Clinical Significance of Matrix Metalloproteinase-7 Expression in Esophageal Carcinoma

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ABSTRACT

Matrix metalloproteinase-7 (MMP-7) is a member of MMP family and has a wide variety of substrate spectra. It is reported to play an important role in carcinoma invasion and metastasis. There is, however, little information on the clinical significance of MMP-7 in human esophageal carcinoma. We thus studied 48 tumor/normal pair samples of human esophagus by Northern blot analysis. The results demonstrated that the tumor tissue (T) of esophageal carcinoma showed a higher expression of MMP-7 mRNA than the corresponding normal tissue (N) in 31 cases (65%). We also statistically evaluated tumor MMP-7 value (T value) corrected for MMP-7-positive control (KYSE150 transfected with the MMP-7 gene). Fourteen cases with T value ≥ 0.3 showed a higher frequency of lymph node metastasis than 34 cases with T value < 0.3 (P < 0.05). The cases with T value ≥ 0.3 showed a significantly poorer prognosis than those with T value < 0.3 (P < 0.01). Multivariate analysis demonstrated that the MMP-7 expression status was the independent factor relating to the prognosis (P = 0.0005). The findings indicated that MMP-7 might be a novel prognostic factor for patients with esophageal carcinoma.

INTRODUCTION

Esophageal carcinoma is one of the most aggressive carcinomas, and the postoperative outcome remains unsatisfactory. On the other hand, cases showing long-term survival were recognized in some patients who underwent a curative operation, and it is therefore very important for surgeons and gastroenterologists to identify effective markers for the postoperative prognosis. In addition to the classical clinicopathological factors, several biological markers for malignant potential have been reported recently to be significant postoperative prognostic factors for the esophageal carcinoma. For example, Sugimachi et al. (1) showed that the DNA distribution pattern could be a new marker for prognosis in esophageal carcinoma. In addition, several factors, such as the coamplification of hst-1 and int-2 genes (2), cyclin D1 (3), DNA ploidy and c-erb-B2 (4), proliferating cell nuclear antigen (5), and Leu-7 and Ki-67 (6), have all been reported previously. These markers, however, are either involved in cell growth or upstream of the signals involved in the transformation of cells, and little has thus far been reported on proteases, which play a crucial role in carcinoma invasion and metastasis.

MMP2 has been demonstrated to be involved in carcinoma invasion and metastasis by degrading the extracellular components (7). MMP-7 is one of the MMP family members and consists of a primordial form of these members (8). It can degrade laminin (9), type IV collagen (10), and entactin (11), which are the main components of the basement membrane, and activate other important MMPs (MMP-1, MMP-2, and MMP-9; Refs. 12 and 13). It can also inactivate α1-antitrypsin (14), which augments the serine protease activity, and thus activates MMPs indirectly. We have demonstrated previously a correlation between MMP-7 and both tumor progression and metastasis in the human colon (15) and gastric carcinoma (16). We thus studied the MMP-7 expression and its clinical significance in human esophageal carcinoma.

MATERIALS AND METHODS

Patients and Sample Collection

The MMP-7 mRNA levels were investigated in a series of 48 esophageal carcinoma specimens from patients who underwent surgery at the Medical Institute of Bioregulation Hospital, Kyushu University and Saitama Cancer Center. All 48 cases were clearly identified based on the clinicopathological findings. All patients underwent a resection of the primary tumor. None of the patients received chemotherapy or radiotherapy. The patients included 42 males and 6 females. The tumor was located in the upper esophagus (n = 2), the middle esophagus (n = 31), and the lower esophagus (n = 15). Seven tumors were well differentiated, 23 were moderately differentiated, and 13 were poorly differentiated squamous cell carcinomas. Other types of carcinoma include basal cell carcinoma (n = 2), adenocarcinoma (n = 2), and carcinosarcoma (n = 1). The depth of invasion of the tumor was as follows: 6 involving submucosa, 4 with muscularis propria, and 38 with adventitia or over. The cases with lymph node metastases were classified into two groups: the nonmetastatic group (n = 8) and metastatic group (n = 40). The specimens were obtained from the tumor edge.

Received 5/28/99; revised 12/7/99; accepted 12/20/99.

