



## *Original article*

# Role of MMP-7 in the formation of peritoneal dissemination in gastric cancer

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### Abstract

**Background.** Matrix metalloproteinase-7 (MMP-7) is an important matrix-degrading enzyme that has a large role in the invasion and metastasis of cancer. To discover the mechanism of the formation of peritoneal dissemination in gastric cancer, we studied the mRNA and protein expression of MMP-7 in primary gastric cancers and peritoneal dissemination.

**Methods.** MMP-7 expression in primary gastric cancers (136 patients) was studied by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR), and the results were compared with clinicopathological parameters.

**Results.** MMP-7 mRNA was expressed in 28 (53%) of 53 primary gastric cancers, but not in normal gastric mucosa, fibroblasts, or mesothelial cells. An immunohistochemical method demonstrated that MMP-7 immunoreactivity was found on the cell membrane and cytoplasm of cancer cells. Among 136 primary tumors, 70 (53%) tumors overexpressed MMP-7, and MMP-7 tissue status had significant positive correlation with serosal involvement, lymph node metastasis, poor differentiation of cancer, and peritoneal dissemination. Patients with MMP-7-positive tumor had significantly poorer survival and more frequently died of peritoneal recurrence than did those with MMP-7-negative tumors. All 6 examined peritoneal disseminations expressed MMP-7 mRNA, and 13 of 14 peritoneal disseminations showed immunoreactivity to anti-human MMP-7 monoclonal antibody. Logistic regression analysis showed that MMP-7 immunohistological status was an independent risk factor for peritoneal dissemination, and patients with MMP-7 mRNA-positive tumors had a 9.9-fold higher relative risk for peritoneal metastasis.

**Conclusion.** These results strongly suggest that MMP-7 may have a large role in the formation of peritoneal dissemination in gastric cancer, and that clonal selection of cancer cells with MMP-7 overexpression may occur during the invasion of intraperitoneal free cancer cells from the peritoneal surface into the subperitoneal tissue. MMP-7 tissue status in the primary tumor may be a good indicator of peritoneal dissemination.

**Key words** MMP-7 · Gastric cancer · Peritoneal carcinomatosis · Peritoneal dissemination

### Introduction

Peritoneal dissemination is a leading cause of death from gastrointestinal cancer; there is no effective treatment for peritoneal carcinomatosis. About 40% of patients with advanced gastric cancer died of this type of metastasis [1]. Considerable efforts have been made to prevent peritoneal recurrence, but large randomized trials have failed to demonstrate the efficacy of prevention. One of the major reasons for the pessimistic results of clinical trials lies in the poor understanding of the mechanisms of the formation of peritoneal dissemination.

At present, cancer metastasis is considered to be formed through multi-step processes by the activation of metastasis-related genes [2]. Peritoneal dissemination is also formed through several steps [2]: (1) detachment of cancer cells from the primary tumor, (2) attachment to distant peritoneum, (3) invasion into subperitoneal space, and (4) proliferation with vascular neogenesis. The origin of peritoneal carcinomatosis in gastric cancer is considered to be intraperitoneal free cancer cells, exfoliated from the serosal surface of the primary tumor. In gastric cancer, intraperitoneal free cancer cells can be detected by peritoneal wash cytology in 40%–60% of patients with stage III or IV disease [4,5].

Free cancer cells attach to the distant peritoneum or migrate into the submesothelial lymphatic spaces (called milky spots) in the greater omentum or mesentery, and stomata in the diaphragm. In the process of attachment of free cancer cells to the peritoneum [3], we have already clarified the important roles of VLA-2, VLA-3 and several cytokines, such as

interleukin (IL)-6, IL-8, and hepatocyte growth factor (HGF) [6,7]. Intraperitoneal free cancer cells attach to the subperitoneal basement membrane by expressing VLA-2 or VLA-3 on the cell membrane, because VLA-2 and VLA-3 are receptors for the constituents of submesothelial basement membrane, like laminin and collagen.

However, the identity of the molecules that are associated with the process of cancer cell invasion into the submesothelial tissue remains unclear. Recent advances in molecular biology showed that matrix-degrading enzymes, including matrix metalloproteinases (MMPs) and serine proteases, were involved in tumor invasion.

