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*Original article*

# Expression of collagenase-3 (matrix metalloproteinase-13) in human gastric cancer

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**Abstract**

*Background.* **Collagenase-3 (matrix metalloproteinase-13; MMP-13) is a recently identified member of the matrix metalloproteinases (MMPs) with broad substrate specificity, and a potential role in tumor metastasis and invasion has been proposed for this enzyme. To date, in gastrointestinal tract tumors, collagenase-3 expression has been reported only in esophageal carcinoma; the presence and possible implications of this enzyme in the progression of gastric cancer are unknown.**

*Methods.* **In this study, MMP-13 mRNA expression was analyzed in a series of 110 matched gastric adenocarcinomas and the corresponding adjacent normal mucosae as well as in nine gastric cancer cell lines. In addition, the mRNA expression of gelatinase A (MMP-2) and membrane type-1 matrix metalloproteinase (MT1-MMP), two MMPs which have the ability to activate MMP-13 in vitro, was also examined in the same cases and cell lines. The production and localization of MMP-13, MMP-2, and MT1-MMP were investigated by immunohistochemistry, immunofluorescence, Western blot analysis, and zymography.**

*Results.* **MMP-13 mRNA was expressed in 23 of the 110 carcinomas (21%), and MT1-MMP mRNA was expressed in 45 (40%), but no MMP-13 or MT1-MMP mRNA was detected in any of the normal mucosae. Also, eight of the nine gastric cancer cell lines expressed mRNA of MMP-13, and in each cell line there was coordinate expression with either MT1-MMP or MMP-2 mRNA. MMP-13 and MT1-MMP were detected at the bases of invadopodia of the cultured cancer cells as well as in the invasive front of the tumors, as shown by immunofluorescence and immunohistochemistry, respectively. Western blot analysis revealed the presence of MMP-13 protein in those cell lines and carcinomas that expressed its mRNA. On zymography, almost all cell lines that expressed MMP-13 showed gelatinolytic bands corresponding to the active form of MMP-13 or one of its intermediate forms. Also, zymographic analysis of the tumor specimens revealed strong gelatinolytic bands of MMP-13 and MMP-2, whereas these bands in normal mucosa were weak. There was no significant relationship between MMP-13 mRNA expression and histologic type, lymph node metastasis, wall invasion, or distant metastasis. However, patients with MMP-13 mRNA-positive tumors had a poorer prognosis than those with MMP-13-mRNA-negative cancer. Furthermore, patients with simultaneous expression of MMP-13 and MT1-MMP mRNA showed the poorest prognosis, as compared with those having tumors expressing either MMP-13 or MT1-MMP, or neither MMP-13 nor MT1-MMP mRNA.**

*Conclusion.* **These findings suggest that MMP-13 expression may contribute to the progression of gastric cancer, and its coordinate overexpression with MT1-MMP and/or MMP-2 may have a cooperative effect in the progression of gastric cancer.**

**Key words** Collagenase-3 · Extracellular matrix components · Invasion · Prognosis

## Introduction

The incidence of gastric carcinoma is the highest of all carcinomas, and it is the leading cause of death from cancer in Japan. In spite of improvements in surgical treatment and chemotherapy, the prognosis is still poor, owing to local recurrence or metastasis [1–3]. Degradation of the extracellular matrix during tumor invasion and metastasis is thought to result from a combined action of several proteolytic enzyme systems, including the collagenases and other matrix metalloproteinases (MMPs) [4,5] and serine proteases, such as plasmin generated by the urokinase pathway of plasminogen activation [6].

Human collagenase-3 (MMP-13) is a recently identified member of the matrix MMP family that was originally isolated from breast carcinoma [7]. Expression of

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collagenase-3 has been detected in squamous carcinomas of the head and neck [8], chondrosarcoma [9], transitional-cell carcinoma of the urinary bladder [10], oral mucosal epithelium of chronic inflammation [11], rheumatoid synovium, and developing bone [12,13], but not in normal adult tissues. Biochemical characterization of collagenase-3 has revealed that it is a very potent enzyme that, after activation through a proteolytic cascade mechanism, displays a broad spectrum of activity against connective tissue components [14,15]. Thus, collagenase-3 degrades very efficiently the native helix of all fibrillar collagens, with preferential activity on type II collagen. In addition to its proteolytic activity on fibrillar collagens, collagenase-3 is also a powerful gelatinase and thus may contribute to further degrade the initial cleavage products of collagenolysis to small fragments suitable for further metabolism [15]. Because fibrillar collagens are the most abundant structural components of human connective tissues, it is conceivable that the ability to degrade collagen extracellular matrix is crucial for the invasion of neoplastic cells [16]. The wide substrate specificity of MMP-13 also makes it a potent proteolytic tool for invading tumor cells [8–10]. As collagenase-3 widely degrades components of the basement membrane and connective tissue surrounding tumor cells, this collagenase is likely to play crucial roles in modulating extracellular matrix degradation and cell-matrix interactions involved in metastasis.

