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Clinicopathological significance of MMP-2 and its specific inhibitor TIMP-2 in gastric cancer

H. Alakus[1], G. Grass[1], J.K. Hennecken[1], E. Bollschweiler[1], C. Schulte[1], U. Drebber[2], S.E. Baldus[3], R. Metzger[1], A.H. Hölscher[1] and S.P. Mönig[1]

[1]Department of Visceral- and Vascular Surgery, [2]Institute of Pathology, University of Cologne, Cologne, Germany and [3]Institute of Pathology, University of Düsseldorf, Düsseldorf, Germany

Summary. Matrix metalloproteinases (MMPs) can degrade type IV collagen of extracellular matrices and basal membranes and thus play a key role in the migration of malignant cells. In vivo, MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs). Since in a previous study we showed that the expression of MMP-2 correlates with clinicopathological parameters in gastric cancer, we have now investigated a possible correlation of MMP-2 and TIMP-2 expression with survival in gastric cancer, as well as the possible association of TIMP-2 with clinicopathological parameters.

Tissue samples were obtained from 116 gastric cancer patients who underwent gastrectomy with extended lymphadenectomy. MMP-2 and TIMP-2 expression was analysed using immunohistochemical staining and was graded semiquantitatively (score 0 – 3). High epithelial MMP-2 immunoreactivity was significantly associated with tumor stage and poor survival using the Kaplan-Meier log-rank statistical method (log-rank statistics). However, using Cox regression analysis, high epithelial MMP-2 immunoreactivity was not an independent prognostic factor. TIMP-2 showed no association with survival in gastric cancer, but the intensity of TIMP-2 staining in tumor cells correlated significantly with tumor differentiation based on the WHO and Lauren and Ming classifications, as well as with presence of distant metastasis.

Our results show that high epithelial MMP-2 expression in gastric cancer is associated with poor survival, although it is not an independent prognostic factor, and that aggressive forms of gastric cancer are associated with low TIMP-2 expression.

Offprint requests to: Stefan P. Mönig, MD, Department of Surgery, University of Cologne, Joseph-Stelzmann-Strasse 9, 50924 Cologne, Germany. e-mail: Stefan.Moenig@uk-koeln.de

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Introduction

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent neutral endopeptidases, capable of degrading various components of the extracellular matrix (ECM) (Egeblad and Werb, 2002). One important step in tumor invasion and metastasis is the degradation of collagen IV, which is a basic element of basement membranes (Liotta el al., 1991). MMP-2, also known as gelatinase A or 72 kDa collagenase IV, is a member of the MMP family and is able to degrade gelatin and type IV collagen (Chen, 1992; Ala-aho and Kahari, 2005) suggesting that it has a role in tumor metastasis and may be of prognostic significance for the survival of cancer patients. In contrast to other MMPs, MMP-2 is expressed by a large number of cell types and overexpressed in a wide variety of human cancers, including gastric, prostate, ovarian and bladder cancers (Murray et al., 1998; Davidson et al., 1999; Upadhyay et al., 1999; Vasala et al., 2003; Zhou et al., 2004).

MMP-2 is secreted as an inactive proenzyme and activated by N-terminal proteolytic cleavage (Stetler-Stevenson et al., 1989; Chen, 1992). The activity of MMP-2 is modulated by transcriptional regulation, as well as by its interaction with tissue inhibitors of metalloproteinases (TIMP). These inhibitors can form complexes either with latent or activated MMPs (Emmert-Buck et al., 1995). Particularly, TIMP-2, a non glycosylated 21 kDa protein, has been reported to be a very effective inhibitor of MMP-2 (Howard et al., 1991). During the last few years levels of MMP expression in human carcinoma tissues and their correlations with clinicopathological parameters have been intensively studied. For example Curran et al. evaluated the MMPs and TIMPs collectively in colorectal cancer, identifying

a group of colorectal cancers with poor prognosis (Curran et al., 2004). Investigation of MMP-1, MMP-2 and MMP-9 in esophageal cancer showed that the presence of MMP-1 was an independent prognostic factor (Murray et al., 1998). MMP-9 has been suggested to be a prognostic marker in stomach cancer (Sier et al., 1996; Mrena et al., 2006).