Regarding MMPs, more than 17 subfamilies have been identified. Among them, MMP-7 is known to be expressed by the glandular epithelium [8] and by cancer cells [9,10], and other metalloproteinases are expressed not only by the stromal component [10] but also by the cancer cells. MMP-7 can degrade a wide range of substrates, including proteoglycans, fibronectin, gelatin, and casein. MMP-7 has a stronger enzymatic activity than any other members of the MMP family [11,12]. In addition, MMP-7 activates other proMMPs such as proMMP-1, -3, -8, and -9 [13,14]. Consequently, MMP-7 can cleave almost all kinds of stromal elements. ProMMP-7 is activated by 4-aminophenylmercuric acetate (APMA), trypsin, and MMP-3 following the stepwise mechanisms proposed for other proMMPs [15].

We report here the close relationship between MMP-7 expression and peritoneal metastasis in gastric cancer. To the best of our knowledge, there has been no article published previously on this relationship.

## Subjects, materials, and methods

### *Cell lines*

Seven gastric cancer cell lines (KATO-III, KKLS, MKN-45, MKN-28, TMK-1, NKPS, and NUGC-3) and a cell line of fibroblasts (KMST-6) were cultured in RPMI-1640 medium with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD, USA).

### *Patients and tumor samples*

One hundred and thirty-six patients with primary gastric cancer diagnosed and treated at the Second Department of Surgery, Kanazawa University Hospital, between 1990 and 1997, were entered into the study. All the patients underwent gastrectomy with lymph node dissection. Immediately after resection of the primary tumor, samples, about 5-mm in diameter, were taken from 53 primary tumors, each adjacent normal mucosa,

and 6 metastatic tumors on the peritoneal surface, and these samples were stored at  $-80^{\circ}\text{C}$ . In addition, small pieces of tissue, about 5- to 8-mm in diameter, were sampled from the 136 primary tumors and 14 peritoneal metastases, fixed in acetone at  $-20^{\circ}\text{C}$  overnight, and prepared to make paraffin-embedded blocks by the AMeX method [17]. All the resected primary tumors and regional lymph nodes were histologically examined by H&E staining, according to the general rules of the *Japanese classification of gastric cancer* [16].

### *Antibodies and immunohistochemistry*

Immunohistochemistry was done as described previously [6,7]. Deparaffinized sections were incubated overnight at  $4^{\circ}\text{C}$  with anti-MMP-7 antibody (Fuji Pharmaceutical, Takaoka, Japan) diluted 1:100 in phosphate-buffered saline (PBS). This monoclonal antibody was developed using a synthetic peptide corresponding to the amino acid sequence of the COOH-terminal peptide of human MMP-7 (residues 253–267) [9]. Next, the slides were incubated with biotinylated goat anti-mouse IgG for 20 min (LSAB kit; Dako, Copenhagen, Denmark) and then with premixed Avidin-Biotin complex (ABC) (Dako) reagent for 20 min. Immunostaining was done with diaminobenzidine (Dako) solution with hydrogen peroxide for 1 min. Negative controls were done in all cases by omitting the first antibody. Only cases in which at least 20% of the tumor cells were immunoreactive were scored as positive.

### *Reverse transcription-polymerase chain reaction (RT-PCR)*

RT-PCR analysis was done according to Ng et al. [19] and Conboy et al. [20], modifications. Briefly, total RNAs were extracted from primary gastric cancer, normal gastric mucosa, the cell line of fibroblasts (KMST6), human mesothelial cells, and the seven gastric cancer cell lines, using Isogen (Nippon Gene, Tokyo, Japan) [18]. Human mesothelial cells were isolated from human greater omentum, as we have already reported [21]. The prepared RNA was mixed with oligo-dT primer and was reverse-transcribed with avian myelocyt leukemia virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, FL, USA), followed by PCR amplification (Perkin-Elmer Cetus, Norwalk, CT, USA) with specific primers (Table 1). PCR amplification was done for 1.5 min at  $94^{\circ}\text{C}$ , 2 min at  $48^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$  for three cycles, then with 25 cycles of 40 s at  $94^{\circ}\text{C}$ , 1.5 min at  $48^{\circ}\text{C}$ , and 1.3 min at  $72^{\circ}\text{C}$ . The PCR products were electrophoresed on 2% agarose gels and transferred to a nylon membrane filter. The transferred products were hybridized overnight to a  $^{32}\text{P}$ -end-labeled probe specific for the target cDNA fragment (Table 1).

