Expression of Matrix Metalloproteinase-7 on Cancer Cells and Tissue Endothelial Cells in Renal Cell Carcinoma: Prognostic Implications and Clinical Significance for Invasion and Metastasis

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Abstract

Purpose: The expression of matrix metalloproteinase-7 (MMP-7) correlates with the malignant potential of various tumors and patient survival. We investigated the clinical and prognostic significance of MMP-7 expression in cancer cells and endothelial cells in human renal cell carcinoma (RCC).

Experimental Design: We reviewed tissue samples of 156 patients with RCC who had undergone radical operation. MMP-7 expression was examined by immunohistochemistry. Sections containing MMP-7-positive vessels were also stained for CD34. The density of MMP-7-positive vessels was determined by a computer-aided image analysis system. Multivariate analysis was done to assess relevant variables for invasion, metastasis, and cause-specific survival.

Results: The proportion of MMP-7-expressing tumor cells were significantly higher ($P < 0.001$) than that of normal cells. MMP-7-positive vessels were considered blood vessels based on staining for CD34, and their density was increased in tumor areas. The proportion of MMP-7-expressing cancer cells and density of MMP-7-positive vessels correlated with grade, pathologic tumor stage, and metastasis. Multivariate analysis showed that MMP-7 expression on cancer cells correlated with pathologic tumor stage only, whereas MMP-7-positive vessel density correlated with metastasis only. The elevated status of MMP-7 in cancer tissues was an independent predictor for cause-specific survival (odds ratio, 8.61; $P = 0.040$) by multivariate analysis.

Conclusions: Our results showed that MMP-7 influences tumor progression by regulating invasion and angiogenesis. Multivariate analysis showed that MMP-7 status of cancer tissues was strong predictor of poor prognosis. Our results suggest that MMP-7 targeting treatment may be a potential target against RCC.

Systemic dissemination of cancer cells influences prognosis in the majority of malignancies. Many factors are associated with this process, and cancer cell invasion into surrounding tissue is one of the early and crucial steps. In addition, the formation of new capillaries (neoangiogenesis) is also an important process in tumor growth and metastasis in solid tumors (1, 2). Degradation of the extracellular matrix and destruction of the basement membrane by cancer cells are important processes for direct invasion. Likewise, degradation of extracellular matrix of endothelial cells is one of the initial steps in neoangiogenesis. Thus, these processes are important for tumor progression and prognosis, and full understanding is essential in the planning of treatment and observation strategy.

Matrix metalloproteinases (MMP) are zinc-dependent proteolytic enzymes capable of cleaving extracellular matrix components. Several investigators have paid special attention to the pathologic significance of MMPs in cancer cells, and it is a well-known fact that MMPs are overexpressed in a variety of cancers and play important roles in cancer invasion and metastasis (3–5). In addition to cancer cells, several investigators found that some MMPs are secreted by endothelial cells and can modulate angiogenesis by regulating endothelial cell proliferation and migration (6–8). This fact is of interest in that MMPs may influence tumor progression and survival via angiogenesis. Among the MMPs, MMP-2 and MMP-9 have been the most investigated and are overexpressed in cancer cells and/or stromal cells, and their expression levels correlate with the grade and stage of various tumors (9–11). However, the clinical significance and pathologic roles of other MMPs in other types of cancers are not fully understood.

Human renal cell carcinoma (RCC) is the most common malignant tumor of the kidney, and ~25% of patients present

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with invasion of the tumor to the surrounding tissues and distant metastasis (12). One of the features of this disease is resistance to radiotherapy and chemotherapy. With regard to MMPs, MMP-2 and MMP-9 are also known to affect malignant aggressiveness in RCC (13–15). In addition to MMP-2 and MMP-9, other MMPs (MMP-1, MMP-3, MMP-11, MMP-12, and MMP-14) are overexpressed and known to be associated with the biological behavior of RCC (16). However, with the exception of MMP-2 and MMP-9, little is known about the pathologic roles of other MMPs in patients with RCC. In addition to tumor invasion, angiogenesis is also an important process in tumor progression and prognosis in RCC (15, 17). However, to our knowledge, there is little or no information on the expression of MMPs in endothelial cells in human RCC tissues and the pathologic significance of such expression on angiogenesis. Furthermore, the relationships between MMP expression in endothelial cells and clinicopathologic features and survival in patients with RCC have not been reported.

