

Matrix Metalloproteinase Matrilysin in Cancers of the Digestive Organs

〈Review〉

Hiroyuki Yamamoto, Fumio Itoh*, Yasushi Adachi, Shouhei Iku, Hiroshi Fukushima, Shina Horiuchi, Yongfen Min, Shigeru Sasaki, Yoshiaki Arimura, Takao Endo, and Kohzoh Imai

*First Department of Internal Medicine, Sapporo Medical University School of Medicine
South 1, West 16, Chuo-ku, Sapporo, Hokkaido, 060-8543 Japan*

**Corresponding author: Fumio Itoh*

ABSTRACT

Matrix metalloproteinase (MMP) matrilysin has been implicated in tumor invasion and metastasis as well as in tumor initiation and growth in gastrointestinal and other cancers. As a MMP, matrilysin is unique in its minimum MMP structure, wide spectrum of substrate specificity, potency for starting an activation cascade of MMPs, and, most notably, in its production by cancer cells themselves. The production by cancer cells themselves could be an advantage as a biological marker of the malignant phenotype. Indeed, we have found that matrilysin expression at the invasive front is correlated with the progression of gastric, colorectal, hepatocellular, and pancreatic carcinomas as well as esophageal squamous cell carcinoma. We have also revealed the correlation of

matrilysin expression with recurrence and/or poor prognosis in carcinomas of the colorectum, liver, pancreas, and esophagus. Another advantage is its susceptibility to direct therapeutic intervention. Inhibition of matrilysin by an antisense expression vector or antisense oligonucleotides has been demonstrated to suppress the *in vitro* invasive potential or *in vivo* metastatic potential of gastric, colonic, and pancreatic cancer cells. The crucial roles of MMPs, especially those of matrilysin, in cancers have thus generated considerable interest in the use of synthetic MMP inhibitors as potential therapeutic agents. Matrilysin could be a promising therapeutic molecular target for cancers of the digestive organs.

Key words: Matrix metalloproteinases, Matrilysin, Tumor invasiveness, Tumor metastasis

1. Introduction

In the process of tumor invasion and metastasis, degradation of extracellular matrix (ECM) mediated by matrix-degrading enzymes is one of the most important step¹⁾. Evidence has emerged that proteolytic degradation of ECM is required for cancer cells to invade basement membranes, stromal matrices, and cell junctions (Fig. 1). Cancer cells secrete var-

ious matrix-degrading enzymes, including matrix metalloproteinase (MMP), serine proteinases, thiol proteinases, and aspartic proteinases. MMPs are zinc ion requiring proteolytic enzymes (Fig. 2) and have been implicated in several normal processes of tissue remodeling, such as embryonic development, wound healing, trophoblast implantation, and organ morphogenesis, as well as in the development of

