Original article

Protein levels of tissue inhibitor of metalloproteinase-1 in tumor extracts as a marker for prognosis and recurrence in patients with gastric cancer

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Abstract

Background. Tissue inhibitor of metalloproteinase-1 (TIMP-1) correlates with tumor progression in patients with gastric cancer; however, the clinical significance of TIMP-1 as a marker for prognosis and recurrence has not been fully clarified.

Methods. TIMP-1 protein was measured by an enzyme-linked immunosorbent assay in tumor samples from 86 patients who had undergone surgical resection. An intratumoral TIMP-1 value of 10.0 ng/mg protein or more was defined as positive. Patients were followed up for more than 5 years prospectively.

Results. Thirty-one of the 86 patients (36.0%) were positive for TIMP-1. Kaplan-Meier curves for overall survival were significantly different between patients who were positive and those who were negative for TIMP-1. Univariate analysis of factors affecting overall survival showed that depth of tumor invasion; lymph node metastasis; peritoneal dissemination; lymphatic invasion; venous invasion; Lauren classification of histology; curability; and TIMP-1 were statistically significant. Stepwise multivariate analysis for overall survival demonstrated that depth of tumor invasion, nodal metastasis, peritoneal dissemination, and TIMP-1 remained independent prognostic factors. Kaplan-Meier curves for disease-free survival were significantly different between patients who were positive and those who were negative for TIMP-1. The incidence of recurrence was significantly higher in patients positive for TIMP-1 than in those who were negative for TIMP-1. The frequency at each site of recurrence was higher in patients positive for TIMP-1.

Conclusion. These results suggested that the protein concentration of TIMP-1 in the tumor extracts was a useful marker for overall survival, disease-free survival, and disease recurrence in patients with gastric cancer. Thus, tumor TIMP-1 may serve to identify a high-risk group, for whom optimal surgical and medical treatment can be given.

Key words TIMP-1 · Gastric carcinoma · Prognostic value · Recurrence

Background

Gastric cancer is the most frequent malignancy in Japan [1] and the second leading cause of death in the world [2]. Although the mortality has gradually decreased with an increasing incidence of early-stage disease in Japan, more than 50% of advanced cases recur, even after curative resection [3]. Prognosis is dismal in patients with recurrent or unresectable gastric cancer, and their median survival time is less than 1 year, even if they receive chemotherapy [4]. Therefore, reliable biomarkers to predict relapse and prognosis are much needed to optimize surgical and medical management for a high risk group.

Recent advances in molecular biology have revealed some mechanisms involved in tumor progression. The family of matrix metalloproteinases (MMPs) plays a significant role in matrix degradation which is an essential step in tumor invasion and metastasis [5,6]. These proteinases are produced not only by tumor cells but also by stromal cells [5–9]. In addition to these proteinases, the tissue inhibitors of metalloproteinases (TIMPs) play an important role in the extent of matrix degradation [5–8]. Once MMPs are activated, the synthesis of TIMPs is induced in stromal cells in response to the proteinase reactions [6]. The TIMP family currently consists of four members: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [7]. Of these known TIMPs, TIMP-1 strongly inhibits various types of MMPs [7–9]. In addition to its inhibitory properties, TIMP-1 has additional activities, such as growth stimulation [10–13] and the inhibition of apoptosis [14,15] in carcinoma cells.

Although these observations suggest that TIMP-1 is important in stimulating tumor growth, the clinical role of TIMP-1 in terms of its prognostic value remains largely unknown in patients with gastric cancer. Previously, Nomura et al. [16] and Murray et al. [17] reported on the expression of TIMP-1 protein in gastric tumors. Mimori et al. [18] observed a positive correlation

between elevated TIMP-1 mRNA concentrations and tumor progression. Recently, we [19] found that high levels of TIMP-1 protein in tumor extracts correlated with tumor aggressiveness in patients with gastric cancer. In that study, we [19] also showed, by analyzing the short-term survival in these patients, that tumor TIMP-1 could be a candidate prognostic factor [19]. Based on these findings, the present study was conducted to further clarify by analyzing the complete 5-year follow-up observations, the clinical significance of tumor TIMP-1 as a marker of prognosis and recurrence in patients with gastric cancer.