Other studies, including our own study, showed a significant correlation of MMP-2 expression in gastric cancer with depth of tumor infiltration, lymph node metastasis, distant metastasis and UICC stage (Nomura et al., 1995; Sier et al., 1996; Monig et al., 2001). However, the prognostic value of MMP-2 for gastric carcinoma survival has been analyzed in only a few studies (Allgayer et al., 1998; Kubben et al., 2006) and the results of these studies are contradictory.

Little has been published in relation to the clinicopathological and prognostic significance of TIMP-2, the specific inhibitor of MMP-2, in gastric carcinoma. In vitro studies have suggested that there may exist a correlation between TIMP-2 expression and clinicopathological parameters (Koyama, 2004).

The aim of this study was to investigate the correlation between TIMP-2 expression and the current classification systems for gastric carcinoma. In addition, we were interested in determining whether MMP-2 and TIMP-2 levels in gastric carcinomas can be used as prognostic factors for survival in gastric cancer.

Materials and methods

Patients

Patients were recruited from a prospective study between May 1996 and July 2000. All 116 patients were treated surgically for primary gastric adenocarcinoma at the Department of Visceral- and Vascular Surgery, University of Cologne. Tumor samples were routinely fixed in 5% phosphate-buffered formalin, embedded in paraffin and categorized according to tumor differentiation, UICC-, WHO-, Lauren-, Goseki- and Ming-classification. One hundred and four patients (88.6%) underwent total gastrectomy and 12 patients (10.5%) subtotal gastrectomy with extended lymphadenectomy (compartment I and II). 50 (43.1%) tumors were located in the proximal part of the stomach including the cardia. 17 (14.6%) tumors were located in the middle part of the stomach. 49 (42.6%) tumors were infiltrating the antrum.

Gastroscopic examination, endoscopic ultrasound and CT of abdomen were performed in all patients for clinical staging. Neoadjuvant chemotherapy was applied in 16 cases (13.8%) with locally advanced tumor.

Mean age was 64 years (range 33-85), 69 patients were male and 47 were female (ratio 1.47).

Two patients were not included in survival analysis since a complete follow-up was not available for these two patients. Follow up of surviving patients was at least 5 years.

MMP-2 immunohistochemistry

Tissue specimens were cut (5 µm thick) and deparaffinized according to standard histological techniques. Endogenous peroxidase activity was blocked by 3% H_2O_2/methanol for 30 min at room temperature (RT). Non-specific binding sites were blocked by normal swine serum (X 901,Dako, Copenhagen, Denmark), diluted 1:20 (v/v) in Tris-buffered saline pH 7.2 (TBS), for 30 min at RT. The sections were incubated with the primary monoclonal anti-human MMP-2 antibody (Clone CA-4001, Oncogene, Cambridge, MA, USA; clone CA-4001) overnight. The antibody reacts with the amino acids at the N-terminus of the ~72 kDa latent form of MMP-2. It was diluted 1:100 (v/v) in TBS containing 2.5% bovine serum albumin (BSA). Between each of the following steps, specimens were washed three times in TBS. After the incubation with the primary antibody biotinylated rabbit anti-mouse immunoglobulin E413 (Dako), diluted 1:400 (v/v) in TBS/2.5% BSA, was added for 30 min at RT. All sections were incubated for 30 min at RT with streptavidin-peroxidase conjugate P397 (Dako), diluted 1:400 (v/v) in TBS/2.5% BSA. The reaction was visualized by 200 µg/ml 3-amino-9-ethyl-carbazol (Sigma, St. Louis, MO, USA) in 50 mM sodium acetate buffer containing 5% dimethyl-formamide and 0.01% H_2O_2 for 30 min at RT. Slides were counterstained with haematoxylin for 2 min and mounted in glycerol jelly.