In the present study, we focused on the role of MMP-7 (matrilysin) in human RCC tissues for the following three reasons. (*a*) MMP-7 is overexpressed in a variety of cancers, and it plays important roles in tumor progression (18–28). However, clinical significance of MMP-7 in human RCC cells is not well understood. (*b*) Although MMP-7 is secreted by endothelial cells and can affect their activity (13–15), its expression on endothelial cells in RCC tissues has not yet been reported. (*c*) The independent role of MMP-7 on cancer cells and endothelial cells in human RCC tissues with regard to tumor progression and survival is not investigated. In this study, we determined the correlation between MMP-7 expression in cancer cells and blood vessels with tumor invasion, metastasis, and survival using multivariate analysis. The results showed the involvement of MMP-7 in cancer cell invasion and angiogenesis in RCC. These findings suggest the therapeutic usefulness of MMP-7 for prevention of cancer cell progression and improvement of survival in patients with RCC.

Materials and Methods

Patients and pathologic materials. We reviewed retrospectively 165 consecutive patients diagnosed with conventional RCC between 1990 and 2004. Patients who received neoadjuvant therapy were excluded from this study. Staging was assessed using the 1997 tumor-node-metastasis classification. Nuclear grading was based on the criteria of Fuhrman et al. (29). For examination of metastasis, all patients underwent ultrasonography, computed tomography of the abdomen, bone scanning, and lung X-ray photography. Magnetic resonance imaging of the bone and abdomen and computed tomography of the lung and brain were done as necessary. Tumors were divided into two groups based on the tumor stage: early stage (T_1 or T_2) and late stage (T_3 or T_4). The nuclear grade was divided into low and high grade, reflecting G_1 to G_2 and G_3 to G_4 grades, respectively. The mean follow-up period was 42.6 ± 32.7 months (±SD), and 30 patients died of RCC during this period. Two pathologists did all the pathologic examinations, and the final diagnosis was approved by the chief pathologist. The study design was in accordance with the guidelines of the Human Ethics Review Committee of Nagasaki University School of Medicine (Nagasaki, Japan).

Immunohistochemistry. Tissue sections (5 μm) from formalin-fixed and paraffin-embedded specimens of primary tumors were deparaffinized in xylene and rehydrated. Antigen retrieval was done for all antibodies used. All sections were then immersed in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Primary antibodies for anti-MMP-7 antibody and anti-CD34 antibody were obtained from Lab Vision Corp. (Fremont, CA) and DAKO Corp. (Glostrup, Denmark), respectively. Sections were incubated overnight with anti-MMP-7 antibody (1:150) and anti-CD34 antibody (1:70) at 4°C. They were then treated with peroxidase using the labeled polymer method with DAKO EnVision+ Peroxidase (DAKO Corp., Carpinteria, CA) for 60 minutes. The peroxidase reaction was visualized with liquid 3,3′-diaminobenzidine substrate kit (Zymed Laboratories, Inc.,

