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ORIGINAL PAPER

Induction of matrix metalloprotease-7 is common in mucinous ovarian tumors including early stage disease

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Matrix metalloproteases are known to play an important role in tumor invasion by mediating degradation of the extracellular matrix. In this study, we have investigated the immunohistochemical expression of matrix metalloprotease -7 (MMP-7) in 44 mucinous ovarian tumors (9 adenomas, 13 low malignant potential tumors, 22 adenocarcinomas) and 6 normal ovaries. Positive staining of MMP-7 is observed in all mucinous ovarian tumors, whereas little or no staining was observed in surface epithelium as well as the epithelial cells of germinal inclusion cyst of the normal ovary. Positive immunostaining of MMP-7 is also observed in the secreted mucin in the tumor glands, which suggests the secretion of the MMP-7 protein from tumor cells. mRNA expression of MMP-7 was confirmed using RT-PCR. The MMP-7 gene was amplified in parallel with an internal control gene β-tubulin using a thermal cycler. mRNA expression levels of MMP-7 were significantly elevated in mucinous tumor samples compared with that in normal ovaries. Our results suggest that MMP-7 is frequently overexpressed in mucinous ovarian tumors and secreted with the mucin which is produced from the tumor cells. MMP-7 may therefore contribute to mucinous ovarian tumor development or enhanced growth capacity of mucinous ovarian tumors. MMP-7 may also serve as a target for therapeutic intervention in the down regulation of tumor progression.
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Introduction

Matrix metalloproteases (MMPs) mediate the degradation of the extracellular matrix, destroy the basement membrane components and connective tissue and allow shedding of tumor cells into the surrounding environment. Therefore MMPs play a critical role for tumor cell invasion and dissemination.[1–3] A marked increase

of metalloprotease gene expression may lead to an enhanced ability of tumor cells to break down extracellular matrix and collagenous structures.[2]

The MMP-7 gene (originally known as the pump-1 gene) was first identified by screening a mixed human tumor cDNA library in an effort to clone stromelysin-related genes that may be involved in tumor invasion and metastasis.[4] Although the predicted domain structure of the MMP-7 protein shares the conserved catalytic domain of the matrix metalloprotease family, MMP-7 is unique in that it lacks the hemopexin-like carboxyl terminal domain encoded by other MMP genes.[4] It has been shown that recombinant MMP-7 degrades casein, gelatins of types I, III, IV, V, and fibronectin.[5] Increased expression of MMP-7 has been reported in several tumor types including squamous cell carcinoma of the head and neck and the lung,[6] prostate carcinomas[7] and stomach and colorectal carcinomas.[8,9] MMP-7 mRNA has been detected in human adenomas as well as adenocarcinomas of the colon.[8–11] We previously reported that MMP-7 mRNA is frequently overexpressed in both ovarian LMP tumors and carcinomas compared to normal ovaries by Northern blot analysis and quantitative PCR.[12] Furthermore, ectopic expression of MMP-7 cDNA in a colorectal carcinoma cell line was found to increase its tumorigenicity in nude mice.[13] Wilson *et al* reported that intestinal tumorigenesis was suppressed in mice lacking MMP-7.[14] The secretion and activation of MMP-7 was up-regulated during malignant conversion of colorectal epithelium.[15] These results suggest that MMP-7 may contribute not only to tumor invasion but also to early tumor development in some neoplasms. In order to investigate the potential contribution of MMP-7 to mucinous ovarian tumor development and progression, we performed the present study. We investigated the immunohistochemical expression of MMP-7 in mucinous ovarian tumors. In addition, mRNA overexpression of MMP-7 in mucinous ovarian tumor tissues was confirmed by RT-PCR.

Materials and methods

Samples for immunohistochemistry were as follows: 44 mucinous ovarian tumor surgical specimens (9 adenomas, 13 low malignant potential tumors, 22 carcinomas) were obtained from Hiroshima University Hospital. Staging was determined according to the criteria of the TNM method. Six normal ovaries were obtained from patients who underwent surgery for benign gynecological disease with their informed consent. The ovaries were fixed in 10% neutral-buffered formalin for more than 24 h and thereafter embedded in paraffin.

Samples for RT-PCR were as follows: fresh surgical specimens of 9 mucinous ovarian tumors (1 mucinous adenoma, 1 LMP tumor, 7 cases of carcinoma) and 6 normal ovaries were in the same series of samples used for immunohistochemistry. The materials were obtained immediately after the surgical procedure and frozen in liquid nitrogen and stored at $-80^{\circ}C$ prior to mRNA isolation. Two mucinous ovarian cancer cell lines, OVK-18 (obtained from the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan) and MCAS (obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan) were also used for mRNA expression analysis of MMP-7.