TIMP-2 immunohistochemistry

For the detection of TIMP-2, monoclonal mouse anti-human TIMP-2 antibody, clone 67-4H11, (Diagnostic International, Schriesheim, Germany) was used at a dilution of 1:200. The antibody of clone 67-4H11 recognizes the COOH-terminal domain of TIMP-2 but not TIMP-2 complexed with the precursor of human MMP-2. Before exposure to the antibody, sections were microwaved in citrate buffer (pH 6.0) for 3x5 min at 700 W. All other steps were performed as described above.

Semiquantitative analysis

The level of expression of MMP-2 and of TIMP-2 was estimated by semiquantitative evaluation and divided into four groups according to the number of positively stained tumor cells: score 0 = negative; score 1 = 30% positive tumor cells; score 2 = 30-70% positive tumor cells; score 3 = ≥ 70% positive tumor cells. Sections with score ≥ 1 were considered positive. Additionally, the dichotomization between low expression (score 0/1) and high expression (2/3) of MMP-2 and TIMP-2, as well as the dichotomization between negative (score 0) and positive (score 1/2/3) expression was performed for statistical analyses.

Immunhistochemistry and semiquantitative analysis was performed by two experienced staff pathologists, who were blinded for all other clinical data.

Statistical analysis

Associations between clinicopathological parameters and TIMP-2 scores were evaluated using Kendall-Tau b-test. Correlations between MMP-2 scores and clinicopathological parameters have been already analyzed and published (Monig et al., 2001). Relations to overall survival were evaluated with univariate analysis according to the Kaplan-Meier approach. To analyze the predictive value of MMP-2 and TIMP-2 compared to other known predictors, Cox's regression analysis was performed. The following variables were included in the conditional forward model: UICC-stage, sex, grading, WHO-type, R-status, and MMP-2. TIMP-2 was not included since univariate prognostic significance could not be shown for TIMP-2

The level of significance was set to $p < 0.05$. Unless otherwise specified, p values were given for 2-sided testing.

All statistical tests were performed using Software Package SPSS for Windows, Version 14.0 (Chicago, IL).

Results

Correlation of TIMP-2 with clinicopathological tumor parameters

Demographic characteristics, tumor grade, and stage for all patients, as well as for the groups based on intensity of TIMP-2 staining in tumor epithelia are presented in Table 1. The TIMP-2 staining pattern was positive (score 1-3) in 88 (75.86%) specimens and negative (score 0) in 28 (24.14%) samples.

The intensity of TIMP-2 staining in tumor epithelia did not correlate with sex (p=0.78), Borrmann classification (p=0.88), pT (p=0.76), pN (p=0.62) or the UICC classification (p=0.78).

A significant correlation was seen for distant metastasis stage (p=0.039), where lower staining scores (0 and 1) were associated with a positive M stage. A significant correlation (p=0.011) was also seen for Ming stages: Infiltrative tumors had less TIMP-2 staining scores than expanding tumors. Additionally there was a significant correlation of TIMP-2 expression and WHO classification (p=0.002).

A statistical association was also seen between the intensity of TIMP-2 staining and the classification according to Goseki (p=0.048).

Correlation of MMP-2 with clinicopathological tumor parameters

In 21 (18.1%) cases MMP-2 expression was negative (score 0). Positive MMP-2 expression was seen in 95 (81.9%) tumor samples. 46 of these 95 (39.66%) samples had a high expression of MMP-2 (scores 2 and 3).

We have previously reported that MMP-2 tissue status correlated with pT (p<0.01), pN (p<0.01), pM (p<0.01) and with the UICC stages (p<0.01) (Monig et al., 2001).