*To whom requests for reprints should be addressed

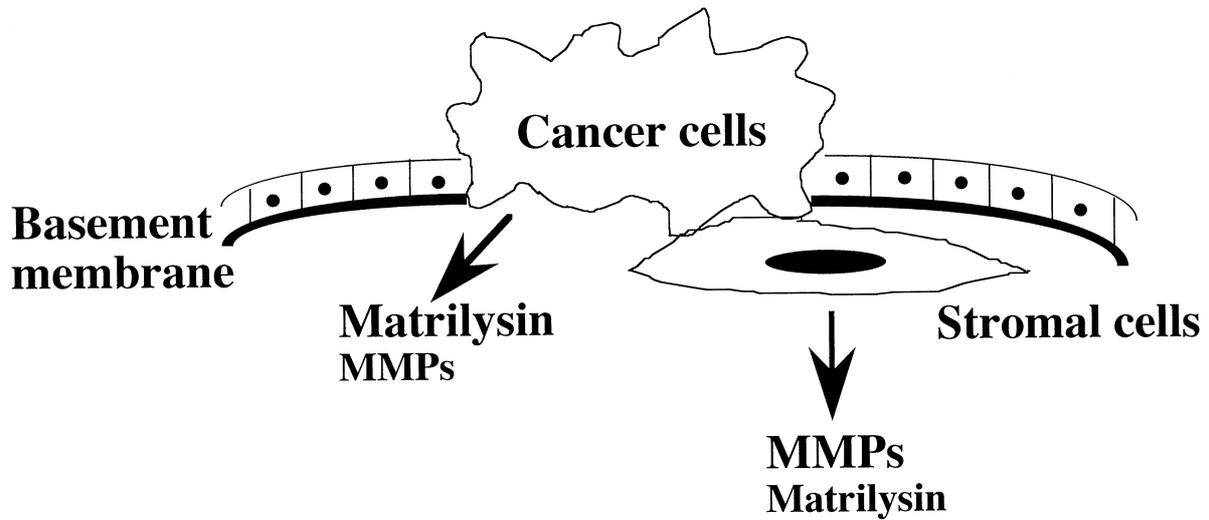


Fig. 1 Production of MMP for the degradation of basement membrane

Soluble MMP

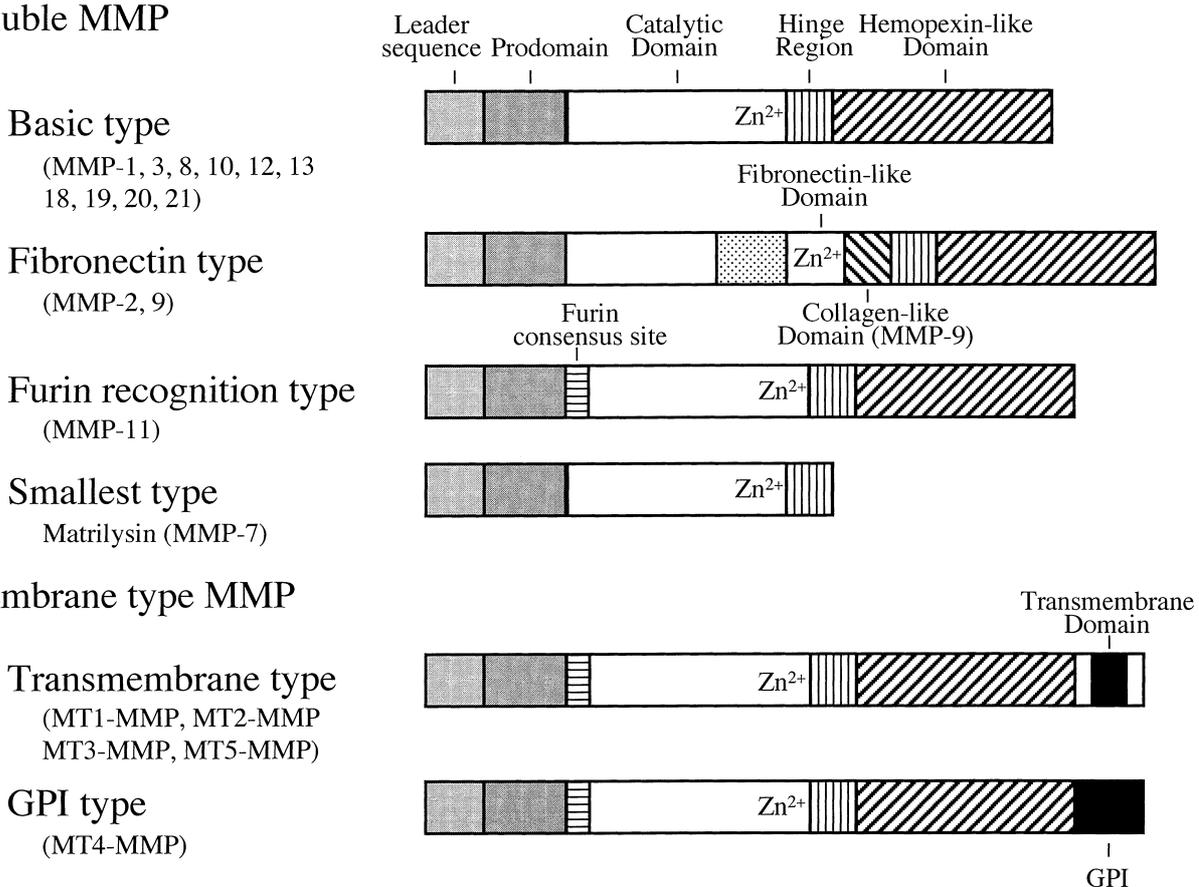


Fig. 2 Structure of the MMP gene family

various disease¹⁻⁴). MMPs are often active during tumor invasion, resulting in an excessive proteolytic degradation of ECM. There is substantial evidence that MMPs play a particular-

ly important role in tumor progression¹⁻⁴).

The MMP family is secreted as a latent proenzyme requiring the removal of an amino-terminal domain to attain enzyme activity (Fig.