**Table 1.** List of primers and probes used

	DNA fragment	DNA sequences	
<i>MMP-7</i>	418 bp	5'-ACTTTAAACTCCCGCGTCATA-3'	Sense
		5'-TGCCAGATGTTGCAGAATACT-3'	Antisense
		5'-CCAATCATGATGTCAGCAGTT-3'	Probe
<i>β-Actin</i>	592 bp	5'-TTCAAGGTAGTTTCGTGGAT-3'	Sense
		5'-GAAAATCTGGCACCCACACCTT-3'	Antisense
		5'-ACTGACTACCTCATGAAGAT-3'	Probe
<i>TFR</i>	415 bp	5'-ACAGACTCTACATGTAGGAT-3'	Sense
		5'-AAACCTTGAAGTTGCTGGTA-3'	Antisense
		5'-TATCCCTCTAGCCATTCAGT-3'	Probe

MMP-7, Matrix metalloproteinase 7; TFR, transferrin receptor

The autoradiogram was exposed for 4–5 h with two intensifying screens at  $-80^{\circ}\text{C}$ . Specific primers for the *MMP-7* gene, *β-actin* gene, and transferrin receptor (*TFR*) gene are shown in Table 1 [19,20].

#### Data presentation and statistical analysis

All statistical calculations were done with SPSS statistical software (Chicago, IL, USA). Values are shown as means plus or minus SD. The  $\chi^2$  test and Student's *t*-test for unpaired samples were used to analyze data. The outcomes of the different groups of patients were compared by the generalized Wilcoxon test. To clarify *MMP-7* tissue status as a predictor of peritoneal dissemination, we did logistic regression analysis.

## Results

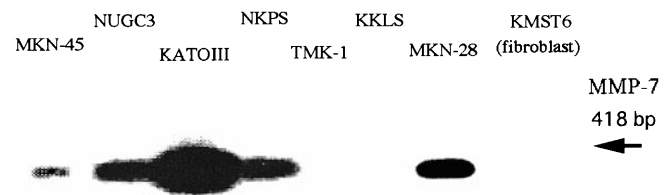
#### *MMP-7* expression in gastric cancer cell lines, fibroblasts, and primary gastric cancer

*MMP-7* mRNA was expressed in MKN-45, NUGC-3, KATO-III, NKPS, and MKN-28, but TMK-1 and KKLS did not express *MMP-7* mRNA (Fig. 1). KMST6, which is an immortalized cell line of fibroblasts, did not express *MMP-7* mRNA (Fig. 1).

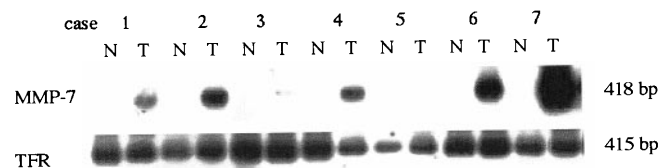
In the clinical specimens, *MMP-7* mRNA was mainly expressed in primary tumors (Fig. 2). *MMP-7* mRNA was detected in 28 (53%) of 53 primary gastric cancers, but not in normal gastric mucosa.

#### Tissue localization and expression of *MMP-7* in gastric cancer and peritoneal dissemination

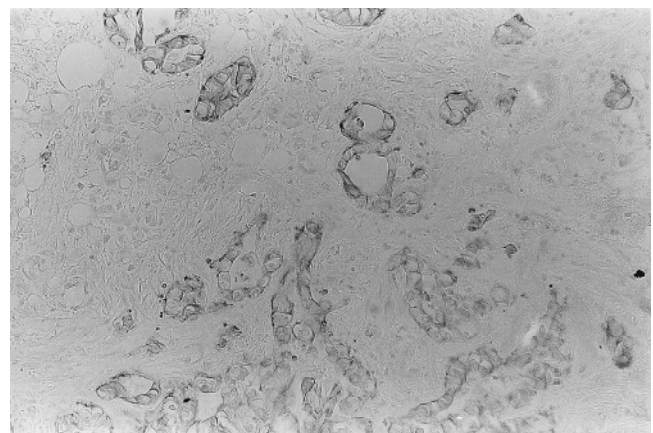
As shown in Fig. 3, immunohistochemical study showed *MMP-7* expression on the cytoplasm or cell membrane of gastric cancers, but not in the normal gastric mucosa or stromal tissue. *MMP-7* immunoreactivity was expressed in 70 (51%) of the 136 primary gastric cancers.