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections, 4 μm thick, were cut and mounted on aminopropyltriethoxysilane-treated slides. Slides were routinely deparaffinized with xylene and rehydrated with a series of ethanol washes. Nonenzymatic antigen retrieval was performed by processing using microwave heat treatment in 0.01 M sodium citrate buffer (pH 6.0). Immunohistochemical staining was performed manually using the avidin–biotin peroxidase complex technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). This indirect immunoperoxidase staining procedure was performed at room temperature. Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with methanol with 0.3% H_2O_2 for 30 min. After washing with phosphate-buffered saline (PBS) for 10 min, slides were blocked with normal horse serum for 30 min, followed by incubation with the anti-MMP-7 mouse monoclonal antibody (Ab-1; Cat# IM40L; dilution 1:20; Oncogene Research Products, Cambridge, MA) for 1 h. After rinsing with PBS for 10 min, sections were incubated with biotinylated anti-mouse Ig-G for 30 min. After washing with PBS for 10 min, slides were incubated with ABC reagent for 30 min. The final products were visualized by using AEC substrate system (DAKO

Corporation, Carpinteria, CA) and sections were counterstained with Mayer hematoxylin for 20 s before mounting. Positive controls and negative controls were used for each section. Normal endometrium was used as positive control. Negative controls were performed by using normal serum instead of the primary antibody. All experiments were duplicated. The stained slides were examined microscopically by 3 observers. More than 10% of positive tumor cells was the criterion for a 1+ positive staining and more than 50% of positive tumor cells was the criterion for a 2+ positive staining. The χ^2 test of significance was to test for a relationship between dichotomous variables. Values were considered statistically significant if $P < 0.05$.

RT-PCR

Extraction of mRNA from the tissue specimen and cDNA synthesis were carried out by the methods described previously.[16–18] mRNA was isolated by using a RiboSep™ mRNA isolation kit (Becton Dickson Labware, Bedlord, MA). cDNA was synthesized with 5.0 μg of mRNA by random hexamer priming using 1st strand™ cDNA synthesis kit (CLONTECH, Palo Alto, CA).

The mRNA overexpression of MMP-7 was determined using RT-PCR. RT-PCR was performed according to the procedure previously reported[12,16–18] with some modifications. Oligonucleotide primers used were as follows: MMP-7 forward (sense) 5′-CATGAGTGAGCTACAGTGGG-3′ and reverse (antisense) 5′-CGATCCACTGTAATATGCGG-3′; β-tubulin forward (sense) 5′-TGCATTGACAACGAGGC-3′ and reverse (antisense) 5′-CTGTCTTGACATTGTTG-3′. β-tubulin was amplified as an internal control. The predicted sizes of the amplified genes were 304 bp for MMP-7 and 454 bp for β-tubulin. The primer sequences used in this study were designed according to the cDNA sequences described by Muller *et al* (MMP-7)[4] and Hall *et al* (β-tubulin).[19] The PCR reaction mixture consisted of cDNA derived from 50 ng of mRNA, 5 pmol of sense and antisense primers for both the MMP-7 gene and the β-tubulin gene, 200 μmol of dNTPs and 0.625 unit of Taq DNA polymerase with reaction buffer (Takara Shuzo Co. Ltd, Kyoto, Japan) in a final volume of 25 μl. The target sequences were amplified in parallel with the β-tubulin gene. Thirty cycles of PCR were carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA). Each cycle of PCR included 30 s of denaturation at 94°C, 1 min of annealing at 60°C and 1 min of extension at 72°C. The PCR products were separated on 1.6% agarose gels and the density of each PCR product was determined by using a Printgraph-Densitograph system (ATTO Corporation, Tokyo, Japan). In the present study, we used the expression ratio (MMP-7/β-tubulin) as measured by densitometry to evaluate gene expression.

Results

Immunohistochemistry

Table 1 summarizes the data obtained on the histological type, stage, grade and immunohistochemical overexpression of MMP-7 in all the cases studied. MMP-7 antigen was positive in all mucinous ovarian tumors. Intense cytoplasmic tumor cell staining was

Table 1 Number of cases with positive expression of MMP-7 protein

		*Immunohistochemical Expression of MMP-7**		
	N	*Negative*	*1+*	*2+*
Normal ovary	6	6(100%)	0	0
Mucinous tumor	44	0	23(52%)	21(48%)
Adenoma	9	0	4(44%)	5(56%)
LMP tumor	13	0	8(62%)	5(38%)
Carcinoma	22	0	11(50%)	11(50%)
Stage 1/2	14	0	8(57%)	6(43%)
Stage 3/4	8	0	3(38%)	5(63%)
Grade 1	15	0	8(53%)	7(47%)
Grade 2	7	0	3(43%)	4(57%)