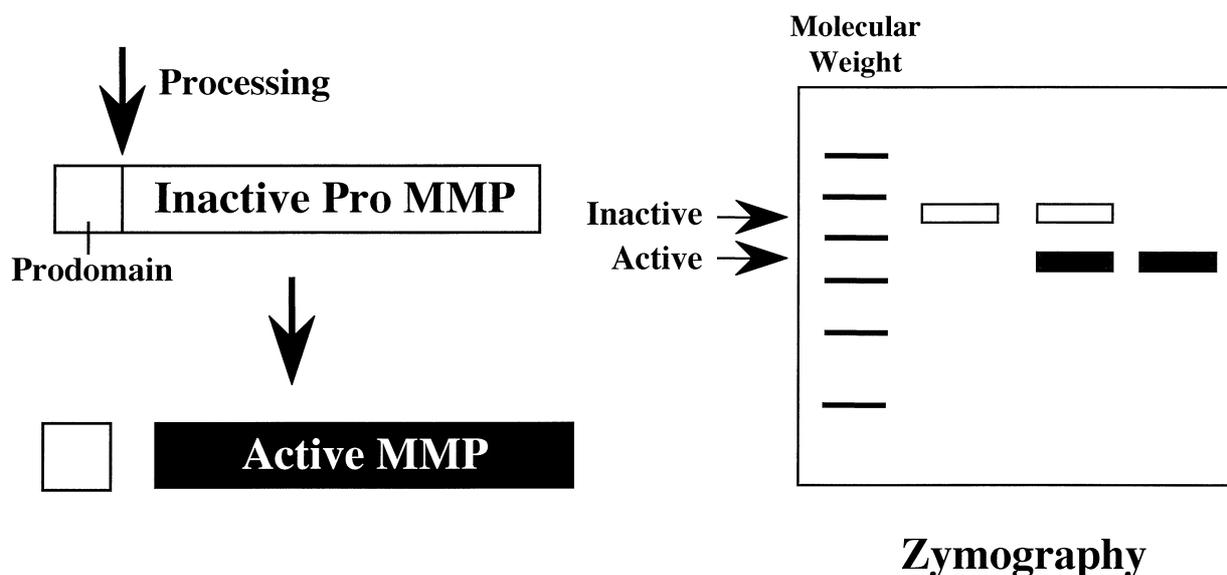


Fig. 3 Activation of MMPs and its detection by zymography

3). Total MMP activity is determined by the amount of proenzyme expressed, the extent to which the proenzyme is activated, and by a specific class of natural inhibitors, designated as tissue inhibitors of metalloproteinases (TIMPs)⁵⁾. Zymography can separate proteinases on the basis of molecular weight under denaturing conditions (Fig. 3). The conversion of latent inactive proMMP to active form involves the proteolytic removal of about 10 kDa of amino-terminal domain. Thus, inactive and active forms can be distinguished according to molecular weight by zymography.

It is thought that the balance between MMPs and TIMPs determines the proteolysis *in vivo*. The close ratio of TIMPs to MMPs that is required to neutralize enzymatic activity means that small changes in the levels of either leads to biologically significant changes in net proteolytic activity. If MMP expression increases and/or TIMP expression decreases, the balance favors proteolysis. Thus, a disruption in the balance between MMPs and TIMPs has been implicated in the progression of many types of cancer.

2. Characteristics of matrilysin

Matrilysin (MMP-7) is a member of the stromelysin subclass of the MMP gene family (Fig. 4). After being activated, matrilysin has a broad proteolytic activity against a variety of ECM substrates, including collagens, proteoglycans, elastin, laminin, fibronectin, and casein⁶⁻⁹⁾. Matrilysin was first identified in the postpartum rat uterus and has been detected in several normal tissues, such as endometrium, bronchial mucosa, monocyte, and mesangial cell⁶⁻⁹⁾. Compared with other MMPs, matrilysin is distinguished by its lowest molecular weight of 28,000 (Fig. 2). This smallest metalloproteinase has only two structural domains and lacks a C-terminal domain, which may contribute to the binding of TIMPs to proMMPs. Thus, inactive promatrilysin may escape inhibition by TIMPs, but active matrilysin, like the other active MMPs, has been shown to be inhibited by TIMPs possibly via the active-site of the enzyme¹⁰⁾ (Fig. 5). Matrilysin has been shown to activate MMP-2 *in vitro*¹¹⁾ and to inactivate serine proteinase inhibitors; the latter results in upregulation of a cascade of metalloproteinase and serine proteinases^{11,12)}. The activation mechanism of matrilysin *in vitro* appears to be similar to that of the other MMPs, which were

CHARACTERISTICS OF MATRILYSIN

The smallest member of the MMP family

Degradation of a **wide spectrum** of extracellular matrix elements

Activation by trypsin, plasmin, leukocyte elastase, and MMP-3

Expression in the **epithelial component** of both **benign** and **malignant** stages of many carcinomas

Expression in **vascular endothelial cells** adjacent to matrilysin-producing tumors

