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Matrix metalloproteinase-7 increases resistance to Fas-mediated apoptosis and is a poor prognostic factor of patients with colorectal carcinoma

Wei-Shu Wang[1,4], Po-Min Chen[1,4], Huann-Sheng Wang[4,2], Wen-Yih Liang[3,4] and Yeu Su[5,*]

[1]Division of Medical Oncology, Department of Medicine, [2]Division of Colorectal Surgery, Department of Surgery, [3]Department of Pathology, Taipei Veterans General Hospital, Taipei, Taiwan, Republic of China, [4]National Yang-Ming University School of Medicine and [5]Institute of Biopharmaceutical Science, College of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China

*To whom correspondence should be addressed. Tel: +886 2 2826 7143; Fax: +886 2 2825 0883;
Email: yeusu@ym.edu.tw

The ability of tumor cells to resist apoptosis triggered by immune cells results in their escape from immune surveillance of the host. A critical effector of apoptosis is the Fas/Fas ligand (FasL) system that mediates the tumoricidal effects of cytotoxic T cells. Recently, *in vitro* cleavage of Fas expressed in various tumor cells by matrix metalloproteinase-7 (MMP-7) was demonstrated. In the present study, we first analyzed the influence of this metalloproteinase on Fas signaling in SW480, HCT-15 and HT-29 colorectal carcinoma (CRC) cells by assessing their responses to either an agonistic Fas antibody (CH11) or the FasL-bearing Jurkat cells after they were pretreated with MMP-7. Interestingly, both antibody- and Jurkat cell-induced apoptosis in three different CRC lines were significantly reduced by MMP-7 pretreatment. Additionally, immunohistochemical (IHC) staining was used to examine the expression levels of MMP-7 and Fas in tumor samples of 54 CRC patients. In agreement with our *in vitro* observation, the expression of MMP-7 in tumor tissues was inversely correlated with those of Fas ($P < 0.001$; χ^2-test). Moreover, shortened survival was found in patients with a higher MMP-7 and a lower Fas expression, respectively, in their tumor tissues ($P < 0.0001$). Finally, by multivariate analysis, we discovered that MMP-7 ($P = 0.001$) and Fas levels ($P = 0.036$) were independent prognostic factors for CRC patients. These results suggest that Fas downregulation and a consequential increased resistance to FasL-triggered apoptosis resulting from upregulated MMP-7 in colorectal cancer cells could be a key mechanism for their escape from the immune surveillance, thereby predicting a poor survival in CRC patients.

Abbreviations: CEA, carcinoembryonic antigen; CRC, colorectal carcinoma; ECM, extracellular matrix; EGF, epidermal growth factor; FasL, Fas ligand; IGFBP-3, insulin-like growth factor binding protein 3; IHC, immunohistochemical staining; LAK, lymphokine-activated killer; MMP, matrix metalloproteinase; TNM, Tumor-Node-Metastasis; OPN, osteopontin.

Introduction

The incidence of colorectal carcinoma (CRC) in Taiwan has increased over the past few decades, and recently, CRC has become one of the leading causes of cancer-related mortality in this country. Blood-borne metastasis, the major cause of death from CRC, is a stepwise process that starts when cancer cells segregate from a primary tumor, migrate across blood vessel walls into the blood stream and disperse throughout the body to generate new colonies (1). During the transit into the circulating system, tumor cells are exposed to fluid mechanical forces, plasma proteins and, especially, the vascular immune cells, all of which may decrease the survival of tumor cells as well as their extravasations from blood vessels (1).

Matrix metalloproteinases (MMPs), a large group of secreted proteinases that require divalent cations for their catalytic activities, play important roles in a variety of physiological and pathological processes such as tissue remodeling, morphogenesis, tumor invasion, as well as distant metastasis (2). MMPs degrade extracellular matrix (ECM) components in the basement membrane, thereby facilitating growth, invasion and metastasis of tumor cells (3). Additionally, several studies have shown that the functions of various plasma membrane molecules are modulated by MMPs, including MMP-7 (4–7). For example, a selective cleavage of beta-4 integrin by MMP-7 results in the loss of its expression in invasive prostate carcinoma (8). Moreover, MMP-7-mediated activation of intestinal α-defensin may be involved in a host defense mechanism (9). In contrast to other MMP family members, MMP-7 is usually overproduced by carcinoma cells instead of stromal cells, and involvement of this proteinase in colorectal tumorigenesis has been proposed because of its tumor-associated expression (10–12). Besides its prometastatic activity, MMP-7 also plays a critical role in early tumor development (13). For example, overexpression of MMP-7 significantly promotes tumor formation in mouse mammary glands (14). Concordantly, ablation in MMP-7 expression results in a dramatic reduction in intestinal cancer growth in animals (15).

Immune privilege is a crucial factor for the survival and metastasis of various types of cancers whose maintenance

relies on the collective production of a number of negative immune modulators, including Fas ligand (FasL) (16). In addition to inducing apoptosis of activated lymphocytes via interaction with its receptor (Fas/Apo-1/CD95), expression of FasL in hepatic metastatic tumor cells may be crucial for their liver colonization because it can trigger the apoptosis of the surrounding hepatocytes (17). In contrast, colon cancer cells seem to be capable of escaping from FasL-mediated cytotoxicity of T, NK, lymphokine-activated killer (LAK) and FasL-bearing tumor cells by downregulating Fas (18). In fact, decreased sensitivity to FasL-mediated apoptosis is a common trait shared by many different types of cancer cells, which provides them with critical survival advantages, ultimately leading to malignant progression (19). Loss of Fas and the expression of soluble Fas have been reported to contribute to the immune evasion of tumor cells and are correlated with poor prognosis in patients with renal cell carcinoma (20).