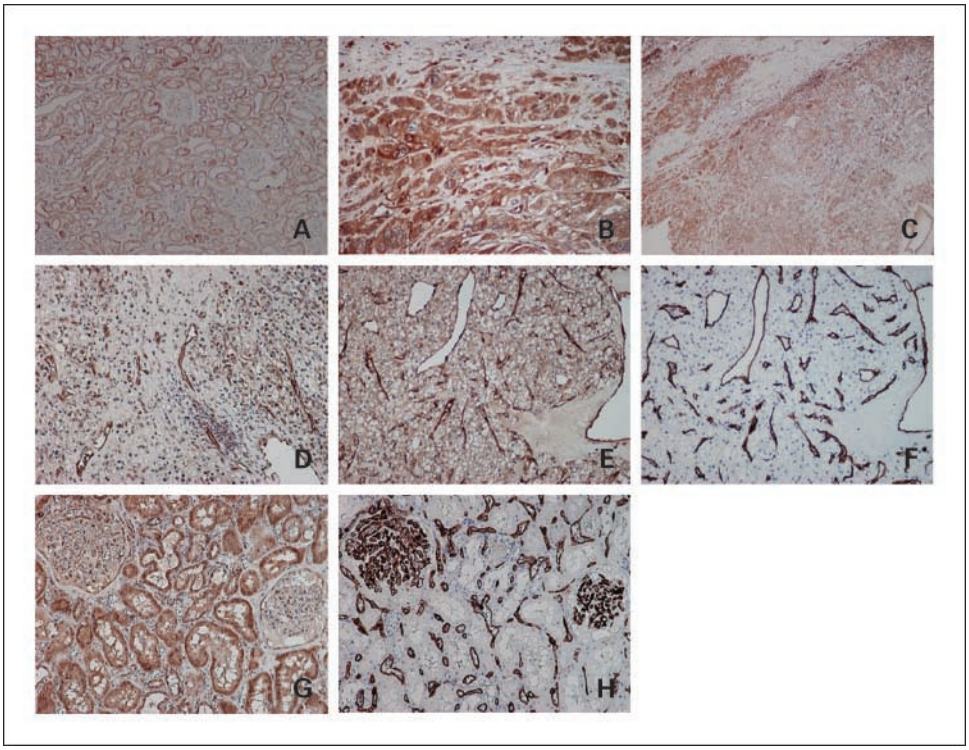

Fig. 1. Expression of MMP-7 in normal and cancer tissues. *A,* staining for MMP-7 expression was weak to moderate in almost all normal tubules. Strongly stained normal tubular cells were relatively rare. Magnification, ×100. *B,* note the immunohistochemical staining for MMP-7 in the cytoplasm of cancer cells. Magnification, ×200. *C,* MMP-7 immunostaining of cancer cells at the invasive front and tumor margin. Magnification, ×100. *D,* MMP-7-positive vessels in tumor area. Magnification, ×200. *E,* some MMP-7-positive vessels were concordant with CD34-positive vessels (*F*) in tumor regions. Magnification, ×200. *G,* MMP-7-positive vessels were rare in normal regions. Magnification, ×200. *H,* CD34-positive vessels in similar area of (*G*). Magnification, ×200.

Table 1. Proportions of MMP-7-positive cancer cells and blood vessels according to various clinicopathologic variables

	No. patients	MMP-7-positive cancer cells (%)	Density of MMP-7-positive blood vessels (/field)
Sex			
Male	125	21.7 ± 9.0	17.6 ± 7.3
Female	40	22.3 ± 8.9	15.7 ± 6.6
P		0.452	0.131
Age (y)			
≤63	83	20.2 ± 7.8	16.4 ± 6.4
>63	82	22.5 ± 9.9	17.9 ± 7.9
P		0.100	0.172
Pathologic tumor stage			
T_1	95	16.3 ± 5.4	14.0 ± 5.5
T_2	21	21.6 ± 5.1	19.1 ± 8.1
T_3	42	30.0 ± 5.7	22.2 ± 6.4
T_4	6	40.9 ± 10.7	24.5 ± 7.3
Early (T_1 + T_2)	116	17.3 ± 5.7	15.0 ± 6.5
Late (T_3 + T_4)	48	31.4 ± 7.3	22.5 ± 6.5
P		<0.001	<0.001
Metastasis			
Absence	138	19.5 ± 7.8	15.2 ± 5.2
Presence	27	31.0 ± 8.3	27.3 ± 7.4
P		<0.001	<0.001
Grade			
1	72	17.3 ± 6.4	14.4 ± 6.0
2	68	21.0 ± 7.2	18.0 ± 7.3
3/4	25	34.0 ± 7.9	22.7 ± 6.4
Low (G_1 + G_2)	141	19.1 ± 7.9	16.2 ± 6.9
P		<0.001	<0.001

San Francisco, CA). We also examined these factors in normal kidney tissue obtained from a region at least 2 cm distant from the tumor margin. A consecutive section from each sample processed without the primary antibody was used as a negative control. Breast cancer tissues and normal renal vein served as positive controls for anti-MMP-7 antibody and anti-CD34 antibody, respectively.

Semiquantitative analysis and evaluation of staining. The staining intensity of MMP-7 expression was classified semiquantitatively into three grades: weak, moderate, and strong. In the present study, the expression was considered positive if staining intensity was strong, and the percentage of positively stained cancer cells were determined using a continuous scale. In addition, when the percentage of positive stained cells were >20%, the specimen was rated as overexpression for MMP-7 as described previously (30, 31).