Patients and methods

Patients

Tumor samples were obtained from 86 patients who had undergone surgical resection for gastric cancer at the Kanagawa Cancer Center (Yokohama, Japan) between August 1996 and March 1998. None of the patients had undergone any prior treatment. Tumor classification and pathologic diagnosis were conducted according to the *Japanese classification of gastric carcinoma* [20].

Preparation tissue samples

Tissue samples were immediately frozen after excision and stored at -80°C until processed. Frozen tissue samples (200 mg each) were homogenized in phosphate-buffered saline (PBS) and centrifuged at 8000g for 20 min. Supernatants were then assayed for TIMP-1 (description of assay follows). The DC protein assay (Bio-Rad, Hercules, CA, USA) was used to measure the total protein concentration of each sample.

Measurement of TIMP-1 protein

A one-step sandwich enzyme-linked immunosorbent assay (ELISA) kit (Fuji Chemical, Takaoka, Japan) was used to measure the protein concentration of TIMP-1 [21,22]. Serially diluted human TIMP-1 standards and 5 μl of each properly diluted tumor sample homogenate were added to separate wells of a 96-well plate, together with 75 μl of anti-human TIMP-1 antibody conjugated to peroxidase. Samples were incubated for 30 min at 30°C , and then washed with PBS supplemented with 0.05% (v/v) Tween 20. The peroxidase reaction was carried out in an H_2O_2 solution containing *o*-phenylenediamine, a colorimetric marker, at 30°C for 15 min, and was stopped by adding H_2SO_4 . A microplate reader was used to measure absorbance at 492 nm. The sensitivity limit of the assay was 50 ng/ml and the assay was linear from 51 to 2000 ng/ml. The coefficient of

variation was less than 5.0%. TIMP-1 concentrations were expressed as nanograms TIMP-1 per milligram of total protein in each supernatant. The cutoff value for tumor TIMP-1 protein was set at 10 ng TIMP-1/mg protein, based on the results of our previous study [19]. Tumor TIMP-1 concentrations of 10 ng/mg protein or more were defined as positive [19].

Follow-up examinations

After curative surgery, patients were examined at the outpatient clinic every 3 months for the first 3 years, and then every 6 months for the subsequent 2 years. The patients were physically examined and assessed for serum levels of the tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 at each examination, and underwent computed tomography (CT) examinations every year. When the physical examinations or tumor marker results suggested a recurrence of cancer, the patients underwent CT, barium enema, and/or diagnostic laparoscopy.

Medical treatment

Patients received chemotherapy including 5-fluorouracil after noncurative surgery and after recurrence of the disease when chemotherapy was feasible. Best supportive care was selected when the tumor did not respond to chemotherapy or when chemotherapy was not appropriate because of the patient's poor condition.

Statistical analyses

Either an unpaired Student's *t*-test or a χ^2 test was used to compare results between the TIMP-1-positive and -negative groups. The survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Cox's proportional hazard model was used to perform univariate and multivariate survival analyses. A *P* value of <0.05 was defined as statistically significant, and the data values were expressed as means $\pm$ SDs. SPSS software (v11.0J Win, SPSS, Chicago, IL, USA) was used for all statistical tests.

Results

Positive tumor TIMP-1 was observed in 31 of the 86 patients (36.0%). When clinicopathologic factors were compared between patients positive and those negative for TIMP-1, significant differences were observed in depth of tumor invasion, lymphatic invasion, and tumor stage (Table 1).

All 86 patients were followed for more than 5 years and the outcomes were obtained through clinical ap-

Table 1. Background of patients

	TIMP-1 negative (<i>n</i> = 55)	TIMP-1 positive (<i>n</i> = 31)	<i>P</i> value ^a
Sex; male/female	37/18	22/9	0.723
Age (years)	60.0 ± 10.6	63.2 ± 8.6	0.148
Macroscopic type			
T1 Type 0	17	0	NA
T2 Type 1	3	2	0.552
Type 2	4	6	
Type 3	7	9	
Type 4	10	7	
Type 5	14	7	
Depth of invasion			0.002
T1	17	0	
T2	13	8	
T3	25	23	
Histological type			0.121
Intestinal	22	7	
Diffuse	33	23	
Lymph node metastasis			0.264
N0	21	6	
N1	14	12	
N2	13	7	
N3	7	6	
Peritoneal metastasis			0.658
Negative	44	26	
Positive	11	5	
Liver metastasis			NA
Negative	55	31	
Lymphatic invasion			0.003
Negative	25	4	
Positive	30	27	
Venous invasion			0.661
Negative	36	19	
Positive	19	12	
Curability			0.810
AB	45	26	
C	10	5	
Stage			0.002
I	24	2	
II	4	7	
III	14	14	
IV	13	8	