Reduction in both number and size of intestinal adenomas in *Min / +* mice lacking matrilysin

One of the target genes of **β -catenin/TCF-4** in the Wnt pathway

Fig. 4 Characteristics of matrilysin

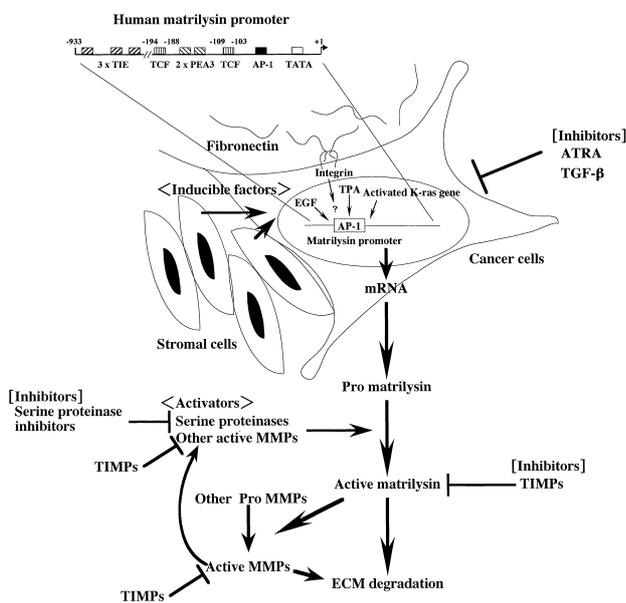


Fig. 5 Regulation mechanisms of matrilysin activity

capable of being activated by activator proteinases such as serine proteinases^{13,14}. Trypsin is another of the promising candidates for activation of matrilysin *in vivo*¹⁵. Previous studies have shown that matrilysin mRNA is overexpressed

in various cancers, including squamous-cell carcinoma of the head and neck and the lung, as well as cancers of the breast, prostate, stomach, and colon^{4,9,16}.

3. Colorectal cancer

Expression of matrilysin in colorectal cancers and adenomas

In colorectal cancer, mRNA for various MMPs such as MMP-1, MMP-2, matrilysin, MMP-9, MMP-11, and membrane type-1 MMP (MT1-MMP) has been shown to be overexpressed^{16,17}. It is noteworthy that only matrilysin and MT1-MMP are expressed in colorectal cancer cells themselves, whereas the other MMPs are mainly expressed in stromal cells. We examined the expression of matrilysin in 10 colorectal cancer tissues by RT-PCR¹⁷. In 9 of the 10 cases, matrilysin mRNA was detected in cancer tissues, but not in adjacent normal colon tissues. These results suggest that matrilysin is expressed in a tumor-associated manner in colorectal cancers¹⁷. It seemed significant to

analyze matrilysin expression in various disorders of colorectal epithelium, where different genetic alterations and different interactions between epithelial components and stromal environments may give rise to different profiles of matrilysin expression. We examined the expression of matrilysin mRNA in various colorectal disorders and its localization using RT-PCR and *in situ* hybridization¹⁸⁾. Matrilysin mRNA was detected in all adenoma tissues examined and its message was localized in adenoma cells themselves. In contrast, matrilysin mRNA was not detectable in hyperplastic polyps, mildly inflamed regions of ulcerative colitis, or normal colorectal tissues from patients without cancer.

We then analyzed the levels of secreted matrilysin using casein zymography. The secretion of matrilysin was detected in both cancer and adenoma tissues but not in hyperplastic polyps, mildly inflamed regions of ulcerative colitis, or normal colorectal tissues. Total activities of secreted matrilysin in adenomas were lower than those in cancers, suggesting that the secretion of matrilysin is enhanced during malignant conversion of colorectal epithelium. In addition, striking differences in the activation of matrilysin were found between cancers and adenomas. While the active form of matrilysin was hardly detectable in adenoma tissues, both inactive and active forms were detected in cancer tissues. Thus, the activity of matrilysin seems to be differentially regulated between cancer and adenoma by means of the secretion levels and the activation machinery. Previous reports have shown that inactive promatrilysin does not form a complex with TIMP, but that active matrilysin does¹⁰⁾. Thus, the activation of promatrilysin and the following complex formation with TIMPs are likely to be key regulatory steps in the control of matrilysin activity. Large quantities of active matrilysin overwhelming the inhibition of TIMPs seem to play a role in colorectal cancer progression.