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2The abbreviations used are: MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Significance of MMP-7 in Esophageal Carcinoma

RNA Extraction and Northern Blot Hybridization

Total RNA Isolation. Frozen-tissue specimens or cultured cell lines in the state of subconfluency were homogenized in 0.85 mol/L guanidinium thiocyanate, and total RNAs were obtained by ultracentrifugation through a cesium chloride cushion as described previously (17, 18).

RNA Analyses. An equal amount (15 μg) of total cellular RNA was loaded onto each lane of 1.2% agarose-formaldehyde gels and electrophoresed for 7 h. The RNAs were transferred to nylon membranes (Hybond-N+; Amersham Pharmacia Biotech UK, Inc., Little Chalfont, United Kingdom). The membranes were UV cross-linked with 120,000 mJ/cm² using an UV Stratalinker 1800 (Stratagene, Inc., La Jolla, CA). After overnight hybridization at 42°C, the blots were washed to a final stringency of 65°C in 0.1× SSPE and 0.1% SDS. Autoradiography was performed at room temperature with an intensifying screen. To insure that comparable amounts of mRNA from both tumor and adjacent normal tissue had been transferred, blots were rehybridized with GAPDH probe.

Probe Preparation. The DNA probes for MMP-7 and GAPDH were previously described (15, 19). They were labeled with 32P deoxyctydine triphosphate using a random-primed DNA labeling kit (Takara, Inc., Otsu, Japan).

Quantitation and Statistical Analysis

MMP-7 mRNA levels were quantitated by measuring the intensities of the appropriate bands in autographs using the Bioimage Analyzing System 1000 (Fuji Film, Inc., Tokyo, Japan). The fold-increase of MMP-7 transcript in each tumor relative to its corresponding adjacent normal tissue was calculated after normalizing for GAPDH expression in the following manner: $T/N = [\text{MMP-7} (T)/\text{GAPDH} (T)]/[\text{MMP-7} (N)/\text{GAPDH} (N)]$, where $T$ means tumor and $N$ means corresponding normal tissue specimens. To evaluate MMP-7 alone, we also calculated the $T$ value using the MMP-7 value of transfectant as an internal control of each membrane in the following manner: $T$ value $= [\text{MMP-7} (T)/\text{GAPDH} (T)]/(\text{MMP-7 of MMP-7 transfectant})/\text{GAPDH of MMP-7 transfectant}$. A multivariate analysis was performed. The BMDP statistical computer program (BMDP, Los Angeles, CA) for the main frame computer (IBM, Inc., Armonk, NY) was used for all analyses. Associations between the variables were tested by Student’s $t$ test or Fisher’s exact probability test. The BMDP P2L program was used for the multivariate adjustments for all covariates simultaneously, with a backward stepwise logistic regression analysis.

Immunohistochemistry

To identify the localization of MMP-7 in the esophageal carcinoma tissue specimens, an immunohistochemical analysis was performed in 11 cases by using a method described previously (16). Six normal mucosal tissues were also immunostained similarly. Briefly, 5-μm-thick sections were cut from the formalin-fixed, paraffin-embedded block. The primary monoclonal antibody used was purchased by Fuji Pharmaecochemical Inc. (Toyama, Japan; Refs. 9, 20, and 21). The recommended dilution was 1:100. Immunostaining was done by the avidin-biotin-peroxidase method.

Western Blot Analysis

Total protein was extracted from clinical samples ($n = 20$) by RIPA buffer. This same aliquots (60 μg) of total protein were applied to 10% acrylamide gradient gels. After electrophoresis, samples were electrophoressed onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 A for 2 h at 4°C. MMP-7 was detected by the use of mouse anti-MMP-7 antibody. The membrane was stripped and rehybridized with a probe to GAPDH as an internal control. Western blot analysis (Fig. 1) was performed as described in the Materials and Methods. The 20,000 molecular weight protein band representing MMP-7 was detected by the use of mouse anti-MMP-7 antibody.