To visualize the blood vessels, sections stained with anti-CD34 antibody were first examined under a microscope (Nikon E400, Nikon, Tokyo, Japan), and digital images were captured using a digital camera (Nikon DU100) at ×200 magnification. The density of MMP-7-positive vessels (defined as the number of blood vessels per field examined at ×200 magnification) was calculated using the method described above. To determine these variables, we used a computer-aided image analysis system (Win ROOF, version 5.0, Mitani Corp., Fukui, Japan). Slides were blindly evaluated twice at different times by three investigators who were blinded to the pathologic features, and average densities were used for statistical analysis.

Statistical analysis. Normality was evaluated by normal distribution and histograms for each variable, and all data were expressed as mean ± SD. The Student's *t* test was used for analysis of continuous variables. Pearson's correlation was used to evaluate relationship between continuous variables by computing the correlation coefficient (*r*) and corresponding *P*s. Survival was evaluated by Kaplan-Meier analysis and the log-rank test. Variables that achieved statistical significance ($P < 0.050$) in the univariate analysis were subsequently entered into a multivariate analysis using Cox proportional hazards analysis [described as odds ratios (OR) with 95% confidential intervals (95% CI), together with the *P*s]. In this multivariate analysis model, if one or both of MMP-7 status on cancer cells and density of MMP-7-positive vessel were positive, MMP-7 status was judged as elevated. The crude and adjusted effects on immunohistochemical staining and vessel density, as well as other risk factors, were estimated by logistic regression analysis. All statistical tests were two sided, and significance was defined as $P < 0.050$. All statistical analyses were done using the statistical package StatView for Windows (version 5.0).

Results

Expression of MMP-7 in normal and cancer tissues. Staining for MMP-7 expression was weak to moderate in most normal tubules (Fig. 1A). Strongly stained normal tubular cells were relatively rare, and their proportion was only 5.3 ± 4.1%. On the other hand, immunohistochemical staining for MMP-7 was noted in the cytoplasm of cancer cells (Fig. 1B), and the proportion of MMP-7-positive cancer cells (21.4 ± 8.9%) were significantly higher than that of normal cells ($P < 0.001$). In addition, cancer cells at the invasive front and margin showed strong MMP-7 immunostaining (Fig. 1C), and such finding was observed in 9.5% (11 of 116) of low pathologic tumor stage and in 66.7% (32 of 48) of high pathologic tumor stage.

Examination of immunostained section also showed MMP-7-positive vessels in tumor area (Fig. 1D). The density of these vessels was 17.1 ± 7.2 per field. These vessels were relatively small and identified by the presence of RBCs within their walls. Comparison of MMP-7-positive vessels with those stained by anti-CD34 antibody showed that some CD34-positive vessels were concordant with MMP-7-positive vessels (Fig. 1E and F). On the other hand, MMP-7-positive vessels were relatively rare (3.4 ± 2.4 per field) in normal areas (Fig. 1G), whereas many CD34-positive vessels were noted in such areas of the same

Table 2. Independent role of MMP-7 expression in cancer cells and blood vessels for high pathologic tumor stage (model A) and metastasis (model B)

MMP-7	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Model A (high pathologic tumor stage)				
Cancer cells (positive)	30.00 (8.77-102.68)	<0.001	13.21 (3.64-51.62)	<0.001
Positive vessels (high)	5.38 (2.49-11.62)	<0.001	2.13 (0.77-5.92)	0.417
Model B (metastasis)				
Cancer cells (positive)	10.04 (2.99-36.18)	<0.001	3.11 (0.78-12.37)	0.106
Positive vessels (high)	11.03 (2.49-38.40)	<0.001	6.32 (1.64-24.34)	0.007

section (Fig. 1H). Based on these features, it was considered that MMP-7-positive vessels were newly formed blood capillaries through the process of carcinogenesis.

Clinical significance and survival implication. Table 1 lists the distribution of MMP-7-positive cancer cells and blood vessels according to various clinicopathologic variables. The proportion of MMP-7-positive cancer cells were significantly higher in patients with high pathologic tumor stage ($P < 0.001$), presence of metastasis ($P < 0.001$), and high grade ($P < 0.0001$) compared with those with low stage and grade and no metastasis. Similar findings were also found with regard to the density of MMP-7-positive vessels (Table 1). On the other hand, the expression pattern in cancer cells and blood vessels did not correlate with age or gender. There was a significant correlation between the proportion of MMP-7-expressing cancer cells and the density of MMP-7-expressing blood vessels ($r = 0.529$; $P < 0.001$).