No correlation (p=0.094) was observed between the level of TIMP-2 expression and the level of MMP-2

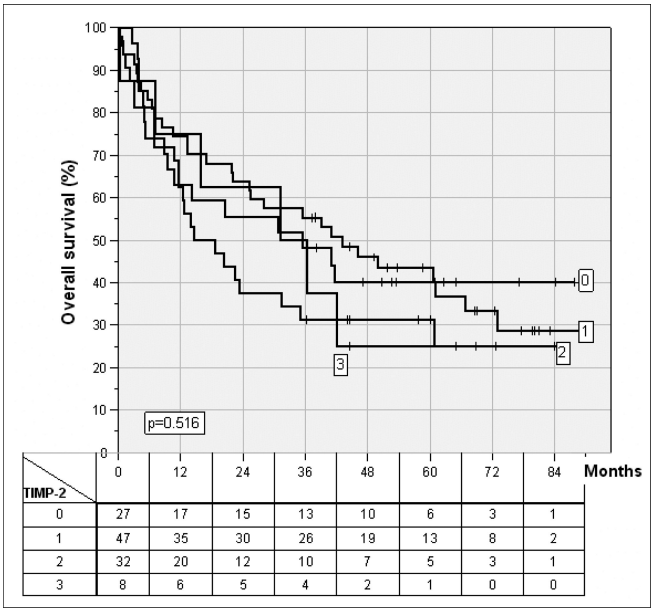

Fig. 1. Overall survival of 114 resected gastric cancer patients according to semiquantitative scoring (0-3) of immunohistochemically detected TIMP-2 levels in tumor cells (Kaplan-Meier analysis), p=0.516 (Mantel-Cox log-rank test). The table below shows the numbers of patients at risk.

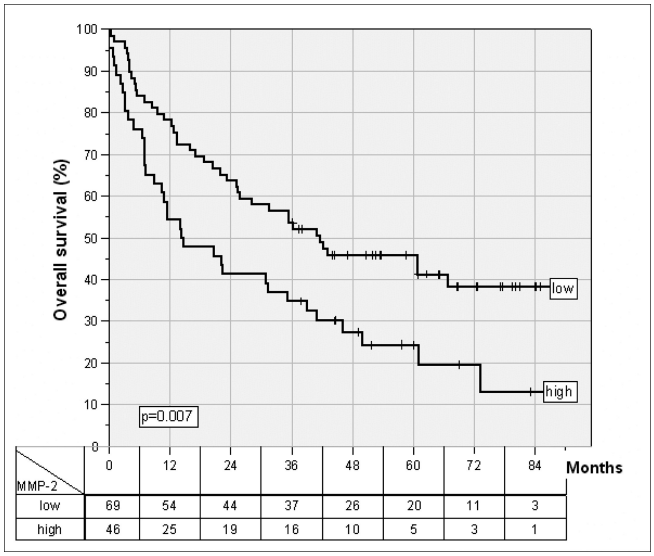

Fig. 2. Overall survival of 114 resected gastric cancer patients according to low (score0/1) and high levels (score 2/3) of MMP-2 expression. The patients at risk are shown in the table below

expression.

Survival analysis for TIMP-2 expression

Kaplan-Meier survival analysis of TIMP-2 expression did not show significant differences in overall survival (all patients: p=0.516) (Fig.1). Dichotomization of TIMP-2-negative (score 0) versus TIMP-2-positive (score1-3) cases, as well as dichotomization between low (score 0/1) and high TIMP-2 (score 2/3) levels also did not show any significant correlation (p=0.138).

Association of immunohistochemical TIMP-2

Table 1. TIMP-2 tissue status and clinicopathological parameters.