Induction of matrilysin expression in colon cancer cells

Previous studies have shown that matrilysin, like other members of the MMP family, is up-regulated by TPA and EGF¹⁹⁾ (Fig. 5). We examined the effects of activated K-ras oncogene on the expression of matrilysin in colon cancer cells²⁰⁾. We found that both mRNA and enzyme activity of matrilysin were induced by the introduction of activated K-ras gene into SW 1417 colon cancer cells. To understand the mechanisms regulating this induction, we analyzed alterations of AP-1 activity induced by activated K-ras gene using the chloramphenicol acetyltransferase assay. AP-1 activity in SW1417 cells expressing activated K-ras gene was higher than that in control cells. The gel-shift assay showed higher levels of AP-1 binding protein in SW1417 cells expressing activated K-ras gene than in control cells. These results suggest that activated K-ras gene plays a role in inducing expression of matrilysin through the AP-1-dependent pathway in colon cancer cells (Fig. 5).

The host-tissue-environment factors, such as interactions between cells and the matrices have been shown to influence MMP activities and metastasis of cancer cells. Fibronectin, a major component of ECM, is one of the most suitable substrates for matrilysin. Recent studies have shown that some fibronectin fragments and native fibronectin have various functions. Attachment of cells to immobilized fibronectin and occupancy or clustering of fibronectin receptors by antibodies are signals for regulating gene expression of several genes including stromelysin. We found that incubation of colon cancer cells on immobilized fibronectin could induce matrilysin mRNA¹⁸⁾ (Fig. 5). In addition, when immobilized, truncated fibronectin and RGD peptide induced matrilysin mRNA. These results suggest that truncation of fibronectin is a requisite for transfer of information, and that conformation of the binding site in fibronectin is likely to be altered when fibronectin is degraded by enzymes such as

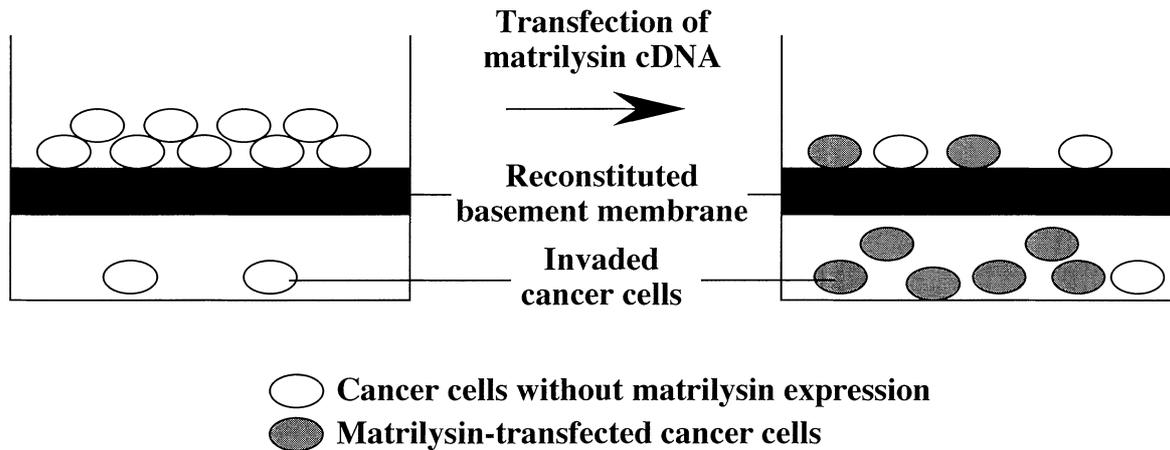


Fig. 6 Enhanced *in vitro* invasive potential of cancer cells transfected with matrilysin

fibronectinase, stromelysin, and matrilysin itself. In other words, some initial degradation of ECM may evoke further degradation through induction of matrilysin.

Effects of matrilysin on the invasive potential of colon cancer cells

Overexpression of matrilysin in a human prostate cancer cell line DU-145 has been shown to increase its invasive potential in a SCID mice model^{4,9)}. A recent study has shown that the expression of matrilysin mRNA in colorectal cancer tissues increases with advanced Dukes' stage and is the greatest in the metastatic liver lesions²¹⁾. These findings suggest that the roles of matrilysin in colorectal cancer are associated with late events in carcinogenesis, such as invasion and metastasis. We examined the effects of matrilysin on the invasive potential of colon cancer cells²²⁾. We introduced matrilysin cDNA into a CHC-Y1 colon cancer cell line which expressed no matrilysin mRNA and established transfectants secreting various levels of matrilysin. Matrilysin-transfected CHC-Y1 cells were more invasive than mock-transfected control cells as assessed by an *in vitro* invasion assay (Fig. 6). These results suggest a causal relationship between matrilysin secretion and invasiveness of colon cancer cells.