**Fig. 1.** Matrix metalloproteinase-7 (*MMP-7*) mRNA expression in gastric cancer cell lines and a fibroblast cell line (KMST6)



**Fig. 2.** *MMP-7* mRNA expression in primary gastric cancer and adjacent normal mucosa. T, Tumor; N, normal counter-part; *TFR*, transferrin receptor



**Fig. 3.** *MMP-7* expression in primary gastric cancer (immunohistochemistry).  $\times 200$

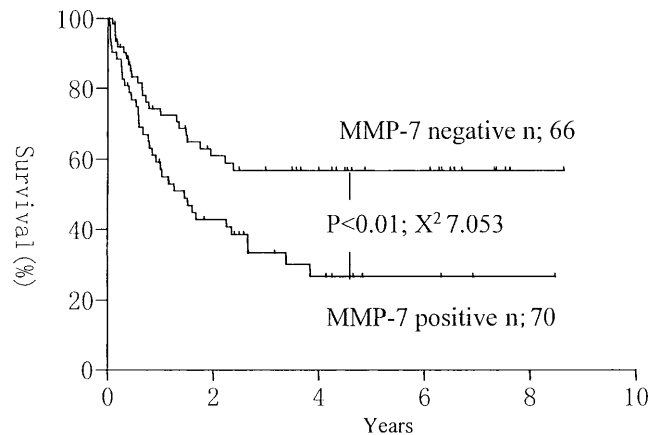
### Relationship between clinicopathological parameters and MMP-7 expression

MMP-7 mRNA expression in primary tumors had a significant relationship with positive serosal involvement (Table 2). Peritoneal dissemination was found in 2 (8%) of 25 MMP-7 mRNA-negative tumors, but 9 (32%) of 28 MMP-7 mRNA-positive tumors had peritoneal dissemination ( $P < 0.05$ ). Tissue status of MMP-7 protein expression was significantly associated with lymph node metastasis, serosal involvement, peritoneal dissemination, and lymphatic invasion (Table 3). In addition, MMP-7 immunoreactivity was expressed in a significantly higher proportion of poorly differentiated adenocarcinomas compared with that in differentiated adenocarcinomas (Table 3). Infiltrating type of cancer tended to overexpress MMP-7 more than did localized tumors, and the proportion of MMP-7 overexpression in early gastric cancer (21%; 7/33) was significantly lower than that in advanced gastric cancer (61%; 63/103) ( $P < 0.05$ ).

### Survival of patients in terms of MMP-7 tissue status, and recurrence mode after operation

As shown in Fig. 4, the survival rate was lower for patients with gastric cancer with positive MMP-7 status than for those with MMP-7-negative status.

Recurrence developed in 50 patients. Of 70 patients with tumors showing positive MMP-7 immunoreactivity, 25 (36%) developed peritoneal recurrence (Table 4). This was significantly higher than the proportion (8%; 5/66) in patients with MMP-7-negative tumors.



**Fig. 4.** Survival of patients with gastric cancer according to MMP-7 tissue status. In terms of immunohistochemical MMP-7 tissue status, patients with MMP-7-positive tumor survived significantly longer than did those with an MMP-7-negative one

**Table 2.** Relationship between clinicopathologic parameters and MMP-7 mRNA expression in primary gastric cancer

	MMP-7 mRNA expression in primary tumors		Significance
	Positive	Negative	
Liver metastasis			
Positive	2 (7%)	0 (0%)	NS
Negative	26 (93%)	25 (100%)	
Peritoneal dissemination			
Positive	9 (32%)	2 (8%)	$P < 0.05$
Negative	19 (68%)	23 (92%)	
Serosal invasion			
Positive	22 (79%)	10 (40%)	$P < 0.05$
Negative	6 (21%)	15 (60%)	
Lymph node metastasis			
Positive	21 (75%)	15 (60%)	NS
Negative	7 (25%)	10 (40%)	
Histologic type			
Differentiated type	11 (39%)	12 (48%)	NS
Poorly differentiated type	17 (61%)	13 (52%)	
Lymphatic invasion			
Positive	16 (57%)	14 (56%)	NS
Negative	12 (43%)	11 (44%)	
Venous invasion			
Positive	15 (54%)	13 (52%)	NS
Negative	13 (46%)	12 (48%)	
Total	28	25	