Fig. 1 a, positive control of MMP-7 mRNA and protein. MMP-7 transfectant was constructed using geneticin selection. This cell line was confirmed to express both mRNA and protein of MMP-7 by Northern blot hybridization (top and middle) and Western blotting (bottom). P, positive control (KYSE150 stably transfectd with the MMP-7 gene). N, negative control (KYSE150, which did not express MMP-7 constitutively). The upper arrow is at $M_r$ 30,000 and the lower arrow is at $M_r$ 20,000. b, Northern blot hybridization analysis is representative of six cases. Top, MMP-7 mRNA expression in esophageal carcinoma (T) and adjacent normal mucosa (N). Middle, the same membranes were stripped and rehybridized with a probe to GAPDH as an internal control. Bottom, MMP-7 mRNA $T$ corrected for positive control of the MMP-7 each membrane.

thus avoiding the necrotic center, immediately after resection. They were quick-frozen in liquid nitrogen and stored at $-80^\circ$C until processing. Corresponding normal mucosa specimens distant at least 5 cm away from the tumor edge were also obtained by sharply dissecting the mucosa off the muscularis propria.

Used Cell Line and MMP-7 Transfection

Eleven esophageal carcinoma cell lines (TE1, TE2, TE3, TE4, TE5, TE7, TE8, TE11, TE12, TE13, and KYSE150) were obtained from the Cell Response Center for Biomedical Research Institute of Department, Aging and Cancer, Tohoku University (Sendai, Japan). KYSE150 stably transfected with MMP-7 was prepared as follows. PCR product of full-length MMP-7 cDNA was ligated into pCR. 3.1 expression vector (In Vitrogen, Inc. Carlsbad, CA), and sequencing confirmed MMP-7 was prepared as follows. PCR product of full-length MMP-7 cDNA was ligated into pCR. 3.1 expression vector (In Vitrogen, Inc. Carlsbad, CA), and sequencing confirmed MMP-7 using DNA sequencing kit-Dye terminator cycle sequencing ready reaction (PE Applied Biosystems, Inc., Foster, CA), and no mutation was recognized. After being transiently transfected with MMP-7 using Lipofectamine (Life Technologies, Inc., Rockville, MD), MMP-7 stable transfectant was selected using geneticin (G418; Life Technologies), and the expression of MMP-7 mRNA and protein was ensured by Northern blot hybridization and Western blot analysis (Fig. 1a).

All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The membranes were UV cross-linked with 120,000 mJ/cm² using an UV Stratalinker 1800 (Stratagene, Inc., La Jolla, CA). After overnight hybridization at 42°C, the blots were washed to a final stringency of 65°C in 0.1× SSPE and 0.1% SDS. Autoradiography was performed at room temperature with an intensifying screen. To insure that comparable amounts of mRNA from both tumor and adjacent normal tissue had been transferred, blots were rehybridized with GAPDH probe.

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A multivariate analysis was performed. The BMDP statistical computer program (BMDP, Los Angeles, CA) for the main frame computer (IBM, Inc., Armonk, NY) was used for all analyses. Associations between the variables were tested by Student’s $t$ test or Fisher’s exact probability test. The BMDP P2L program was used for the multivariate adjustments for all covariates simultaneously, with a backward stepwise logistic regression analysis.

Immunohistochemistry

To identify the localization of MMP-7 in the esophageal carcinoma tissue specimens, an immunohistochemical analysis was performed in 11 cases by using a method described previously (16). Six normal mucosal tissues were also immunostained similarly. Briefly, 4-μm-thick sections were cut from the formalin-fixed, paraffin-embedded block. The primary monoclonal antibody used was purchased by Fuji Pharmaecochemical Inc. (Toyama, Japan; Refs. 9, 20, and 21). The recommended dilution was 1:100. Immunostaining was done by the avidin-biotin-peroxidase method.

Western Blot Analysis

Total protein was extracted from clinical samples ($n = 20$) by RIPA buffer. This same aliquots (60 μg) of total protein were applied to 10% acrylamide gradient gels. After electrophoresis, samples were electrophoressed onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Inc., Bedford, MA) at 0.5 A for 2 h at 4°C. MMP-7 was detected by the use of mouse anti-MMP-7 antibody. The membrane was stripped and rehybridized with a probe to GAPDH as an internal control. Western blot analysis (Fig. 1) was performed as described in the Materials and Methods. The 20,000 molecular weight protein band representing MMP-7 was detected by the use of mouse anti-MMP-7 antibody.

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monoclonal primary antibodies to MMP-7 used in an immunohistochemical analysis at a dilution of 1:1000. The blots were developed using antimouse immunoglobulin, horseradish peroxidase-linked whole antibody (Promega, Inc., Madison, WI). Signals for MMP-7 were detected using Supersignal (Pierce, Inc., Rockford, IL). The prestained high molecular weight markers were run on gels (Amersham Life Science, Inc., Little Chalfont, United Kingdom).