NA, not applicable

^a Unpaired Student's *t*-test or the χ^2 method was used to compare the data between the two groups

pointments at the outpatient clinic or by telephone contact. The median observation period was 60.1 months (range, 60.0–69.4 months). Of the 86 patients, 45 died during the follow-up period; 15 were in the TIMP-1-negative group and 30 in the TIMP-1 positive group. Kaplan-Meier curves for overall survival were significantly different between patients who were positive and those who were negative for TIMP-1 (Fig. 1). Overall survival rates were 65.5% at 3 years and 58.2% at 5 years in patients negative for TIMP-1, while these values were 41.9% at 3 years and 35.5% at 5 years in patients positive for TIMP-1. Overall survival was compared between patients who were negative and those

who were positive for TIMP-1, stratified by disease stage (Table 2). Overall survival rates were lower in the positive TIMP-1 group with stage II/stage III than in the negative group with stage II/III, although the difference did not reach significance. Univariate analysis of factors affecting overall survival demonstrated that depth of tumor invasion; lymph node metastasis; peritoneal dissemination; lymphatic invasion; venous invasion; Lauren classification of histology; curability; and TIMP-1 were statistically significant (Table 3). Stepwise multivariate analysis for overall survival revealed that depth of tumor invasion, metastasis to lymph nodes, peritoneal dissemination, and TIMP-1 remained independent

Fig. 1. Kaplan-Meier curves for overall survival showed a significant difference between patients who were negative (*solid line*) and those who were positive (*broken line*) for tumor tissue inhibitor of metalloproteinase-1 (*TIMP-1*) ($P = 0.0097$)

prognostic factors, although the statistics for depth of tumor invasion, nodal metastases, and TIMP-1 were marginally significant (Table 4).

The disease recurred in 22 of the 71 patients who underwent curative resection among the 86 patients. Kaplan-Meier curves for disease-free survival were significantly different between patients who were positive and those who were negative for TIMP-1 (Fig. 2). The incidence of recurrence was significantly higher in patients positive for TIMP-1 than in those negative for TIMP-1 (Table 5). The frequency at each site of recurrence was higher in patients positive for TIMP-1; however, there was no significant difference in the recurrent patterns (Table 6).

Discussion

This study indicated that gastric cancer patients who were positive for intratumoral TIMP-1 had a poor prognosis. Moreover, multivariate analysis revealed that

Table 2. Survival rates of patients negative and those positive for TIMP-1, stratified by stage of disease

Stage	Negative for TIMP-1			Positive for TIMP-1			<i>P</i> value
	No.	3-Year	5-Year	No.	3-Year	5-Year	
I	24	91.7%	87.5%	2	100%	100%	0.556
II	4	100%	100%	7	57.1%	57.1%	0.154
III	14	64.3%	50%	14	42.9%	35.7%	0.379
IV	13	7.7%	0%	8	12.5%	0%	0.341
II/III	18	72.2%	61.1%	21	47.6%	42.9%	0.238

Table 3. Univariate Cox proportional hazards analysis of clinicopathologic factors

Factors (category)	No. of patients	<i>P</i> value	Hazard ratio
Depth of invasion (T1 vs T2 vs T3-T4)	17, 21, and 48	0.000	3.327
Lymph node metastasis (N0-1 vs N2-4)	53 and 33	0.000	5.001
Peritoneal metastasis (negative vs positive)	70 and 16	0.000	5.109
Lymphatic invasion (negative vs positive)	28 and 57	0.005	3.030
Venous invasion (negative vs positive)	53 and 31	0.049	1.829
Lauren classification (intestinal vs diffuse)	29 and 56	0.040	2.038
Curability (AB vs C)	71 and 15	0.000	5.652
TIMP-1 (negative vs positive)	55 and 31	0.027	1.943