Previous experiments using recombinant human procollagenase-3 showed that MT1-MMP, as well as MMP-2, may be able to activate progelatinase-3, alone or in concert, thereby establishing a new activation cascade consisting of three members of the MMP family; these experiments have also shown that active collagenase-3 can activate gelatinase B (MMP-9) [14,17].

In gastrointestinal tract malignancies, only esophageal cancer was shown to produce collagenase-3 and its activator MT1-MMP [18]; however, to our knowledge, there is no report about collagenase-3 expression in gastric cancer.

In the present study, using nine human gastric cancer cell lines and resected specimens of 110 gastric adenocarcinomas, we investigated the mRNA and protein expression, as well as the enzymatic activity, of MMP-13, MMP-2, and MT1-MMP. In addition, we studied whether collagenase-3 plays an important role in tumor aggressiveness in association with MT1-MMP.

## Materials and methods

### *Cell lines and culture conditions*

The gastric cancer cell lines TMK-1, KATO-III, AZ-521, NUGC-3, MKN-28, MKN-45, MKN-45-P, and KMST-6 cells, a human immortalized fibroblast cell line were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The gastric cancer cell lines KKLS and NKPS were a kind gift from Dr. M. Mai, Cancer Institute Hospital of Kanazawa University, Kanazawa, Japan. MKN-45-P cells were subcloned from human gastric cancer MKN-45 cells and maintained by intraperitoneal passage in nude mice. This cell line has high metastasis ability in the peritoneal cavity of nude mice [19]. Except for the AZ-521 cell line, which was maintained in MEM medium, all cell lines were maintained in RPMI-1640 medium, supplemented with 2mM L-glutamine (Nissui Pharmaceutical, Tokyo, Japan); 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA); and 100U/ml penicillin and 100μg/ml streptomycin (Gibco BRL, Karlsruhe, Germany). Cells were grown at 37°C in an atmosphere of 95% air and 5% CO2. All experiments were performed within 20 passages of the frozen stocks from which the cells were periodically recovered.

### *Clinical specimens*

Primary gastric adenocarcima specimens, as well as the adjacent normal mucosae, of 110 patients were obtained fresh from routine surgical procedures performed at the Second Department of Surgery, Kanazawa University Hospital from 1990 to 1994, following a protocol approved by the Kanazawa University Ethics Committee. Clinical staging was determined according to the *General rules for study of gastric cancer* [20]. The pathological stages of the 110 cases studied were: stage Ia, 14; Ib, 9; II, 14; IIIa, 22; IIIb, 12; and IV, 38. Tumors were well ($n$ = 20), moderately ($n$ = 24), or poorly ($n$ = 66) differentiated. Tissues were immediately frozen in liquid nitrogen, and stored at −80°C until use for RNA extraction. Parallel tissue samples were fixed in 10% formalin overnight at room temperature, embedded in paraffin, and cut into 4-μm-thick sections for immunhistochemistry.

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

RT-PCR was performed as described elsewhere [21,22]. Approximately $10^7$ human gastric adenocarcinoma cells or 50–100mg of gastric adenocarcinoma tissue and normal mucosa were homogenized with 1.5ml RNAzol reagent (Biotex Laboratories, Houston, TX, USA), and total RNA was isolated according to the manufacturer's protocol. cDNA was synthesized by extension of oligo dT primers with M-MLV reverse transcriptase (Toyobo Biomedicals, Tokyo, Japan), in a mixture containing 2μg of total RNA, for 60min at 37°C. PCR of the cDNA was performed in a final volume of 20μl contain-

**Table 1.** Primers and probes used for polymerase chain reaction (PCR)/Southern blotting

| Primer name | Nucleotide sequence | Amplified segment (bp) | AT (°C) |
|---|---|---|---|
| GAPDH, F | 5′-GCGCTGCCAAGGCTGTGGGCAAG-3′ | 475 | 60 |
| GAPDH, R | 5′-ATTCAGTGTGGTGGGGGACTGAG-3′ | | |
| GAPDH, P | 5′-TGGTCGTTGAGGGCAATGCCAGCCCC-3′ | | |
| MT1-MMP, F | 5′-GCTTGCAAGTAACAGGCAAA-3′ | 604 | 63 |
| MT1-MMP, R | 5′-AAATTCTCCGTGTCCATCCA-3′ | | |
| MT1-MMP, P | 5′-CTACATCCGTGAGGGCCATGAGA-3′ | | |
| MMP-2, F | TTCTTCAAGGACCGGTTCATT | 511 | 57 |
| MMP-2, R | AGTCGGATTTGATGCTTCCAA | | |
| MMP-2, P | TTGATGCGGTATACGAGGCCCCACA | | |
| MMP-13, F | TTGATGCGGTATACGAGGCCCCACA | 566 | 62 |
| MMP-13, R | TGTAGATAGGAAACATGAAGTGCTCC | | |
| MMP-13, P | CGAACTCATGCGCAGCAACAAGAAAC | | |