RESULTS

MMP-7 Expression by Northern Blot Hybridization and Its Clinical Significance. On the basis of our present analysis, the esophageal carcinoma and normal mucosa showed variable levels of MMP-7 mRNA signals by Northern blot hybridization. In 31 (65%) of the 48 cases, the expression of MMP-7 mRNA was greater in T than in N. However, among them, only 25 cases expressed an expression of MMP-7 in the normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases. The T:N ratio of MMP-7 mRNA was calculated in 25 cases with expression of MMP-7 in normal esophageal mucosal tissue specimens. Fig. 1b showed representative cases.

As shown in Table 1, all 14 cases with T ϳ 0.3 showed lymph node metastasis, whereas 26 of 34 cases with T < 0.3 showed lymph node metastasis; a significant difference was observed between the two groups (P < 0.05). On the other hand, no significant differences were seen regarding age, sex, histological differentiation, the depth of wall invasion, or lymphatic or vascular permeation between these groups. As shown in Table 2, a multivariate analysis demonstrated that MMP-7 expression did not influence lymph node metastasis (P = 0.087). Nevertheless, MMP-7 did influence lymph node metastasis (P = 0.04) at step zero after lymphatic permeation (P = 0.025).

Regarding prognosis, the patient with T ϳ 0.3 (n = 14) showed a significantly poorer prognosis than those with T < 0.3 (n = 34; P = 0.002; Fig. 2). Multivariate analyses showed MMP-7 overexpression to be a significant independent prognostic factor (P = 0.0005) in comparison with other clinicopathological factors.

All investigated esophageal carcinoma cell lines hardly expressed MMP-7 mRNA, based on the Northern hybridization findings (data not shown).

MMP-7 Expression by Immunohistochemistry. An immunohistochemical study was performed in 17 cases, including 6 normal mucosal tissues and 11 carcinoma tissues. Slight staining was seen in the distant normal mucosal tissue in four cases (Fig. 3a). All 11 carcinoma tissues were immunostained as to MMP-7. Carcinoma cells facing the stroma also showed an intense staining, despite almost nonstaining in the stromal components (Fig. 3b). The esophageal proper glands among the nests were also immunostained.

MMP-7 Protein by Western Blot Analysis in Esophageal Carcinoma. Fig. 4 showed a representative band at Mᵣ 29,000 (proMMP-7). Six cases showed this band in 20 carcinoma tissue specimens, whereas only 4 cases in 20 normal esophageal tissue specimens demonstrated this band. Cases expressing proMMP-7 showed either equal or more intense signals in carcinoma tissue specimens than the normal tissue specimens. No band at Mᵣ 19,000 (activated MMP-7) was recognized in this analysis. Positive control revealed our used monoclonal MMP-7 antibody to recognize a band at Mᵣ 19,000 and 29,000 alone, which can correspond to activated and latent MMP-7, respectively.

DISCUSSION

MMPs in esophageal carcinoma were reported on MMP-2 and MMP-3 to be crucial for tumor invasion and metastasis (22). According to Shima et al. (22), MMP-2 and MMP-3 were immunolocalized in carcinoma cells of invasive margin either in 14 (47%) of 29 cases, respectively, and their expression was correlated with the depth of invasion, vessel permeation, and lymph node metastasis. MMP-11 was demonstrated recently to correlate with tumor size, invasive depth, and the relapse-free survival by Northern blot hybridization in esophageal carcinoma (23). However, the significance of MMP-7, which contains only