Down-regulation of matrilysin expression in colon cancer cells

The suppression of matrilysin activity could be an excellent target for inhibiting colorectal cancer progression. We found that TGF- β and all-trans retinoic acid (ATRA) decreased the expression of matrilysin in colon cancer cell line BM314 (Fig. 5), suggesting a possible application of these compounds in the treatment of invasive and metastatic colon cancer. The matrilysin promoter contains sequences with a high homology to the TGF- β inhibitory element originally identified in the rat stromelysin promoter¹⁹⁾ (Fig. 5). Therefore, the suppressive effect of TGF- β on matrilysin gene expression in BM314 cells may be regulated by this element. Since ATRA is a more appropriate choice for possible application in clinical settings, we examined the effects of down-regulation of matrilysin by ATRA on the invasive potential of BM314 cells. BM314 cells treated with ATRA demonstrated about one-third the invasive potential of control cells. This suggests that the use of ATRA as anti-matrilysin therapy is effective in the treatment of colon cancer. Since we could not rule out the possibility that ATRA modulated other invasion-related genes in BM314 cells, we directly down-regulated the expression of matrilysin by the introduction of an antisense matrilysin. Antisense matrilysin-transfected cells were significantly less invasive than mock-transfected control cells and the difference was

eliminated by a metalloproteinase inhibitor. These results confirm that matrilysin contributes to the invasive phenotype of colon cancer cells.

Matrilysin expression in metastasis of colon cancer

Matrilysin's unique structure and its localizing pattern suggest that this enzyme functions in a manner distinct from other MMPs and contributes directly to the invasive and metastatic potential of colorectal cancers *in vivo*. To assess the clinical impact of matrilysin protein expression in colorectal cancers, we performed an immunohistochemical examination of 78 surgically resected colorectal cancer specimens and five metastatic liver tumors²³). In 46% of primary and all of metastatic liver tumors, over 10% of cancer cells were stained positively for matrilysin. Metastatic liver tumors expressed matrilysin equally or stronger than did the corresponding primary site. Immunohistochemical expression of matrilysin was more frequent in Dukes' stage C-D tumors than in stage A-B tumors. These results suggest that matrilysin plays a crucial role in metastasis of colon cancer. We then examined the effects of matrilysin on the *in vivo* invasive and metastatic potential of colon cancer cells six weeks after subcutaneous injection into SCID mice. Only matrilysin-transfectants formed invasive tumors and multiple liver metastases in SCID mice. Casein zymography revealed that the invaded and metastasized tumors had enhanced active matrilysin activity which correlated with the number of metastatic lesions. These results suggest that not only expression but also activation of matrilysin is important for colon cancer cells to efficiently invade and metastasize. Activation of MMPs is one of the most critical steps in controlling their enzyme activities and is supposed to be mediated by host-tumor interactions and/or by several proteinases such as serine proteinases, MMP-3, and MT1-MMP^{14,24}). How the proteolytic activation of matrilysin occurs *in vivo* is very important for understand-

ing the metastatic pathway in colorectal cancer.

There are at least two explanations for the enhancement of colon cancer invasion and metastasis by overexpression of matrilysin *in vivo*. One is the direct effects of matrilysin, as this proteinase exhibits a wide spectrum of substrate specificity and efficiently degrades ECM⁴⁻⁹). Another explanation is the indirect effects of matrilysin, such as an activation of MMP-2 and/or MMP-9^{11,14}). To address this issue, we examined the gelatinase activity of tumor extracts and we found no apparent differences between the activities of tumors from matrilysin-transfectants and those from mock-transfectants. Therefore, the latter explanation is unlikely. However, we can not rule out other indirect effects of matrilysin, such as an activation of other MMPs or inactivation of serine proteinase inhibitors.

4. Gastric cancer

There are a few reports documenting the expression of MMPs in gastric cancers. Expression of MMP-1, MMP2, MMP-9, and MT1-MMP has been detected in gastric cancers at various frequencies. We examined the expression of matrilysin mRNA in 46 gastric cancers by RT-PCR²⁵). Overexpression of matrilysin was observed in 61% of the gastric cancer tissues. Expression of matrilysin was significantly correlated with depth of invasion. Immunohistochemical study with anti-matrilysin monoclonal antibody revealed that matrilysin was mainly expressed in cancer cells both of intestinal type and of diffuse type but not, or only very weakly, expressed in other noncancerous cells. In addition, zymographic analysis detected the active form of matrilysin in gastric cancer tissues that overexpressed matrilysin mRNA but not in noncancerous tissues. We have previously shown enhanced secretion of matrilysin during malignant conversion of colorectal epithelial cells²⁶). This may also be the case in the stomach because our preliminary data showed a modest level of matrilysin expression in intesti-

nal metaplasia. Quantitative analysis of matrilysin expression in intestinal metaplasia and gastric adenoma tissues in comparison with that in gastric cancer tissues would be necessary to clarify the issue.