F, Forward primer; R, reverse primer; P, probe; AT, annealing temperature; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MT1, membrane type-1; MMP, matrix metalloproteinase

ing all four dNTPs (each at 200 μM), 0.5 units Taq polymerase (Takara, Kyoto, Japan), and each primer at 0.5 μM. PCR was initiated in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). Amplification was done with an initial denaturation for 3 min, followed by 30 cycles of 94 °C for 45 s, varying annealing temperatures (Table 1) for 45 s, and 72 °C for 1 min, and then a final extension step for 5 min. The PCR products were separated by electrophoresis on a 1.2% agarose gel. As described earlier [23], gels were depurinated with 0.1 N HCl for 20 min and were then denatured with 0.5 N NaOH/1.5 M NaCl for 30 min. Denatured gels were applied directly to wicks; 20× standard saline citrate (SSC) was used as a transfer buffer. Dry amphoteric nylon membranes (Biodyne A; Pall BioSupport, East Hills, NY, USA) were placed on the gel for transfer overnight. The transferred membrane was hybridized to a fluorescein-11-dUTP-end-labeled probe specific for the target cDNA fragment (Southern blotting), using a 3′-oligolabelling and detection system (Amersham International, Buckinghamshire, England) according to the supplier's protocol. Specific primers for the investigated targets are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

*Immunoprecipitation and Western blotting*

Subconfluent cell cultures from all cell lines and surgical specimens were washed twice with ice-cold phosphate-buffered saline (PBS), and were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM ethyline diamine tetraacetic acid [EDTA], 50 mM NaCl, 0.02% $NaN_3$, 1% Nonidet P-40, 0.25 mM dithiothreitol). Lysates (200 μg protein/sample) were precleaned and incubated with monoclonal antibodies (mAbs) against MMP-13, MMP-2, and MT1-MMP (mAbs were provided by Fuji Chemical Industries, Takaoka, Japan). Antigen-antibody complexes were precipitated with protein G coupled to Sepharose beads according to the supplier's protocol (Boehringer Mannheim, Germany). After a washing with lysis buffer containing protease inhibi-tor mix (Boehringer Mannheim), immunoprecipitates were suspended and boiled for 5 min in sample buffer (50 mM Tris/HCl, pH 6.8, 10% glycerin, 1% sodium dodecylsulfate [SDS], 0.1% bromophenol blue, and 5% 2-mercaptoethanol). Equal samples were then run on a 12% SDS polyacrylamide gel. Proteins were blotted onto an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in PBS, 0.05% Tween 20, and 2% skim milk at room temperature for 60 min. Primary antibodies were diluted in Tween-PBS containing 0.04% skim milk. Primary antibodies were used at a dilution of 1:1000 (anti-MMP-13, anti-MMP-2, anti-MT1-MMP mABs; Fuji Chemical Industries), and incubated at room temperature for 120 min. The secondary antibodies (horseradish peroxidase-conjugated anti-mouse IgG) (1:2000; Amersham Life Science, Arlington Heights, IL, USA) were used at a dilution of 1:2000 and incubated at room temperature for 45 min. The signals were detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) according to the supplier's recommendations.

*Gelatin zymography*

Zymography was performed as described [24]. All cell lines were cultured in RPMI 1640 with 10% fetal bovine serum until they reached the subconfluent state. Then the cells were washed with PBS and cultured in RPMI without serum for 48 h. The conditioned media were collected, pH adjusted to 7.6, concentrated 50 times with Ultrafree-MC (Millipore) by centrifugation at

10000 rpm for 30 min, and mixed with SDS sample buffer containing 10 mM Tris/HCl, pH 6.8, 20% glycerin, 2% SDS, and 0.1% bromophenol blue. Also, each surgical specimen was homogenized with the same sample buffer. In the absence of reducing agents, the mixtures were incubated for 20 min to denature MMPs and dissociate MMP-TIMP complexes. Electrophoresis was performed on 12% polyacrylamide gels containing 0.1% SDS and gelatin at a final concentration of 0.1% (w/v). Then the gels were washed in 2.5% Triton X-100 for 1 h to remove the SDS. During this process, proenzymes are activated autocatalytically. The gels were then incubated for 24 h in a reaction buffer (50 mM Tris/HCl, pH 7.6, 0.15 M NaCl, 10 mM $CaCl_2$, 0.02% $NaN_3$) and stained with 0.1% Amido black. The location of gelatinolytic activity was detectable as a clear band in a background of uniform staining. For the positive control, the concentrated supernatant of KMST-6 cells was used.

