularization[40], a phenomenon that is critical for malignant growth ; and recently MT-1 MMP was shown to process directly la-minin-5 r2 chains, which caused a strong migration effect by tumor cells over laminin-5 surfaces[41].

In general, MMPs facilitate the invasion of the tumor. On the other hand, TIMPs play an important role in inhibiting MMPs. TIMPs have been reported to be negative regulators of MMPs in human and mouse tumor models *in vivo* and *in vitro*[42]. However, in another study using clinical samples, the expression of TIMP mRNA was higher in carcinoma tissues. In studies of various carcinoma cases, such as stomach, colorectal, head and neck, and pancreas, both MMPs and TIMPs were found to correlate with an increased metastatic and invasive potential of tumor cells[23)43]. TIMP-1 RNA levels were higher in primary colorectal carcinomas with distant metastasis than in those without it, and the expression of TIMPs increased with the advance of the neoplastic process[23)25]. A discrepancy still exists, however, between the function of TIMP-1 as an inhibitor of tumor cell invasion *in vitro* and the higher expression of TIMP-1 in human carcinoma cells, according to previous reports. There are several possible explanations for this discrepancy. First of all, higher expression of MMPs was observed in tissues with invasive carcinoma cells, which induced macrophages with cytokines and thus elevated the expression of TIMPs.[44)45] Secondly, TIMP-1 has two distinct activities, i.e., a metalloproteinase inhibitory activity and a growth factor activity[21)44)45].

Although both normal and neoplastic cells produce MMPs and other proteinases, only malignant cells are invasive[46]. Therefore, it is more likely that the control of MMP activity by specific inhibitors is one of causes of the different functions of these enzymes in normal and neoplastic tissues. Ko et al[47]. has proven an im-portant role of TIMP-2 in demonstrating inverse corre-lation of TIMP-2 expression in nodal metastasis and found the fact that TIMP-2 expression in EGC is stronger than in AGC indicating that TIMP-2 may play an im-portant role in protection against MMPs. But in another study, it was shown that the expression of TIMP-2 was not associated with variable clinicopathological parame-ters, and that the status of expression of TIMP-2 was variable in many types of cancer tissues. In our study, the level of TIMP-1 and TIMP-2 mRNA expression was not correlated with clinicopathologic parameters of gastric cancer.

In summary, our data supported the previous suggestions of the importance of MT-1 MMP for malignant growth and increased MT-1 MMP mRNA expression by tumor cells in gastric cancer reflected its role in predicting the aggressive behavior of gastric cancer. As an RT-PCR assay can be performed on biopsy specimens obtained before surgery, evaluation of its expression in biopsy specimens by RT-PCR may provide useful preoperative information on tumor aggressiveness.

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