*negative; less than 10% positive tumor cells, 1+; 10% to 50% positive tumor cells, 2+; more than 50% positive tumor cells.

detected in several mucinous tumors (Figure 1A, B, C) while little or no staining was observed in normal surface epithelium of the ovary as well as epithelial cells of germinal inclusion cyst of the normal ovary (Figure 1D, 1E). A secreted product was most noticeable in the mucin of the mucinous tumor glands (Figure 1F), suggesting the secretion of MMP-7 protein from mucinous tumor cells. Relative expression of MMP-7 in ovarian tumors was as follows. Four of 9 mucinous adenomas showed 1+ positive staining of MMP-7. 8 of 13 LMP tumors and 11 of 22 mucinous adenocarcinomas also showed 1+ positive stainings. All the

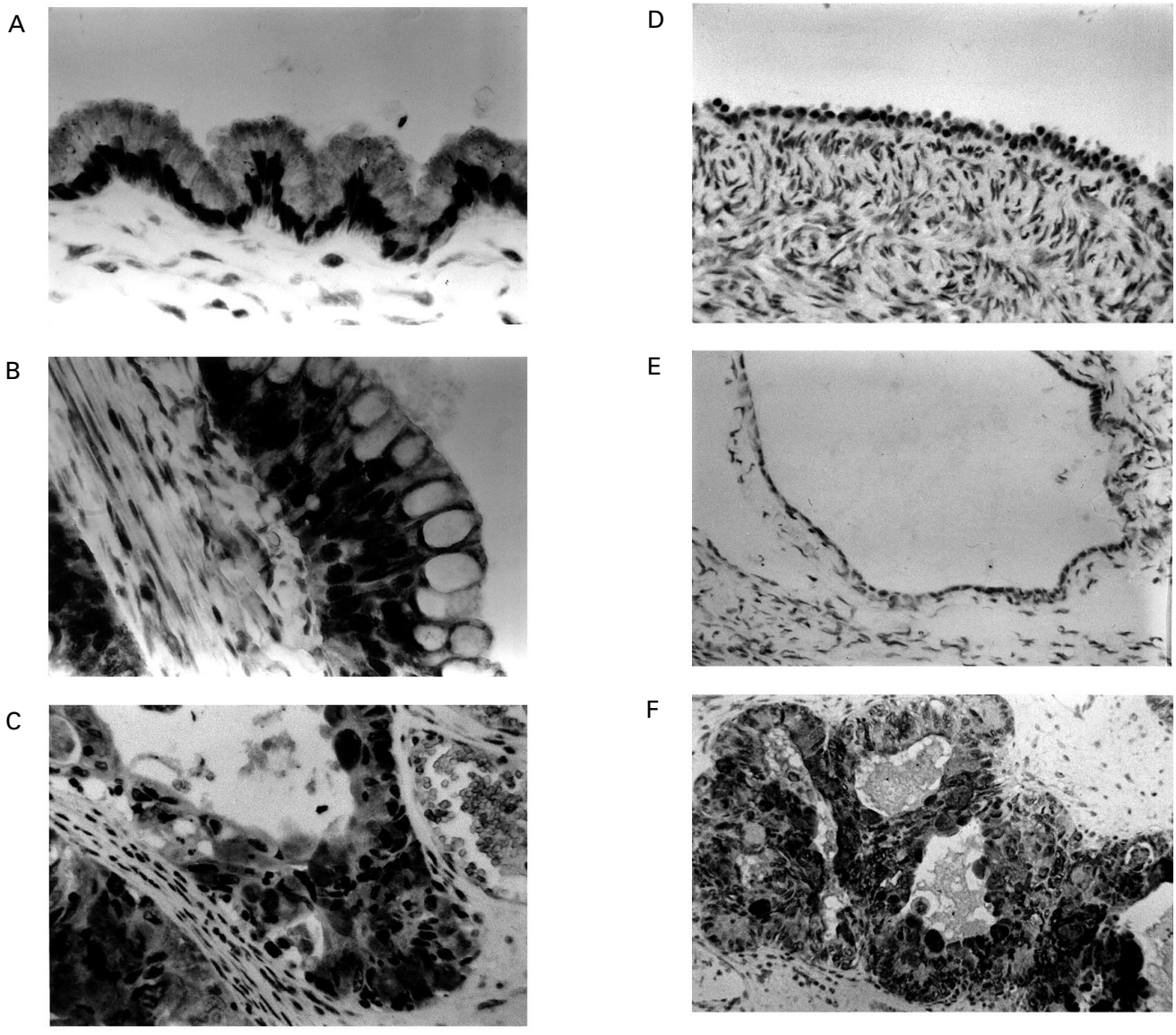

Figure 1 *Immunohistochemical staining of MMP-7 in normal ovary and mucinous ovarian tumor tissues.*
Intense cytoplasmic staining of mucinous adenoma cells (A; immunoperoxidase, ×400), LMP tumor cells (B; immunoperoxidase, ×400) and adenocarcinoma cells (C; immunoperoxidase, ×200). Normal ovarian surface epithelium shows little or no MMP-7 immunoreactivity (D; immunoperoxidase, ×200). MMP-7 was also negative in epithelial cells of germinal inclusion cyst of normal ovary. (E; immunoperoxidase, ×100). Positive stainings were observed both on the mucin in the glands of mucinous adenocarcinoma and in cancer cell themselves. (F; immunoperoxidase, ×100).

remaining tumors showed 2+ staining including 5 of 9 (56%) adenoma cases, 5 of 13 LMP (38%) tumors and 11 of 22 (50%) carcinoma cases. No statistical difference of the 2+ positive rate was found neither with stage nor grade of the diseases.

RT-PCR

In a preliminary study, we confirmed the linearity of the PCR assay[12] using 30 cycles of PCR with primers of MMP-7 and β-tubulin in the same reaction tube. PCR products were analyzed after agarose separation and the ratios of expression of MMP-7/β-tubulin were calculated.