In the next step, we examined the importance of MMP-7 expression on cancer cells and blood vessels in cancer cell invasion and metastasis by using two separate multivariate analysis models that included tumor grade (Table 2). Model A identified the independent roles of MMP-7 expression in cancer cells on high pathologic tumor stage and presence of metastasis. MMP-7 expression in cancer cells correlated with high pathologic tumor stage (OR, 13.21; 95% CI, 3.64-51.62; $P < 0.001$) but not with presence of metastasis. On the other hand, MMP-7-positive vessel density correlated with presence of metastasis (OR, 6.32; 95% CI, 1.64-24.34; $P = 0.007$) but not with pathologic tumor stage (Table 2, model B).

Kaplan-Meier curves of cause-specific survival according to MMP-7 expression on cancer cells and MMP-7-positive blood vessel density status are presented in Fig. 2A and B. Log-rank test showed that both variables were significant predictors of cause-specific survival. To evaluate the prognostic implication of MMP-7, we did multivariate analysis that included pathologic tumor stage, presence of metastasis, grade, and MMP-7 status for cause-specific survival. MMP-7 status was an independent predictor of poor prognosis (OR, 8.61; 95% CI, 1.10-67.28; $P = 0.040$; Table 3).

Discussion

Our results showed that MMP-7 expression level on RCC cells was significantly higher than in normal renal cells. Furthermore, high MMP-7 expression correlated with malignant potential, including high grade, cancer cell invasion, and metastasis. In addition, multivariate analysis showed that MMP-7 expression on cancer cells strongly influenced cancer cell invasion and negatively affected prognosis. To date, only a few studies have examined the clinical significance of MMP-7 in RCC. Our results agree with the findings of Sumi et al. (32) who reported stronger MMP-7 expression in high-grade RCC tumors than in low-grade tumors. However, our results of significant difference in MMP-7 expression between advanced-stage and low-stage RCC were different to that reported by the same group (32). We speculate that the discrepancy is due to differences in methodology and study population ($n = 20$). Apart from RCC, high MMP-7 expression was reported in various malignancies and that it correlated with malignant aggressiveness in various cancers, such as esophageal (22), gastric (18), colorectal (23, 25, 26), pancreas (24, 27, 29), lung (21), breast (28), and prostate cancers (19). On the other hand, other investigators reported that MMP-7 expression did not

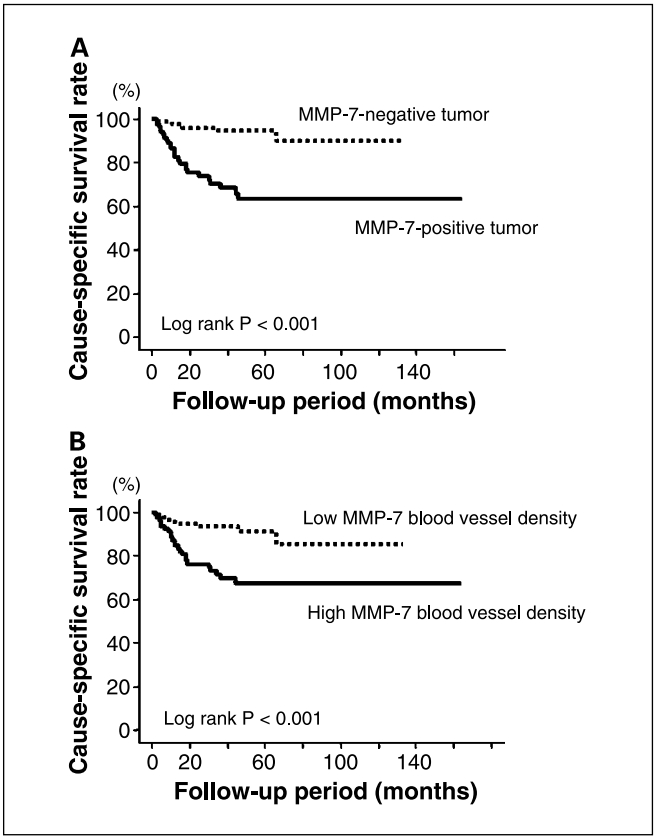

Fig. 2. Kaplan-Meier curves of cause-specific survival according to (*A*) MMP-7 expression in cancer cells and (*B*) MMP-7-positive blood vessel density status.