Table 4. Stepwise multivariate Cox proportional hazards analysis of clinicopathologic factors

Factors (category)	No. of patients	<i>P</i> value	Hazard ratio
Depth of invasion (T1 vs T2 vs T3-T4)	17, 21, and 48	0.061	1.926
Lymph node metastasis (N0-1 vs N2-4)	53 and 33	0.069	2.052
Peritoneal metastasis (negative vs positive)	70 and 16	0.010	2.819
TIMP-1 (negative vs positive)	55 and 31	0.083	1.798

tumor TIMP-1 was one of the independent prognostic factors for overall survival. These data suggested that the protein level of TIMP-1 in tumor extracts was a useful prognostic marker in patients with gastric cancer. Previously, we demonstrated that intratumoral TIMP-1 concentration was an independent prognostic factor for the short-term survival of patients with gastric carcinoma [19]. In that study, however, the observation period ranged from 350 days to 650 days, which was too short for a definitive conclusion on the prognostic value. In the present study, all patients were followed for more than 5 years. This report finally confirmed that tumor

Fig. 2. Kaplan-Meier curves for disease-free survival indicated that the difference was statistically significant between patients who were negative (solid line) and those who were positive (broken line) for tumor TIMP-1 ($P=0.03$). Censored cases were deaths due to causes other than cancer, such as traffic accident, stroke, and myocardial infarction

Table 5. Intratumoral TIMP-1 concentration and disease recurrence in patients who underwent curative resection

Recurrence	TIMP-1 negative	TIMP-1 positive
Negative	36	13
Positive	9	13

$P = 0.008$

Table 6. Site of disease recurrence classified by intratumoral TIMP-1 concentration

Site of recurrence	TIMP-1 negative (% of total cases)	TIMP-1 positive (% of total cases)
Peritoneum	6/45 (13.3%)	7/26 (26.9%)
Liver	0/45	1/26 (3.8%)
Lymph nodes	3/45 (6.7%)	4/26 (15.4%)
Local	0/45	1/26 (3.8%)

$P = 0.672$

TIMP-1 was an independent prognostic factor for overall survival in patients with gastric cancer.

In the present study, a cutoff value of 10 ng/mg protein for the protein level of TIMP-1 in the tumor extracts was determined using the upper value observed in T1 tumors, because MMPs and TIMPs would be less likely to be induced in these tumors, as degradation of the matrix is not initiated in early disease, and because the prognosis of early gastric cancer is excellent [23]. In fact, Nomura et al. [16] reported that MMP-2 was not elevated in patients with early gastric cancer. Mimori et al. [18] also demonstrated that TIMP-1 mRNA was not increased in early gastric cancer. Although other methods of defining a cutoff value can be used, the value we set clearly separated patients with good and bad prognoses.

Apart from our previous study [19], there has been only one other report (Schrohl et al. [24]) of TIMP-1 measurements in tumor extracts done by ELISA. They demonstrated that intratumoral TIMP-1 level was an independent prognostic factor for patients with breast cancer, in a study that was performed as a European Organization for Research and Treatment of Cancer — Receptor and Biomarker Group collaborative study [24]; their cutoff value was set at 11.71 ng/mg protein, using isotonic regression analysis. However, the prognostic significance of TIMP-1 has not been confirmed in other types of malignancies. In the present study, we have, for the first time, demonstrated the prognostic value of tumor TIMP-1 concentrations in patients with gastric cancer. A few investigators have, however, examined the production of TIMP-1 in the tumor tissue of gastric cancer by immunohistochemical analyses [16,25]. Joo et al. [25] demonstrated that TIMP-1 was detected predominantly in the peritumoral stromal cells rather than the tumor cells themselves. Moreover, they reported that the intensity of TIMP-1 immunohistochemical staining correlated positively with tumor stage and survival [25]. Recently, enhanced immunostaining of TIMP-1 has been reported to be correlated with prognosis in patients with urothelial [26], renal cell [27], and non-small-cell lung cancers [28]. Immunohistochemical analyses are convenient and suitable for examining stored samples retrospectively, but the intensity of immunostaining is decreased over time