### *Immunohistochemistry for MMP-13 in gastric cancer tissues*

The sections were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in 0.3% $H_2O_2$ solution in methanol for 20 min to block endogenous peroxidase. The sections were then washed with water and with PBS for 5 min, then boiled in 10 mM sodium citrate solution (pH 6.0) for 10 min in a pressure cooker, and allowed to cool at room temperature. After incubation in 4% skim milk for 2 h at room temperature, the sections were incubated overnight at 4°C with anti-MMP13 (diluted 1:100) in PBS with 1% skim milk. The reaction was visualized using a Vectastain Elite ABC kit and 3,3′-diaminobenzidine solution (Vector Laboratories, Burlingame, CA, USA). The sections were then slightly counterstained with hematoxylin. Negative control slides were prepared by substituting an irrelevant antibody for the primary antibody.

### *Statistical analysis*

The correlations between MMP-13 and MT1-MMP expression and clinicopathological parameters were statistically evaluated by using Fisher's exact probability test. A $P$ value of less than 0.05 was considered significant. Patient survival times were calculated from the date of surgery until an individual's death. The survival rates were estimated by the Kaplan-Meier method [25], and compared by log-rank tests. In multivariate analysis, independent prognostic factors were determined by the Cox proportional hazards model ([26]; StatView 4.5; Abacus Concepts, Berkeley, CA, USA). The clinicopathologic classifications and staging were performed according to the Japanese classification of gastric carcinoma [20].

## Results

### *mRNA expression of MMP-13, MMP-2, and MT1-MMP by RT-PCR and Southern blotting*

MMP-13 mRNA was analyzed by RT-PCR and Southern blotting in nine gastric cancer cell lines. Except for one cell line (KKLS), comparable levels of MMP-13 mRNA were detected in all examined gastric cancer cell lines (Fig. 1). MT1-MMP mRNA expression was analyzed in the same cell lines. Strong expression was detected in eight cell lines, but NKPS cells did not express MT1-MMP. MMP-2 mRNA expression was found in eight cell lines, with no expression in MKN-28. MMP-13 mRNA was expressed in eight cell lines, and all cell lines coexpressed at least one of the activators of MMP-13 (MT1-MMP or MMP-2) (Fig. 1).

In the surgically resected specimens, the expression of these MMPs (MMP-13, MMP-2, and MT1-MMP) was examined in 110 gastric adenocarcimonas and their respective normal mucosae. MMP-13 mRNA was detected in 23 of the 110 tumors (21%). However, no expression was detected in any of the normal samples. MT1-MMP mRNA was detected in 45 of the 110 tumors (40%); however, no expression was observed in the normal counterparts. On the other hand, MMP-2 mRNA was expressed in 89 of the 110 primary tumors, but in 23 of the normal mucosa samples. Figure 2 shows representative results in 5 cases.

The positive rates for MMP mRNA expression in primary cancers in terms of histological stage were as follow: stage Ia, 7% (1/13); stage Ib, 33% (3/9); stage II, 28% (4/14); stage IIIa, 22% (5/22); stage IIIb, 17% (2/12); stage IVa, 20% (1/5)a; and stage IVb, 21% (7/34).

Overall survival curves in terms of MMP-13 and MT1-MMP mRNA expression are shown in Fig. 3. There was no difference in survival between the MMP-13-negative and MMP-13-positive mRNA expression group (Fig. 3a). However, the difference in survival time was significant between the MT1-MMP-negative

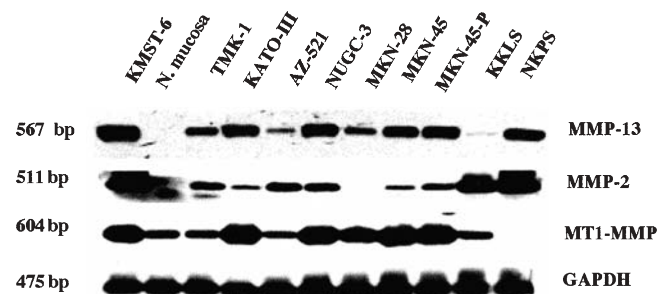


**Fig. 1.** Matrix metalloproteinase-13 (*MMP-13*), MMP-2, and membrane type-1-MMP (*MT1-MMP*) mRNA expression in gastric cancer cell lines. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *N. mucosa*, normal gastric mucosa

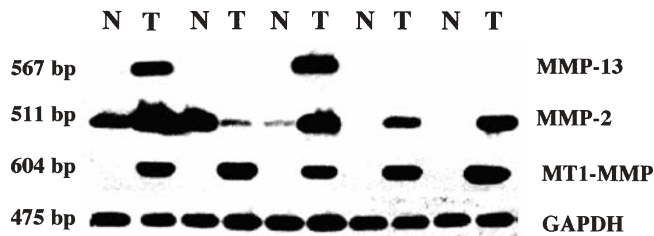


**Fig. 2.** MMP-13, MMP-2, and MT1-MMP mRNA expression in primary gastric cancer tissues (*T*) and their normal counterparts. *N*, Normal gastric mucosa