		TIMP-2 tissue status (score 0-3)				
	ALL	0	1	2	3	p value
N (%)	116	28 (24.1)	48 (41.4)	32 (27.6)	8 (6.9)	
Gender						
Men	69 (59.5)	16 (23.2)	30 (43.5)	19 (27.5)	4 (5.8)	
Women	47 (40.5)	12 (25.5)	18 (38.3)	13 (27.7)	4 (8.5)	0.78
Borrmann						
Early cancer	23 (19.8)	7 (30.4)	8 (34.8)	5 (21.7)	3 (13.0)	
I	9 (7.8)	2 (22.2)	4 (44.4)	3 (33.3)	0 (0.0)	
II	22 (19.0)	4 (18.2)	10 (45.5)	7 (31.8)	1 (4.5)	
III	25 (21.6)	7 (28.0)	7 (28.0)	10 (40.0)	1 (4.0)	
IV	37 (31.9)	8 (21.6)	19 (51.4)	7 (18.9)	3 (8.1)	0.88
WHO						
Papillary	5 (4.3)	0 (0)	2 (40.0)	3 (60.0)	0 (0)	
Tubular	68 (58.6)	13 (19.1)	26 (38.2)	22 (32.4)	7 (10.3)	
Ring cell	37 (31.9)	11 (29.7)	19 (51.4)	6 (16.2)	1 (2.7)	
Mucinous	3 (2.6)	2 (66.7)	0 (0)	1 (33.3)	0 (0)	
Unclassified	3 (2.6)	2 (66.7)	1 (33.3)	0 (0)	0 (0)	0.002
Differentation						
Well	1 (0.9)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	
Moderate	33 (28.4)	5 (15.2)	10 (30.3)	15 (45.5)	3 (9.1)	
Poor	82 (70.7)	23 (28.0)	38 (46.3)	16 (19.5)	5 (6.1)	0.005
Lauren						
Intestinal	45 (38.8)	7 (15.6)	16 (35.6)	18 (40.0)	4 (8.9)	
Diffuse	56 (48.2)	17 (30.4)	26 (46.4)	10 (17.9)	3 (5.4)	
Mixed	15 (12.9)	4 (26.7)	6 (40.0)	4 (26.7)	1 (6.7)	0.033
Goseki						
I	53 (45.7)	10 (18.9)	22 (41.5)	16 (30.2)	5 (9.4)	
II	9 (7.8)	1 (1)	4 (44.4)	4 (44.4)	0 (0)	
III	20 (17.2)	5 (25.0)	7 (35.0)	7 (35.0)	1 (5.0)	
IV	34 (29.3)	12 (35.3)	15 (44.1)	5 (14.7)	2 (5.9)	0.048
Ming						
Expanding	45 (38.8)	7 (15.6)	16 (35.6)	18 (40.0)	4 (8.9)	
Infiltrative	71 (61.2)	21 (29.6)	32 (45.1)	14 (19.7)	4 (5.6)	0.011
Tumor depth						
T1	23 (19.8)	7 (30.4)	8 (34.8)	5 (21.7)	3 (13.0)	
T2	38 (32.8)	7 (18.4)	16 (42.1)	13 (34.2)	2 (5.3)	
T3	45 (38.8)	13 (28.9)	18 (40.0)	12 (26.7)	2 (4.4)	
T4	10 (8.6)	1 (10.0)	6 (60.0)	2 (20.0)	1 (10.0)	0.763
Nodal status						
N0	41 (35.3)	12 (29.3)	17 (41.5)	10 (24.4)	2 (4.9)	
N1	35 (30.2)	6 (17.1)	14 (40.0)	12 (34.3)	3 (8.6)	
N2	18 (15.5)	5 (27.8)	7 (38.9)	4 (22.2)	2 (11.1)	
N3	22 (19.0)	5 (22.7)	10 (45.5)	6 (27.3)	1 (4.5)	0.620
Metastasis						
M0	97 (83.6)	22 (22.7)	37 (38.1)	30 (30.9)	8 (8.2)	
M1	19 (16.4)	6 (31.6)	11 (57.9)	2 (10.5)	0 (0)	0.039
UICC stage						
Ia	19 (16.4)	6 (31.6)	8 (42.1)	3 (15.8)	2 (10.5)	
Ib	18 (15.5)	3 (16.7)	7 (38.9)	7 (38.9)	1 (5.6)	
II	21 (18.1)	5 (23.8)	9 (42.9)	6 (28.6)	1 (4.8)	
IIIa	14 (12.0)	3 (21.4)	3 (21.4)	8 (57.1)	0 (0)	
IIIb	8 (6.9)	2 (25.0)	3 (37.5)	1 (12.5)	2 (25.0)	
IV	36 (31.0)	9 (25.0)	18 (50.0)	7 (19.4)	2 (5.6)	0.780