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<th>Table 1</th>
<th>MMP-7 expression and clinicopathological factor</th>
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<tr>
<td></td>
<td>MMP-7 T value</td>
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<tr>
<td>Age (yr)</td>
<td>&lt;0.3 (n = 34)</td>
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<tr>
<td>Sex</td>
<td>64.5 ± 9.5</td>
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<tr>
<td>Male</td>
<td>29</td>
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<tr>
<td>Female</td>
<td>5</td>
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<tr>
<td>Histological differentiation⁺⁺</td>
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<tr>
<td>Well</td>
<td>4</td>
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<tr>
<td>Moderate</td>
<td>19</td>
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<tr>
<td>Poor</td>
<td>9</td>
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<td>Others</td>
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<tr>
<td>Upper</td>
<td>1</td>
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<tr>
<td>Middle</td>
<td>24</td>
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<tr>
<td>Lower</td>
<td>9</td>
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<tr>
<td>Depth of invasion</td>
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<td>Submucosa</td>
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<td>Muscularis propria</td>
<td>3</td>
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<tr>
<td>Adventitia</td>
<td>26</td>
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<td>Vascular permeation</td>
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<td>Absent</td>
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<td>Lymphatic permeation</td>
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<td>Present</td>
<td>29</td>
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<td>Lymph node metastasis</td>
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<td>Absent</td>
<td>8</td>
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<td>Present</td>
<td>26</td>
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⁺⁺ NS, not significant.
⁺⁺ This analysis includes 43 squamous cell carcinoma. Others include basal cell carcinoma (n = 2), adenocarcinoma (n = 2), and carcinosarcoma (n = 1).
the minimal number of domains and thus has a fundamental function, has not been studied in detail at the mRNA level in esophageal carcinoma. The characteristics of MMP-7 includes:

(a) a broad spectrum of substrate specificity; (b) the localization of carcinoma cells themselves (24); (c) involvement in tumorigenicity (25, 26); and (d) resistance to tissue inhibitor of metalloproteinase attributable to the lack of a COOH-terminal hemopexin-like domain (27).

We investigated a series of 48 archival specimens in esophageal carcinomas, and MMP-7 was demonstrated to be overexpressed in 31 carcinoma tissues as compared with the corresponding normal esophageal mucosa from the esophagus. Western blot analysis also revealed MMP-7 to be expressed in both carcinoma and normal tissue specimens, and an overexpression of MMP-7 was recognized in carcinoma tissue specimens (Fig. 4). Some normal esophageal mucosas also showed a slight expression of MMP-7, and the physiological function thus was suggested in normal esophagus, such as the growth of epithelial cells. Immunohistochemically, Adachi et al. (24) demonstrated MMP-7 to be localized in carcinoma cells in all investigated esophageal carcinomas (13 of 13) as well as in various other carcinomas (16, 28), and we had the same results. However the immunostaining pattern of MMP-7 was heterogeneous among the carcinoma cell nests, and the invasive fronts were especially strongly stained in our cases, which might suggest the involvement of MMP-7 in carcinoma invasion. On contrast, stromal cells in tumor tissue specimens were hardly recognized regarding the localization of MMP-7 proteins.

MMP-7 transcripts in situ were also reported to be expressed in carcinoma cells (21, 29), and this in turn indicated that MMP-7 protein was translated and used in the carcinoma cells themselves in a different manner than MMP-2, which was translated mainly in stromal cells and used in carcinoma cells.

All investigated esophageal carcinoma cell lines hardly expressed MMP-7 mRNA. This pattern greatly differed from the results of surgical samples. This indicated that MMP-7 expression in epithelial cells was not completely independent of the
indicated that MMP-7 might be strongly involved in the degradation of the basement membrane. However, it remains controversial as to whether MMP-7 transfectants augmented in vitro invasion through Matrigel (25, 36, 37). The MMP-7 overexpression did not correlate with vascular permeation in esophageal carcinoma. This may be because of differences in the investigated organ rather than to the small number of investigated cases. An abundant number of lymphatic vessels in the submucosa are characteristic of esophageal carcinoma, and esophageal carcinoma involving submucosa has been demonstrated to show a similar high incidence of gastric carcinoma, involving the muscularis propria, and a similar malignant biology to that of advanced gastric carcinoma (38).

MMP-7 overexpression significantly correlated with a poor prognosis based on the univariate Kaplan-Meier method ($P = 0.002$). Our multivariate analysis also revealed MMP-7 overexpression to be a significant independent factor ($P = 0.0005$) influencing the prognosis, among other clinicopathological factors, as a similar observation made by Yamamoto et al. (39). In conclusion, our analysis revealed that an overexpression of MMP-7 can effect lymph node metastasis via lymphatic permeation and could thus be an independent prognostic factor for esophageal carcinoma. In addition, the inhibition of MMP-7 using substances antagonizing MMP-7 may help control the spread of esophageal carcinoma.

REFERENCES

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