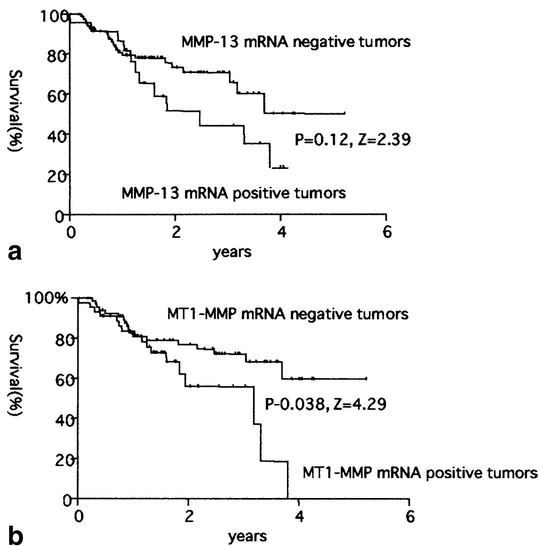


**Fig. 3. a** Survival curves of patients with gastric cancer in terms of the MMP-13 mRNA tissue status measured by a reverse transcription polymerase chain reaction (RT-PCR) method. **b** Survival curves of patients with gastric cancer in terms of the MT1-MMP mRNA tissue status measured by the RT-PCR method

and MT1-MMP-positive mRNA expression groups ($P = 0.038$) (Fig. 3b). Moreover, when analyzed together, MT1-MMP- and MMP-13-positive against MT1-MMP- or MMP-13-negative mRNA expression, the difference in survival was significant ($P = 0.0034$). The median survival time of the former group was 1338 days, while that of latter was only 701 days. The Cox proportional hazard model showed that serosal invasion and MMP-13/MT1-MMP mRNA status emerged as independent prognostic factors ($P = 0.008$, and $P = 0.014$, respectively) (Table 2) for predicting the overall survival. However, univariate and multivariate analysis showed no significant correlation between MMP-13/MT1-MMP mRNA expression and any of the clinicopathological parameters.

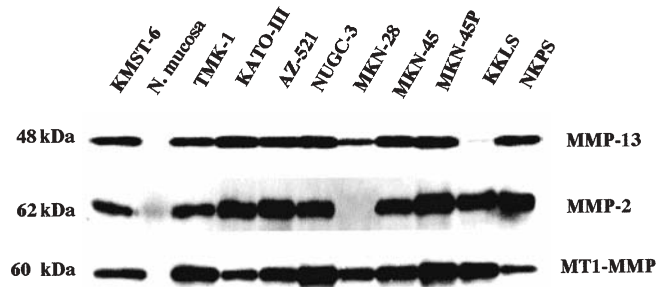


**Fig. 4.** MMP-13, MMP-2, and MT1-MMP protein expression in gastric cancer cell lines by immunoprecipitation (IP)/Western blotting

### *Protein expression of MMP-13, MT1-MMP, and MMP-2*

To determine whether the MMP-13, MT1-MMP, and MMP-2 mRNA were transcribed to protein and to establish the localization of the protein in these tumors, we evaluated protein expression by Western blotting, immunofluorescence, and immunohistchemistry in selected gastric cancer cell lines and in a series of representative tumors and their respective normal mucosae previously examined for mRNA expression. As shown in Fig. 4, immunoprecipitation and Western blotting showed that all examined gastric cancer cell lines, except for KKLS cells, expressed the expected size of MMP-13 protein (48kDa) nearly equal to the positive control KMST-6 cells (Fig. 4). Also, the protein expression of both MT1-MMP and MMP-2 were in parallel to the mRNA expression by Western blotting (Fig. 4). In the surgically resected specimens, MMP-13 and MT1-MMP bands (48 and 60kDa, respectively) were detected in the protein extracted from tumors and not from their corresponding normal adjacent mucosae (Fig. 5). These tumors had shown MMP-13 and MT1-MMP mRNA expression. Immunohistochemically, MMP-13 was detected in tumors with mRNA expression, but not in tumors with no mRNA expression. MMP-13 was expressed at the invasive front predominantly in a cytoplasmic pattern (Fig. 6). Also, MT1-MMP was only expressed by cancer cells; however, MMP-2 was mainly expressed by cancer cells with occasional positive stromal cells in the vicinity of the malignant epithelial cells (data not shown).