Table 2. Association of TIMP-2 expression in gastric cancer with prognosis to Mantel-Cox log-rank.

	p for semiquantitative scores 0-3	p for dichotomization 0/1 (low) and 2/3 (high)	p for dichotomization 0 (negative) and 1/2/3 (positive)
Overall survival for all patients (n=115)	0.516	0.138	0.637
Overall survival for R0- resected patients (n=106)	0.370	0.081	0.716

Table 3. Association of MMP-2 expression in gastric cancer with prognosis to Mantel-Cox log-rank.

	p for semiquantitative scores 0-3	p for dichotomization 0/1 (low) and 2/3 (high)	p for dichotomization 0 (negative) and 1/2/3 (positive)
Overall survival for all patients (n=115)	0.025	0.007	0.020
Overall survival for R0- resected patients (n=106)	0.077	0.027	0.043

expression with prognosis according to Mantel-Cox log-rank tests is summarized in Table 2.

Survival analysis for MMP-2 expression

Association of MMP-2 expression with prognosis according to Mantel-Cox log rank test is shown in Table 3. The highest significance (p=0.007) is seen for overall survival of all patients (n=114) with the low/high scale of MMP-2 expression. A different outcome for the patients is also clearly seen in the Kaplan-Meier survival curve (Fig. 2). The 5-year survival for the low expression of MMP-2 was at 45.8%, whereas patients with a high MMP-2 expression had a 5-year-survival of only 24.4%.

Multivariate analysis was performed to correct these results for known risk factors in gastric cancer (pT, pN, pM, R). As seen in Table 4, the prognostic value of MMP-2 expression disappears in the multivariate analysis. Known prognostic parameters (stage, resectability) keep their independent prognostic value.

Table 4. Multivariate analysis of prognostic covariates of survival in 114 patients with gastric cancer.

Covariate	p value	HR	95 % CI
TNM stage			
IA	<0.001	1	1.14-13.40
IB	<0.001	3.91	1.44-15.87
II	0.001	4.77	3.40-37.62
IIIA	0.001	11.32	2.64-41.46
IIIB	0.247	10.47	5.36-54.57
IV	0.294	17.10	1.24-6.64
Resectability (R0/R+)	0.014	2.87	1.24-6.64
Differentation(well/poor)	NS	0.68	0.38-1.20
Age (≤ 65/>65)	NS	1.51	0.91-2.50
Sex (female/male)	NS	0.67	0.41-1.10
MMP-2 (low/high)	NS	1.29	0.78-2.13

Discussion

Degradation of the basement membrane and the extracellular matrix is essential for tumor invasion and metastasis. Matrix metalloproteinases, such as MMP-2, degrade type IV collagen, gelatin and laminin in the basement membrane and other extracellular matrices and therefore have an important role in tumor invasion and metastasis (Leone et al., 1991). Since both the latent and active forms of MMP-2 are inhibited by TIMP-2, it has been suggested that TIMP-2 may also be involved in tumor invasion and metastasis. We have previously evaluated the expression of MMP-2 in 114 gastric cancer tissues using immunohistochemistry and found that high MMP-2 expression levels correlated significantly with depth of tumor infiltration (T-stage), lymph node metastasis (N-stage), distant metastasis (M-stage) and UICC stage (Monig et al., 2001). In this study we have analyzed the prognostic value of MMP-2 and its inhibitor, TIMP-2, for gastric cancer, as well as the correlations between TIMP-2 expression levels and clinicopathological parameters of gastric cancer.

In 1996 Sier et al. first reported that MMP-2 could be of value as a prognostic parameter for gastric cancer. In their study high levels of MMP-2 were associated with a poor overall survival (Sier et al., 1996). Two years later, Allgayer's et al. (1998) published a study on the immunohistochemical expression of MMP-2 in a series of 139 curatively resected gastric cancer patients. Statistical analysis revealed a positive association between MMP-2 levels and survival. High MMP-2 expression was associated with poor overall survival, but univariate Mantel-Cox log-rank statistics failed to show that high MMP-2 was a significant clinical prognostic parameter (Allgayer et al., 1998).