because of loss of sensitivity secondary to antigenic alterations caused by fixation procedures and the storage of the samples. Immunohistochemical evaluation is objective, but not quantitative or reproducible. It has been reported that elevated levels of TIMP-1 mRNA were associated with poor prognosis in patients with some other tumors, including breast [29], esophageal [30], and non-small-cell lung cancers [31]. The presence of TIMP-1 has also been shown in body fluids (including plasma and urine) in various types of malignancies, including those of the rectum, colon, ovary, and bladder, and in lung cancer [32–37]. To date, we have reported that TIMP-1 was detected in the plasma, and was correlated with tumor progression and survival in patients with gastric and colorectal cancers [38–41].

The finding that TIMP-1 was correlated with poor prognosis seemed surprising, given that TIMP-1 inhibits MMPs, which play a major role in tissue degradation by tumor cells. However, additional activities of TIMP-1 have been reported: stimulating growth in several cell types [10–13] and inhibiting apoptosis [14,15]. Both such activities could increase proliferation and induce the tumor growth. More recently, a regulatory role in angiogenesis has been suggested for TIMP-1 [42,43]. In our study, comparison of clinicopathologic factors between patients who were positive and those who were negative for TIMP-1 revealed a significant correlation with depth of tumor invasion, lymphatic invasion, and stage of disease. Thus, TIMP-1 may play a role in tumor aggressiveness, through the stimulation of cancer cell growth, shortening the survival of patients with gastric cancer.

Generally, in patients with gastric cancer, the most important factors affecting survival have been found to be depth of tumor invasion, lymph node metastasis, and the presence of distant metastases to the peritoneum or the liver. Measurement of the tumor tissue concentration of TIMP-1 has a great advantage when compared with other prognostic factors. Intratumoral TIMP-1 can be determined by assaying biopsy specimens before surgery, which could provide potentially useful prognostic information that can aid in formulating an effective treatment strategy, whereas depth of invasion and lymph node metastases are determined pathologically after surgical resection. For example, if a patient has a gastric tumor that can be treated with surgery, but the tumor has a high intratumoral TIMP-1 protein concentration, pre- or postoperative chemotherapy can be used to attempt to improve the prognosis.

In the present study, multivariate analysis revealed that the statistics for TIMP-1, as well as those for depth of tumor invasion and nodal metastasis, were marginally significant as prognostic factors. Subset analyses of overall survival rates stratified by tumor stage demonstrated that there was some difference between those

positive for TIMP-1 and those negative for TIMP-1 in stages II and stage III, although these statistics too did not reach significance for prognosis. The number of patients examined in this study was too small to reach a definite conclusion on the statistical significance of TIMP-1 as a prognostic factor. Further study will clarify the prognostic value of TIMP-1 in patients with gastric cancer by targeting patients with cancers at stages II and III and using an appropriate sample size to confirm statistical significance.

In patients with gastric cancer, the most frequent sites of recurrence are the peritoneum, lymph node, liver, and local sites [44]. To date, the association between TIMP-1 and disease-free survival or recurrence patterns has not been examined in patients with cancers. TIMP-1 promotes cell proliferation [10–13] and inhibits apoptosis [14,15], actions which may increase tumor aggressiveness and enhance metastases nonspecifically, while TIMP-1 also plays a role in angiogenesis [42,43], which may augment hematological metastases. In the present study, the rate of disease-free survival was significantly lower in patients who were positive for TIMP-1 than in those who were negative for TIMP-1; moreover, the incidence of recurrence was significantly higher in patients positive for TIMP-1. Although the frequency at each site of recurrence was higher in patients positive for TIMP-1, the distribution of the recurrence patterns was not significantly different. These data may suggest that TIMP-1 could be a marker for disease recurrence and disease-free survival in patients with gastric cancer. TIMP-1 may enhance metastases nonspecifically.

In conclusion, our results suggested that the protein concentration of TIMP-1 in tumor extracts was a useful marker for overall survival, disease-free survival, and disease recurrence in patients with gastric cancer. Thus, tumor TIMP-1 may serve to identify a high-risk group, for whom optimal surgical and medical treatment can be given.

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