### *Functional MMP-13, MT1-MMP, and MMP-2 are expressed in gastric cancer as determined by zymographic analysis*

To further evaluate the role of these MMPs in gastric carcinoma, we studied all nine cell lines, as well as five tumor samples from patients with MMP-13 mRNA expression. Zymographic analysis of the concentrated cul-

**Table 2.** Cox proportional hazard model showed that serosal invasion and MMP-13/MT1-MMP mRNA status emerged as independent prognostic factors

| Clinicopathologic factors | $\chi^2$ | *P* value | Risk ratio | 95% CI |
|---|---|---|---|---|
| Histologic type | | | | |
| Differentiated vs undifferentiated | 0.243 | 0.188 | 1.198 | 0.584–2.456 |
| Lymph node status | | | | |
| Negative vs positive | 1.533 | 0.213 | 3.773 | 0.467–30.544 |
| Lymphatic invasion | | | | |
| Negative vs positive | 0.511 | 0.458 | 1.446 | 0.456–3.833 |
| Venous invasion | | | | |
| Negative vs positive | 0.417 | 0.519 | 1.333 | 0.557–3.188 |
| Serosal invasion | | | | |
| Negative vs positive | 6.928 | 0.008 | 4.228 | 1.445–12.369 |
| MT1-MMP mRNA expression | | | | |
| Negative vs positive | 1.732 | 0.188 | 1.613 | 0.791–3.289 |
| MMP-13 mRNA expression | | | | |
| Negative vs positive | 0.723 | 0.395 | 1.374 | 0.660–2.860 |
| MT1-MMP and MMP-13 mRNA expression | | | | |
| MT1(−) and MMP-13 (−) vs MT1(−) and MMP-13 (+) | 1.208 | 0.272 | 0.421 | 0.089–1.969 |
| MT1(−) and MMP-13 (−) vs MT1(+) and MMP-13 (−) | >0.001 | 0.998 | 1.001 | 0.395–2.532 |
| MT1(−) and MMP-13 (−) vs MT1(+) and MMP-13 (+) | 6.004 | 0.014 | 2.895 | 1.235–6.692 |

CI, Confidence interval

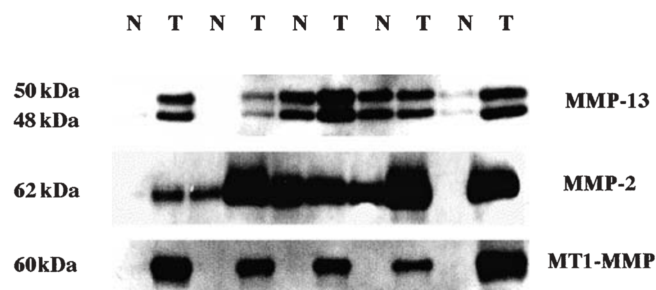


**Fig. 5.** MMP-13, MMP-2, and MT-1-MMP protein expression in five resected tumors and their normal counterparts by IP/Western blotting

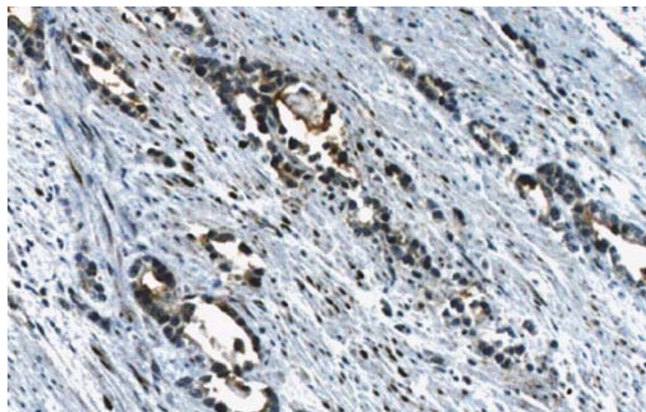

**Fig. 6.** MMP-13 immunoreactivity was detected at the invasion front of primary gastric cancer, and was localized on the cell membrane and in the cytoplasm of gastric cancer cells

ture supernatants of these cell lines revealed a combination of four gelatinolytic bands at 60, 56, 50, and 48 kDa, corresponding to the latent, two intermediate, and final active forms of MMP-13 (Fig. 7). These bands were confirmed to be specific for MMP-13 by Western blotting, using the mAb specific for MMP-13 in the same samples (Fig. 7). Gelatinolytic bands at 68, 64, and 62 kDa, corresponding to the latent, intermediate, and active forms of MMP-2 were detected in all cell lines expressing MMP-2 mRNA, with the highest expression in the NKPS and KKLS cell lines. The appearance of intermediate and active forms of MMP-2 and MMP-13 is consistent with the expression of their activator, MT1-MMP, in these cells. The gelatinolytic activity of MT1-MMP itself [27,28] was too weak to be detected in this condition and was not secreted in the media.