In order to gain more insight into the relationship of matrilysin to invasive potential, we examined the effects of matrilysin on the invasive potential of gastric cancer cells. Overexpression of matrilysin rendered the gastric cancer cells more invasive *in vitro* (Fig. 6). These results suggest that matrilysin contributes to the invasive potential of gastric cancer. We further immunohistochemically analyzed the expression of matrilysin in gastric cancer²⁷. Matrilysin was expressed in the majority (89%) of gastric adenocarcinomas. Matrilysin expression was frequently observed in cancer cells at the invasive front. There was a significant relationship between matrilysin expression at the invasive front and the presence of nodal metastasis or advanced stages. These findings suggest that matrilysin plays an important role in the progression of gastric cancers.

5. Hepatocellular carcinoma

We examined the secretion and activation of MMPs in liver tissues from patients with hepatocellular carcinoma (HCC) and we evaluated its relationship with clinicopathological characteristics. Using zymography, we measured MMP activities in 30 surgical specimen pairs of carcinoma and adjacent nontumoral liver²⁸). We found a significant association between tumor spread, such as portal venous invasion and intrahepatic metastasis, and enhanced secretion of active forms of MMP-2 and matrilysin. This finding is intriguing, because it is well known that these pathological characteristics are the most important factors determining recurrence. It was suggested that patients with enhanced secretion of active MMP-2 or active matrilysin could be a higher risk group for recurrence. This suggestion was supported by a follow-up study showing an association of recurrence within the first postopera-

tive year with enhanced secretion of these active MMPs. Thus, postoperative scrutiny for recurrence and/or more intensive postoperative treatment should be administered to these higher-risk patients. Enhanced mRNA expression of MT1-MMP was observed in 22 of 30 cases and was associated with capsule invasion and the activation of proMMP-2. Taken together, active MMP-2, active matrilysin, and MT1-MMP likely play important roles in tumor spread of HCC.

To gain further clarification, we analyzed mRNA expression of MMP-2, MMP-9, matrilysin, TIMP-1, and TIMP-2 in 30 paired specimens of HCC and adjacent nontumoral liver using semi-quantitative RT-PCR²⁹). The mRNA expression of MMP-2, MMP-9, and matrilysin was increased in about two-thirds of the tumor samples. A modest increase in mRNA expression of TIMP-1 and TIMP-2 in tumors was observed in half of the patients. These results suggest that the balance of MMP/TIMP expression favors MMP in the majority of HCCs. Although neither TIMP alone nor the MMP/TIMP mRNA ratio was correlated with any clinicopathological features, enhanced mRNA expression of MMP-2 or MMP-9 and that of matrilysin showed trends toward the presence of capsular invasion and intrahepatic metastasis, respectively. Concomitant overexpression of MMP-2 and matrilysin was associated with recurrence within the first postoperative year. These results suggest that semiquantitative RT-PCR analysis of MMPs in HCC is useful for predicting which patients are at high risk of developing early recurrences.

6. Esophageal cancer

We immunohistochemically investigated the expression of matrilysin in esophageal squamous cell carcinomas (SCCs). Matrilysin was expressed in all of 13 esophageal SCCs²⁷). Matrilysin expression was frequently observed in cancer cells at the invasive front. We then analyzed the possible association between immunohistochemically detected matrilysin expression at

the invasive front in esophageal SCCs and clinicopathological characteristics and we determined whether matrilysin can predict recurrence and/or survival³⁰). Matrilysin expression at the invasive front was detected in about one-half of 100 carcinoma tissues and was associated with depth of invasion, advanced tumor stage, recurrences, and recurrences within the first postoperative year. Patients with matrilysin-positive carcinoma had a significantly shorter disease-free and overall survival time than those with matrilysin-negative one. Matrilysin remained of significant value for prediction of disease-free and overall survival in multivariate analysis including conventional clinicopathological factors. These results suggest that matrilysin plays a key role in the progression of esophageal carcinoma and its detection is useful for prediction of recurrence, prognosis and possibly for selecting patients for anti-MMP therapy.