The analysis of our test tumor panel of 6 normal ovaries, 1 mucinous adenoma, 1 LMP tumor and 7 mucinous adenocarcinoma is shown in Table 2. Figure 2 also demonstrates mRNA overexpression in mucinous ovarian tumors compared to normal ovaries. All 6 cases of normal ovaries showed relatively low levels of MMP-7 mRNA expression. The ratios of expression of MMP-7/β-tubulin in normal ovaries were in the range $0.02-0.16$ (mean $=0.080$, s.d. $=0.055$). In mucinous ovarian tumors, the ratios of expression of MMP-7/β-tubulin were in the range $0.33-1.86$ (mean $=0.912$, SD $=0.547$). All 7 mucinous ovarian tumor cases exceeded the mean for the normal ovary by more than four standard deviations. MMP-7 mRNA expression was significantly elevated in tumors compared to that in normal ovary for adenoma, LMP tumor and adenocarcinoma. All 7 mucinous tumor cases also showed positive immunostaining of MMP-7. As noted, PCR analysis confirms overexpression of MMP-7 in all the tumor samples tested, in complete agreement with the immunohistochemical staining data. The mucinous ovarian cancel cell lines, OVK-18 and MCAS, were also used for mRNA expression analysis of MMP-7. Significant MMP-7 mRNA overexpression compared to the normal ovary was observed in MCAS. In contrast, MMP-7 mRNA expression was quite low in OVK-18 (Figure 2).

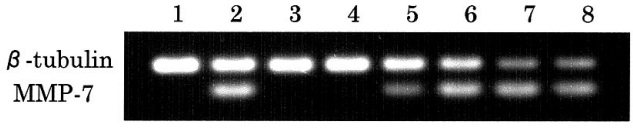

Figure 2 *Expression of MMP-7 mRNA in mucinous ovarian cancer cell lines, mucinous ovarian cancer tissues and normal ovaries.*
β-tubulin was used as internal control. Lane 1 (OVK-18) and Lane 2(MCAS) are mucinous ovarian cancer cell lines. Lane 3 and Lane 4 are normal ovaries. Lane 5 is a mucinous adenoma. Lane 6, Lane 7 and Lane 8 are mucinous adenocarcinomas. mRNA expression levels of MMP-7 were elevated in MCAS cell line and mucinous ovarian tumor tissues compared to that in normal ovaries.