In a recent study, Sier and co-workers (Kubben et al., 2006) reported levels of MMP-2 in a series of 81 gastric cancer patients and concluded that MMP-2 antigen levels, as well MMP-2 activity levels, were significantly associated with worse survival according to univariate Cox proportional hazards analysis. In the multivariate analysis the MMP-2 antigen level kept its independent prognostic value, however, the prognostic value of the MMP-2 BIA activity level was lost. Our

Kaplan-Meier survival analysis of immunohistochemical MMP-2 detection showed significant differences in overall survival for all 114 patients for all MMP-2 staining grades, with the higher MMP-2 expression associated with worse survival. Significant differences in overall survival of curatively resected cases (n=106) could also be shown for the low/high and negative/positive MMP-2 staining grades. However, multivariate analysis that included established risk factors in gastric cancer (pT, N, M) did not establish MMP-2 expression as an independent prognostic parameter.

These findings confirm Mrena´s et al. (2006) findings in which epithelial MMP-2 expression in gastric cancer was associated with poor survival, although MMP-2 was not an independent prognostic factor (Mrena et al., 2006).

Interestingly, these results are in contrast to those of Kubben et al. (2006), who reported that MMP-2 antigen level kept its prognostic value also in the multivariate analysis. A possible reason for the different results may be due to the methods used to determine antigen levels of MMP-2. Kubben at al used ELISA, whereas we and other investigators used immunohistochemistry. And even though both methods are well established, it will be necessary to perform a study comparing the two methods to determine MMP-2 in gastric cancer tissue and to determine whether the two methods give different results for MMP-2 antigen levels. In the present study we also analyzed immunohistochemically levels of TIMP-2, the inhibitor of MMP-2 in gastric cancer tissue, and found that expression of TIMP-2 was not associated with overall survival of patients with resected gastric cancer. This finding confirms Joo´s et al. results of expression of TIMPs in gastric cancer (Joo et al., 2000). Several other studies have shown a correlation of TIMP-2 expression with outcome for some cancers other than gastric cancer (Nuovo et al., 1995; Grignon et al., 1996; Kanayama et al., 1996), suggesting that TIMP-2 expression may be of prognostic value in predicting the behaviour of some malignant cancers, but not others. To our knowledge, our results are the first to show a significant correlation of TIMP-2 levels in gastric cancer with WHO, Lauren and Ming classification, as well as with stage of differentiation. No significant correlation was found in our study between TIMP-2 expression and gender, Borrmann classification, depth of tumor infiltration (T-stage), lymph node metastasis (N-stage) or UICC-stage. These results are in contrast to a previous study that described a significant association of immuno-histochemically detected TIMP-2 expression in gastric cancer with Borrmann´s classification, lymph node metastasis and depth of invasion (Zhang et al., 2005). Other studies did not find any correlation of TIMP-2 expression in gastric cancer with any clinicopathological parameters (Joo et al., 2000; Shim et al., 2007).

Our results show that tissue from infiltrative and poorly differentiated gastric cancers has lower TIMP-2 expression compared to tissue from expanding and well differentiated gastric cancers; tissue from gastric cancers with metastasis (M1) has lower TIMP-2 expression than tissue from gastric cancers without metastasis (M0). The explanation for these findings may simply be that high TIMP-2 inhibits MMP-2 in tumor tissue, and thus suppresses the cancer's ability to degrade the extracellular matrix and prevents infiltration and ability to metastasize. However, since the regulation of TIMP is very complex and the exact role of TIMP-2 and other TIMPs in gastric cancer are not known, there may be a number of explanations for our results.

In conclusion, our results suggest MMP-2 and TIMP-2 play a crucial role in gastric cancer invasion and may be helpful in preoperative identification of gastric cancer patients with a poor clinical outcome.

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