NS, Not significant

**Table 3.** Correlations between MMP-7 immunoreactivity in primary tumors and clinicopathologic parameters

	MMP-7 immunoreactivity in primary tumor		Significance
	Positive	Negative	
Liver metastasis			
Positive	3 (4%)	8 (12%)	NS
Negative	67 (96%)	58 (88%)	
Peritoneal dissemination			
Positive	27 (39%)	5 (8%)	$P < 0.01$
Negative	43 (61%)	61 (92%)	
Serosal involvement			
Positive	63 (90%)	36 (55%)	$P < 0.01$
Negative	7 (10%)	30 (45%)	
Lymph node metastasis			
Positive	51 (73%)	33 (50%)	$P < 0.05$
Negative	19 (27%)	33 (50%)	
Histologic type			
Differentiated type	45 (64%)	28 (42%)	$P < 0.05$
Poorly differentiated type	25 (36%)	38 (58%)	
Lymphatic invasion			
Positive	54 (77%)	38 (58%)	$P < 0.05$
Negative	16 (23%)	28 (42%)	
Venous invasion			
Positive	48 (69%)	36 (55%)	NS
Negative	22 (31%)	30 (45%)	
Patterns of growth			
Localized	5 (7%)	11 (17%)	$P < 0.05$
Intermediate	22 (32%)	34 (51%)	
Infiltrating	43 (61%)	21 (32%)	
Macroscopic type			
Early cancers	7 (10%)	26 (39%)	$P < 0.05$
Borrmann type 1,2	17 (24%)	12 (19%)	
Borrmann type 3,4	46 (66%)	28 (42%)	
Total	70	66	

**Table 4.** Recurrence pattern and MMP-7 immunoreactivity expression in primary tumors

Recurrence mode	MMP-7 immunoreactivity in primary tumor		
	Negative	Positive	
Peritoneum	5 (8%)	25 (36%)	$P < 0.01$
Liver	8 (12%)	4 (6%)	
Lymph node	5 (8%)	8 (11%)	
Bone	2 (3%)	1 (1%)	
Free of recurrence	46 (70%)	32 (46%)	
Total	66	70	

#### *MMP-7 expression in peritoneal dissemination*

MMP-7 immunoreactivity was found in specimens from peritoneal dissemination (Fig. 5), with a positive incidence of 93% (13/14). One tumor that was negative for MMP-7 immunoreactivity was pseudomyxoma peritonei from gastric cancer.

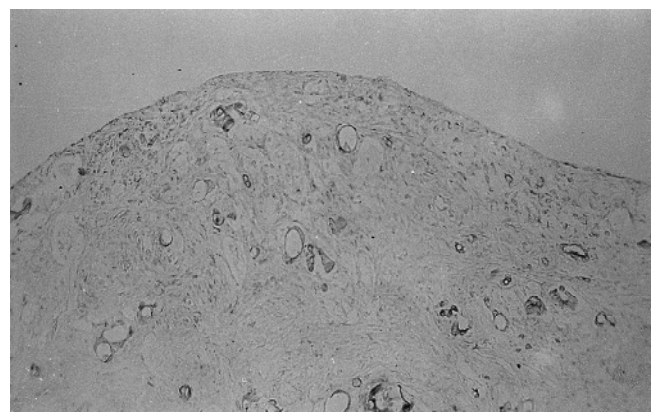
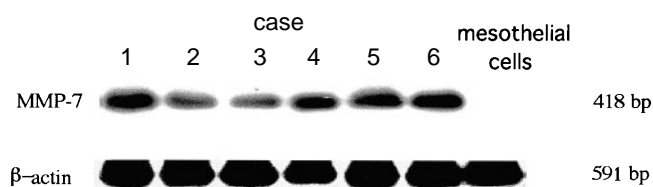
**Fig. 5.** MMP-7 expression in peritoneal dissemination (immunohistochemistry).  $\times 100$

Figure 6 shows the MMP-7 mRNA expression in peritoneal disseminations. Although mesothelial cells did not express MMP-7, bands compatible with MMP-7 mRNA were found in all the samples obtained from peritoneal disseminations.

We did a logistic regression analysis to identify the independent risk factors for peritoneal dissemination. As shown in Table 5, MMP-7 tissue status emerged as an independent risk factor for peritoneal dissemination.

## Discussion

As shown in the logistic regression analysis, MMP-7 tissue status in gastric cancer was an independent



**Fig. 6.** MMP-7 mRNA expression in peritoneal dissemination and mesothelial cells (reverse transcription-polymerase chain reaction method)

predictor of peritoneal dissemination. Accordingly, this study clearly demonstrates a close relationship between peritoneal dissemination and MMP-7 expression in gastric cancer. Some investigators have reported that matrix-digesting enzymes other than MMP-7, such as urokinase type plasminogen activator receptor (uPAR), urokinase type plasminogen activator (uPA), and membrane type (MT1)-MMP [22,23], are also involved in the progression of gastric cancer. Among these matrix-degrading enzymes, MMP-7 is unique, in that it is expressed by tumor cells themselves rather than by the stromal cells [8]. By the in-situ hybridization technique, Yamashita et al. [24] reported that MMP-7 mRNA signals were exclusively found in the cytoplasm of gastric cancer cells. In this present study, KMST-6, a fibroblast cell line, and normal gastric mucosa and mesothelial cells did not express MMP-7 mRNA.