Table 3. Independent role of MMP-7 expression status in tumor tissues for cause-specific survival

Risk factors	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Pathologic tumor stage				
High (T_3 + T_4)	10.46 (4.63-23.64)	<0.001	2.09 (0.78-5.62)	0.145
Metastasis				
Presence	21.43 (9.83-46.69)	<0.001	10.96 (4.40-27.30)	<0.001
Grade				
High (G_3 + G_4)	9.79 (4.64-20.64)	<0.001	2.40 (0.97-5.92)	0.058
MMP-7				
Elevated	23.08 (3.14-169.60)	<0.001	8.61 (1.10-67.28)	0.040

correlate with clinicopathologic features, including grade, invasion, and metastasis, in breast (33) and lung cancers (34). Thus, there is a difference of opinion about the clinical significance of MMP-7 in cancers. Although we cannot explain the difference in clinical significance, we speculate that MMP-7 in cancer cells is an important factor in tumor invasion and it is associated with malignant potential at least in patients with RCC. With regard to MMP-7 expression in malignancy, it was also reported to influence the early stage of carcinogenesis (35, 36) and that MMP-7 was strongly expressed especially in the invasive front in a variety of malignancies, including oral (37), esophageal (38), and colorectal cancers (23, 39), suggesting that it has a direct role in cancer cell invasion. On the other hand, Li et al. (30) speculated that MMP-7 plays an important role not only in the early stage of tumor progression of pancreatic cancer but also in prognosis. We also showed the role of MMP-7 expression in cancer cell invasion of RCC because MMP-7 was strongly expressed in the invasive front. Based on this finding and the results of multivariate analyses, we speculate that the main function of MMP-7 in cancer cells is to regulate direct invasion into the surrounding tissues. Furthermore, the high expression of MMP-7 on cancer cells also correlated with metastasis and poor survival by univariate analysis. Accordingly, it seems that MMP-7 expression on cancer cells plays significant roles in cancer cell invasion and is an independent determinant of survival of patients with RCC.

Another interesting finding of our study was that MMP-7-positive vessels in cancer tissues were part of newly formed blood vessels. Furthermore, the density of such blood vessels in cancer tissues correlated positively with MMP-7 expression levels in cancer cells. Parts of the biological characteristics of tumor cells are closely associated with those of microvascular endothelial cells in tumor tissues (40). In addition, several investigators reported that MMP-7 could stimulate endothelial cell growth (6, 8). In addition, Mignatti et al. (6) reported previously that MMP-7 affected the migration of endothelial cells. Furthermore, Nagashima et al. (7) reported that endothelial cells have the ability to secret MMP-7 and the newly formed vascular endothelial cells adjacent to MMP-7-producing tumors expressed MMP-7. Our results provide support to these findings. About the clinical significance of MMP-7-positive blood vessels, we found that they played important and independent roles in RCC metastasis. In addition, this variable was a significant predictor for cause-specific survival by log-rank test. However, we consider that MMP-7 does not regulate all steps of angiogenesis and metastasis through the newly formed blood vessels because not all CD34-positive blood vessels were stained for MMP-7. Nevertheless, MMP-7 seems to play crucial roles in tumor angiogenesis, metastasis, and survival of patients with RCC.

The above findings have therapeutic potentials by targeting MMP-7 expression in tumor tissue. In fact, numerous preclinical and clinical trials have already tested the effects of certain natural and synthetic MMP inhibitors, such as batimastat, marimastat, and bryostatin (41–45). However, these MMP inhibitors have shown disappointing results about improvement of prognosis of cancer patients. In addition, they induced various side effects, including physiologic reactions, probably because of their broad specificity (45). However, a recent study examined the effect of specific MMP inhibitors on the prevention and treatment of malignancy (46). Furthermore, MMP-7 was reported in *in vitro* and animal model study to be a useful target for the prevention of metastasis in colon cancer (47, 48), and similar conclusion was reported by others for other types of cancers (49). Our results also suggest that MMP-7 can be potentially useful therapeutically through the regulation of both direct invasion of cancer cells and stimulation of angiogenesis in RCC. In addition, we found that elevated status of MMP-7 in cancer tissues (cancer cells and/or blood vessels) was a strong and independent predictor of poor prognosis. Therefore, targeting of MMP-7 may be an additional tool for prevention of tumor development and improvement of survival.

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