Analysis of tumor specimens revealed four strong gelatinolytic bands with molecular weights of 68, 64, and 62 kDa (latent, intermediate, and active forms of MMP-2, respectively), and 48 kDa (active form of MMP-13), whereas these bands in normal tissues were weak (Fig. 8). Western blot analysis using mAb specific to MMP-13 was used to confirm these bands in the same samples (Fig. 8).

## Discussion

Collagenase-3 is a member of the matrix metalloproteinase (MMP) family recently identified on the basis of its differential expression in breast carcinoma versus normal tissues [7]. This novel MMP (MMP-13) has also been implicated in inflammatory and degenera-

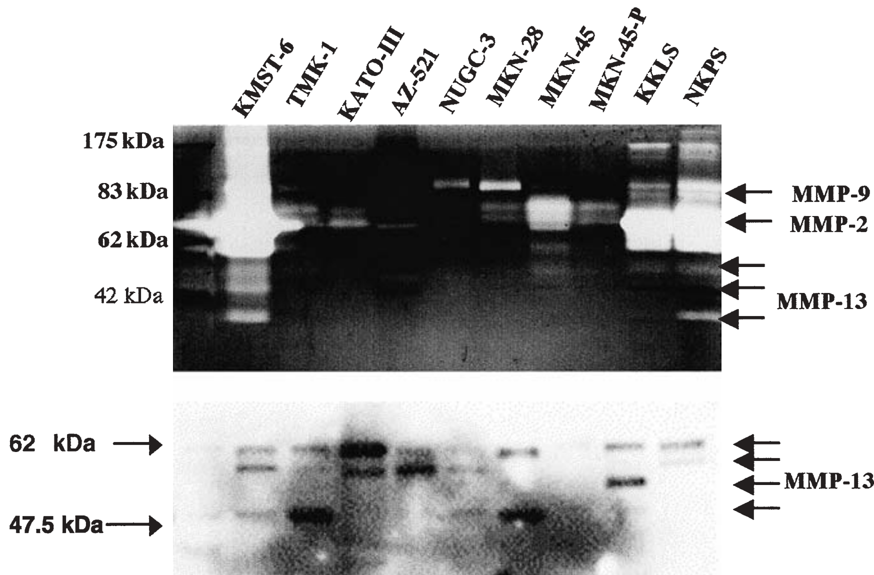


**Fig. 7.** Gelatin zymography (*above*) using culture supernatants of gastric cancer cell lines, and Western blotting (*below*) using monoclonal antibody (mAb) specific for MMP-13 in the same samples from cell lines

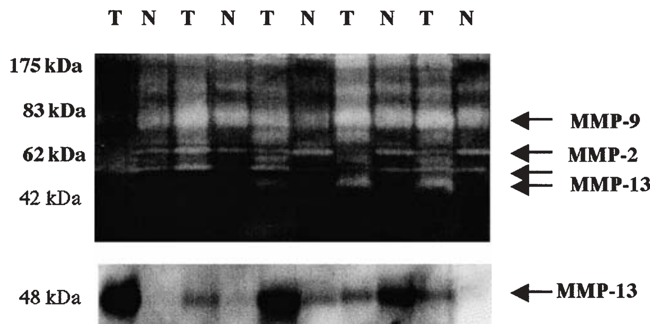


**Fig. 8.** Gelatin zymography (*above*) and Western blotting (*below*) using mAb specific for MMP-13 from the cell lysates of gastric cancer tissues and normal gastric mucosae

tive joint diseases [13,29]. However, although MMPs have been implicated in the mechanisms of progression and invasion of most solid tumors [16,30], there are no previous reports about MMP-13 expression in gastrointestinal tract tumors other than esophageal carcinoma [18]. In the present study, we show for the first time that the recently discovered human collagenase-3, MMP-13, is expressed in eight gastric cancer cell lines, whereas no MMP-13 expression can be detected in normal gastric mucosa, indicating that this enzyme is not constitutively expressed in gastric mucosa and that its upregulation is a phenomenon associated with malignant transformation. This possibility is in agreement with the known biochemical properties of collagenase-3, showing that it is a potent proteolytic enzyme with wide substrate specificity, including different protein components of the extracellular matrix and basement membranes which must be degraded during tumor progression [15]. The expression of MMP-13 could be detected in only 21% of cases (23/110) in vivo in our material, which included well, moderately, and poorly differentiated tumors representing different stages of invasion. The explanation for the more frequent expression in cells lines than in surgical specimens is that the selection of an MMP-13-expressing cell population may occur during the subculturing of gastric cancer cells as a consequence of genomic instability. This notion is also supported by the observation of Johansson et al. [31] that UT-DEC-1, a cell line derived from premalignant vaginal dysplasia, expressed MMP-13 only after it had been selected by subculturing to grow anchorage-independently. These observations provide strong evidence that MMP-13 is specifically activated in gastric cancer as the primary tumor progresses toward an invasive phenotype, and provide further evidence that MMP-13 expression is a marker for the invasive capacity of gastric cancer cells in vivo.