7. Pancreatic cancer

Using immunohistochemistry, we analyzed 70 pancreatic ductal adenocarcinoma tissues for expression of MMP-1, MMP-2, MMP-3, matrilysin, MMP-9, MT1-MMP, TIMP-1, and TIMP-2³¹). The results were matched with clinicopathological characteristics and patients' survival. Expression of MMP-1, MMP-2, MMP-3, matrilysin, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 was detected in either tumor cells or tumor stromal cells, or in both components, at varying frequencies. Among MMPs, matrilysin showed a unique distribution in the tumor nests; its expression was usually most pronounced at the invasive front of the tumors. Matrilysin expression at the invasive front was observed in 57% of cases and was significantly correlated with pT, pN, and pM categories and with more advanced pTNM stages. The significance of matrilysin expression was further substantiated by its correlation with a shorter overall survival time. Moreover, only matrilysin provided a significant predictive value for overall survival in multivariate analysis, suggesting that matrilysin expression could be a powerful

indicator of poor prognosis with a significance equaling or surpassing that of other conventional clinicopathological factors.

To evaluate the potential for using matrilysin as a therapeutic target, the effect of antisense matrilysin on *in vitro* invasive potential of pancreatic carcinoma cells was examined. Antisense matrilysin-transfected CFPAC-1 cells expressed reduced levels of matrilysin and demonstrated a similar growth potential but were less invasive *in vitro* compared to neo-transfected CFPAC-1 cells. Down-regulation of matrilysin by the antisense vector also markedly reduced the invasive potential of a metastasis-derived pancreatic carcinoma cell line, Capan-1. These results suggest that matrilysin plays a key role in the progression of pancreatic carcinoma thereby contributing to a poor prognosis. Matrilysin could be a new prognostic marker that would allow us to identify patients with a poor prognosis who might benefit from more aggressive treatments³²). Immunohistochemical analysis is a technique available in daily clinical settings, and analysis of matrilysin expression could therefore be an important routine part of the management of patients with pancreatic carcinoma.

8. Matrilysin in early carcinogenesis

Matrilysin has been implicated not only in tumor invasion and metastasis but also in tumor initiation and growth³³). Matrilysin-positive carcinoma cells show a unique distribution in the tumor nests. In cancer tissues of the esophagus, stomach, colorectum, and pancreas, matrilysin was not stained homogeneously in the tumor nodules. First, most matrilysin-positive tumor cells showed depolarized diffuse cytoplasmic staining, which was usually most pronounced at the invasive front of the tumors. With respect to the mechanism(s) underlying the depolarized matrilysin expression in tumor cells at the invasive front, genetic alterations in tumor cells and/or tumor-host interactions may play a key role.

In addition, polarized immunoreactivity was

observed on the luminal surfaces of neoplastic glands in cancers of the stomach, colorectum, and pancreas. MMPs reportedly activate luminal or membrane-bound cytokines or growth factors, such as tumor necrosis factor α and heparin-binding epidermal growth factor, to locally perturb the growth of responsive cells. *Min* mice carry a germline mutation in the APC tumor suppressor gene and spontaneously develop premalignant tumors in the small and large intestine. Like human colon tumors, *Min* tumors express high levels of several MMP family members, including matrilysin, MMP-2, MMP-3, MMP-10, and collagenase. It has been demonstrated that the induction of matrilysin expression by tumor cells is critical to the development of adenomas because matrilysin-deficient *Min* mice developed 58% fewer tumors³⁴. In addition, administration of the synthetic MMP inhibitor batimastat to *Min* mice reportedly suppresses tumor multiplicity by nearly 50%, which is similar to the rate of suppression observed in matrilysin-deficient *Min* mice. These findings further support the notion that matrilysin plays a role in early colorectal carcinogenesis.

9. Conclusions

Although a number of molecular prognostic markers in carcinoma have been reported, routine analysis of these markers is not warranted, because they do not have any therapeutic implications. Therefore, identification of a molecular prognostic marker, which is susceptible to or modifiable by direct therapeutic intervention, could give rise to therapeutic and prognostic improvements for patients with carcinoma. In this regard, as we have shown in this review, matrilysin could be a new prognostic marker that would allow us to identify patients with a poor prognosis who might benefit from more aggressive treatments and it could be a potentially useful target for therapeutic intervention either by the use of an antisense strategy or synthetic MMP inhibitors.

Although considerable optimization is nec-

essary in many aspects, the matrilysin antisense strategy offers a feasible possibility as an adjuvant therapy for carcinoma in the future. The use of synthetic MMP inhibitors is the currently used strategy in clinical settings. Some inhibitors have been proven to be of therapeutic significance in clinical trials. Considering our results, matrilysin could be a primary target of such broad-type MMP inhibitors in cancers of the digestive organs. For a specific selection of patients who would benefit from these therapies, examination of MMP expression will be necessary. The diagnostic strategy presented here and advances in therapeutic approaches, including anti-MMP therapy, are expected to improve the prognosis of patients with cancer of the digestive organs.

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