These results indicate that MMP-7 is mainly produced by gastric cancer cells. Accordingly, MMP-7-producing cancer cells have a high matrix-degrading activity in the pericellular space, making them highly invasive.

In the clinical specimens, primary tumors with MMP-7 overexpression were significantly more likely to show positive serosal involvement, lymph node metastasis, and peritoneal dissemination than did the specimens

**Table 5.** Multivariate analysis with respect to peritoneal metastasis in gastric cancer (logistic regression analysis)

Clinicopathologic factors	Multivariate analysis			
	$\chi^2$	<i>P</i> value	Risk ratio	95% CI
Macroscopic type				
Early gastric cancer	1.689	0.194	2.201	0.431–5.395
Advanced gastric cancer				
Lymph node metastasis				
Negative	5.127	0.023	5.375	1.331–18.113
Positive				
Histologic type				
Differentiated type	0.572	0.499	1.475	0.538–4.041
Poorly differentiated type				
Serosal invasion				
Negative	3.869	0.049	3.65	1.012–11.498
Positive				
Infiltrating pattern				
Localized	0.426	0.514	1.524	0.431–5.395
Infiltrating				
Vessel invasion				
Negative	0.324	0.569	1.715	0.268–10.999
Positive				
Lymphatic invasion				
Negative	0.397	0.529	1.489	0.053–4.509
Positive				
MMP-7 immunohistological status				
Negative	7.852	0.005	9.921	1.624–14.996
Positive				

CI, Confidence interval

with MMP-7 negative tumors. Furthermore, MMP-7 overexpression in poorly differentiated adenocarcinoma and the macroscopically infiltrating type was significantly more frequent than that in differentiated adenocarcinoma and the localized type.

The phenotypes of poorly differentiated adenocarcinoma or macroscopically infiltrating tumor are well known as tumors that tend to develop peritoneal recurrence [1]. Poorly differentiated adenocarcinomas tend to grow in an infiltrating type of tumor growth pattern and to exfoliate easily from the serosal surface, because of the loss of cell-to-cell adhesion due to an abnormality of the E-cadherin gene [25].

Accordingly, simultaneous expression of MMP-7 and down-regulation of E-cadherin expression may render gastric cancer cells more invasive and metastatic. As a result, these cancer cells are prone to exfoliate from the serosal surface and to become intraperitoneal free cancer cells.

We previously reported a significant role of integrins  $\alpha 2$  and  $\alpha 3$  in the attachment of intraperitoneal free cancer cells on the peritoneal surface [6]. After such attachment, cancer cells must invade the subperitoneal tissue and move to the perivascular space to get enough nutrition and oxygen from submesothelial blood capillaries [26]. If cancer cells have no ability to invade the subperitoneal tissue, almost all the cells may die off on the peritoneal surface. To accomplish the invasion process smoothly, the production of matrix-degrading enzymes in the pericellular space of the cancer cells is essential. In the early stage of peritoneal dissemination, however, inflammatory reactions may be rare, and cancer cells cannot invade the subperitoneal tissue by using matrix-degrading enzymes, such as MMP-2 and MMP-9, which are produced by fibroblasts or inflammatory cells.

Accordingly, MMP-7, which is exclusively produced by the cancer cells themselves, must have an important role in the completion of this process. Interestingly, this present study demonstrated that MMP-7 mRNA was detected in all specimens of peritoneal dissemination. In addition, logistic regression analysis demonstrated that MMP-7 tissue status was an independent risk factor for peritoneal dissemination.

These results suggest that the clonal selection of cancer cells with MMP-7 overexpression may occur during the invasion of intraperitoneal free cancer cells from the peritoneal surface into the subperitoneal tissue.

Furthermore, these results suggest that MMP-7 has a large role in the establishment of peritoneal dissemination, and that MMP-7 is a reliable predictor of peritoneal recurrence after curative resection of gastric cancer. Accordingly, MMP-7 may be a highly suitable molecular target for gene therapy for peritoneal carcinomatosis. We are now trying to treat experimental

peritoneal carcinomatosis with antisense oligonucleotides against MMP-7 mRNA.

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