The mechanism involved in collagenase-3 activation is complex. It has been observed that collagenase-3 expression is enhanced by cytokines, such as transforming growth factor-alpha and -beta, and tumor necrosis factor-alpha [8]. In a recent report, MMP-2 and MT1-MMP were found to process MMP-13, and the rate of activation by MT1-MMP was enhanced in the presence of a latent form of MMP-2, suggesting that these three MMPs belong to the same activation cascade, leading to the generation of a potent extracellular gelatinolytic activity [14]. Interestingly, our results showed that all nine examined gastric cancer lines simultaneously expressed MMP-13 and at least one of its activators; MT1-MMP and/or MMP-2. This simultaneous expression of different MMPs by gastric cancer cells suggests that collaboration among them is required for degradation of the different extracellular matrix and basal mem-

brane components in the process of neoplastic invasion. Expression of other MMPs has been described previously in other cancers, but their patterns of expression and cellular localization show significant variability. Thus, MT1-MMP mRNA transcripts have been detected in tumor and stromal cells in human lung cancer [32] and in stromal cells of head and neck cancers [33]. Previous reports of gastric cancer demonstrated that MT1-MMP expression was mainly recognized in the cell membrane of cancer cells and fibroblasts in the invasion front [34,35]. In contrast, our results suggest that MT1-MMP may be linked to the expression of MMP-13 by tumor cells, mainly in an autocrine manner, as eight of the nine gastric cancer cell lines themselves produced MMP-13 and MT1-MMP. In summary, these observations lend support to the hypothesis that MMP expression in tumorous versus stromal cells may be controlled, at least in part, by tissue-specific mechanisms, and the breakdown of the extracellular matrix surrounding invasive tumors may not depend on the expression of a single MMP, but may involve interactions between tumorous and stromal cells, including the activation of tumor cell-derived MMP by tumor cells themselves and/or by stromal cells.

MT1-MMP expression was an independent factor influencing both tumor invasion of the gastric wall and lymph node metastasis [34,35,36]. In their report, Kabashima et al. [37] investigated the expression of MMP-2 and MMP-9 in gastric cancer by immunohistochemistry and found that the expression of MMP-9 was positive in 67% of tumors with lymph node-positive findings versus 32% in tumors with lymph node-negative findings; they suggested that these MMPs can degrade the basement membrane, allowing cancer cells to permeate the lymph capillaries and develop lymph node metastases. In the present study, we followed patients and recorded whether collagenase-3 and MT1-MMP mRNA expression were correlated with clinicopathological parameters. We found that the survival of patients with positivity for MMP-13 and MT1-MMP was significantly shorter than that of patients with no MMP-13 and MT1-MMP expression ($P = 0.0034$). The Cox proportional hazard model showed that serosal invasion and MMP-13/MT1-MMP mRNA status emerged as independent prognostic factors.

The expression of MMP-2 was also detected in tumor cells of gastric adenocarcinomas in vivo and in culture, although the expression of MMP-2 in vivo was more often detected in stromal cells (data not shown). It is likely that the combined collagenolytic activity of tumor-derived MMP-13 and MMP-2 produced by tumor or stromal cells plays an important role in gastric cancer invasion. However, our results provide evidence that MMP-13 is the principal collagenase expressed by tumor cells in gastric cancer. Furthermore, in our material of 110 gastric adenocarcinomas, all tumors with serosal invasion at the time of diagnosis showed high levels of MMP-13 mRNA, strongly suggesting a crucial role for MMP-13 in invasion and metastasis of gastric cancer. There was no significant difference between MMP-13 mRNA expression and the clinicopathological parameters involved in tumor invasion and metastasis. In addition, MT1-MMP expression did not correlate with prognostic indicators such as lymph node metastasis, invasion patterns, and serosal invasion. When the analyses were performed in combination with analysis of MT1-MMP and MMP-13 mRNA expression, no significant prognostic indicators could be found for the mRNA status of the two proteinases in the primary tumors. However, the prognosis in patients with tumors expressing both MMP-13 and MT1-MMP was significantly poorer than that of patients with tumors showing no expression of MMP-13 or MT1-MMP or with the expression of either MMP-13 or MT1-MMP. These results indicate that the simultaneous expression of MMP-13 and MT1-MMP in gastric cancer may indicate an independent prognosticator for poor prognosis, which cannot be predicted by the ordinary prognostic indicators.

Our results suggest that the ability of MMP-13 to initiate and continue the degradation of fibrillar collagens makes it a powerful tool for cancer cells and may contribute to their invasive potency in association with MT1-MMP. Finally, it is hoped that identification of individual MMPs, including MMP-13, and regulation of the activation systems associated with MT1-MMP in gastric cancer may provide benefits in establishing novel therapeutic strategies for preventing the invasion and metastasis of these tumor cells.

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