Table 2 mRNA expression of MMP-7 gene in normal ovaries and ovarian tumors

Histological type	*Stage/grade*	*LN**	*Immunohistochemical expression of MMP7+†*	*mRNA expression of MMP-7/β-tubulin‡*
Normal ovary			—	0.05 (N)
Normal ovary			—	0.02 (N)
Normal ovary			—	0.08 (N)
Normal ovary			—	0.13 (N)
Normal ovary			—	0.16 (N)
Normal ovary			—	0.04 (N)
Mucinous adenoma		ND	1+	0.61 (4+)
Mucinous adenoma (LMP)	1a/G1	ND	2+	0.45 (4+)
Mucinous adenocarcinoma	1a/G1	ND	2+	0.60 (4+)
Mucinous adenocarcinoma	1a/G2	—	2+	0.65 (4+)
Mucinous adenocarcinoma	1c/G1	ND	2+	1.10 (4+)
Mucinous adenocarcinoma	1c/G2	ND	2+	0.33 (4+)
Mucinous adenocarcinoma	3a/G1	—	1+	0.89 (4+)
Mucinous adenocarcinoma	3c/G1	ND	2+	1.86 (4+)
Mucinous adenocarcinoma	3c/G2	ND	1+	1.72 (4+)

*; LN; lymph node metastasis, +; positive, —; negative, ND; not determined. †; —; negative (less than 10% positive tumor cells), 1+ 10% to 50% positive tumor cells, 2+; more than 50% positive tumor cells ‡; N; normal range is equal to mean ± 2s.d., 4+; Mean + 4s.d. or greater

Discussion

In many of the neoplastic lesions that have been examined in humans, the expression of most MMPs is restricted to the stromal component.[20] However, MMP-7 localizes primarily to the tumor cells in lesions of epithelial origin.[21] It has been shown that MMP-7 is constitutively expressed in normal human exocrine glands and that MMP-7 may be secreted into the lumen of specific glands in order to keep the lumen patent and the gland functioning property.[22] Strikingly, the MMP-7 protein was positive in all mucinous ovarian tumors examined whereas little or no positive staining was observed on the surface epithelium of the normal ovary. It should be noted that MMP-7 was also negative in the epithelial cells of germinal inclusion cyst of the ovary, which is believed to be the precursor lesion of the epithelial ovarian tumor. Our study showed the cytoplasmic positive stainings of MMP-7 in mucinous tumor cells including adenoma, LMP tumor and adenocarcinoma. It is noteworthy that MMP-7 is expressed in mucinous ovarian tumor cells themselves. In addition, the present study showed that MMP-7 was also positive on the mucin in the mucinous tumor glands. This observation suggests that the MMP-7 protein may be secreted from the mucinous tumor cells. Using RT-PCR, we demonstrated that significant mRNA overexpression occurred in mucinous ovarian tumors including adenoma, LMP tumor and adenocarcinoma compared to the normal ovary. These data confirm both mRNA and protein expression levels of MMP-7 are increased in mucinous ovarian tumors. Furthermore, the fact that MMP-7 message and protein appears in adenoma suggests that MMP-7 may relate to the mucinous differentiation of surface epithelium of the ovary and that induction of the mRNA occurs early in mucinous tumor development. Although it remains unclear whether ovarian adenocarcinomas arise from preexisting benign epithelial lesion such as adenomas,[23] a sizable proportion of benign mucinous tumors are believed to have the potential for malignant transformation.[24] Though it remains unclear what the precise relationship of MMP-7 is to invasion, or its relationship to other proteases, nevertheless MMP-7 is frequently overexpressed and/or secreted in mucinous ovarian tumors and is therefore likely to contribute to the invasive growth of ovarian tumor cells. Furthermore, MMP-7 may be involved in tumor development and progression as well as the tissue degradation which accompanies tumor cell extravasation. We also showed one mucinous ovarian cancer cell line, OVK-18, showed little or no expression of MMP-7 whereas another cell line, MCAS, showed mRNA overexpression of MMP-7. This could be due to the fact that cell lines usually change their phenotypes as the result of long *in vitro* culturing. Another possibility is that host-tissue environment factors, such as cell-to-matrix interaction or some cytokines, may induce the production of MMP-7 and that loss of cell-to-matrix interaction causes the cells to change the level of MMP-7 expression.

Since the mechanism for increased expression of MMP-7 mRNA and protein in mucinous ovarian tumors remains to be determined, here we demonstrate that the overexpression of MMP-7 is a common event in mucinous ovarian tumors, whereas the normal ovary shows very low expression of MMP-7. Of particular note in this study is overexpression of MMP-7 in mucinous adenoma, LMP tumors and stage I carcinomas. Here it is also noted that MMP-7 is produced directly by tumor cells, not by the underlying stromal tissue. Increased levels of secreted MMP-7 produced by early stage mucinous tumors may well allow for the detection of such an early stage of the disease if these MMP-7 levels provide sufficient enhancement of the signal to noise ratio of circulating MMP-7. Further assessment of circulating levels of this antigen relative to tumor burden and an examination of its role in tumor progression will determine its efficacy as a tumor marker. These results would also argue for the use of an MMP-7 inhibitor as a therapeutic agent in the treatment and prevention of mucinous ovarian cancer.

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