In addition to degrading ECM components, MMP-7 may confer apoptosis resistance to chemotherapeutic agents in SW480 colon carcinoma cells as well as SK-N-MC Ewing's sarcoma cells by modulating Fas-mediated death signaling (21,22). Cleavage of FasL by this proteinase may protect SW480 and SK-N-MC cells from apoptosis triggered by doxorubicin. In contrast, inhibition of MMP-7 expression by transfecting its antisense oligonucleotides seemed to sensitize SK-N-MC cells to doxorubicin (21,22). By cleaving FasL, overexpressed MMP-7 has been shown to provide apoptosis resistance and subsequently lead to tumor formation of murine mammary gland cells (23). A specific cleavage of Fas by MMP-7 that results in decreased sensitivity of HT-29 colon carcinoma cells to Fas-mediated apoptosis has been demonstrated (24). In contrast, the susceptibility of these cells to Fas-mediated apoptosis was drastically increased when their MMP-7 expression was suppressed by transient transfection of the antisense oligonucleotides for this proteinase (24).

In a previous study, we have shown that overexpression of thymosin β-4 ($T\beta_4$), a major G-actin sequestering peptide, in SW480 colon carcinoma cells results in a dramatic stimulation of β-catenin/Tcf-4 pathway and subsequently activates one of its downstream targets, *mmp-7* gene (25). These MMP-7-overexpressing cells not only showed increased invasion capabilities but their Fas levels were also drastically reduced (25). Since the ability of tumor cells to resist FasL-mediated apoptosis may contribute to their escape from the host immune surveillance, and MMP-7 appears to be capable of cleaving Fas, we hypothesized that overexpression of MMP-7 in colon cancer cells, by cleaving Fas, may result in their immune evasion and a poor prognosis in CRC patients. In the present study, we not only examined the relationship between Fas levels and the susceptibility of three different colon cancer cell lines to the apoptosis induced by the FasL-bearing Jurkat T cells as well as the influence of exogenous MMP-7 on the tumoricidal effects of Jurkat cells but also analyzed the correlation between MMP-7 and Fas levels in tumor samples of CRC patient by immunohistochemical (IHC) staining as well as their respective impacts on survival.

Materials and methods

Cell culture and reagents

Human colon carcinoma cell lines SW480, HCT-15, as well as HT-29 were purchased from the American Type Culture Collection (ATCC). They were maintained in L-15 medium (SW480) and RPMI (HCT-15 and HT-29), respectively, supplemented with 10% fetal calf serum (Life Technologies, Rockville, MD, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C with (HCT-15 and HT-29) or without (SW480) CO_2. The culture media were replenished every other day and passage was performed twice a week to maintain the viability of cells and to keep them in exponentially growing status. Recombinant MMP-7 was obtained from BioTrend (Koln, Germany). Polyclonal antibody against MMP-7 (clone C-17) was purchased from Santa Cruz (CA, USA). An agonistic Fas antibody (clone CH11) and a monoclonal anti-Fas antibody (clone ZB4) were purchased from Upstate (Lake Placid, NY, USA). Polyclonal neutralizing antibody for FasL was purchased from R&D (Minneapolis, MN, USA). Monoclonal anti-human β-tubulin antibody was purchased from BD Transduction Laboratories (San Diego, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Sigma (St Louis, MO, USA). Biotin-conjugated secondary antibodies and peroxidase-conjugated streptavidin IHC staining system were purchased from BioGenex (San Ramon, CA, USA). MMP inhibitor GM6001, propidium iodide, RNase A, and MTT were purchased from Sigma (St Louis, MO, USA).

Effects of MMP-7 on Fas-mediated apoptosis resistance

For examining the effect of MMP-7 treatment on the alterations in Fas-mediated apoptosis of colon carcinoma cells, SW480, HCT-15, as well as HT-29 colon cancer cells were seeded at a density of 1×10^6/well in 6-well plates and treated with MMP-7 (250 ng/ml). Twenty-four hours later, cells were incubated with either an agonistic Fas antibody (clone CH11, 40 ng/ml) or 1×10^6 FasL-bearing Jurkat cells (prefixed by 2% paraformaldehyde at 4°C for 1 h) for 48 h. In some experiments, an MMP inhibitor GM6001 (20 μM) was added together with MMP-7 to examine if MMP-7-induced apoptosis resistance can be reversed by blocking its proteolytic effects. A neutralizing antibody for FasL (1 μg/ml) was added together with Jurkat cells in some experiments to examine whether apoptosis induced by Jurkat cells was indeed mediated by Fas. Apoptosis and viability of these cells were analyzed by flow cytometry (Becton Dickinson & Co., Oxford, CA) and MTT assay, respectively.

Western blotting

Whole-cell lysate was prepared according to a protocol described previously (25). Fifty micrograms of protein was separated on a 10% SDS-polyacrylamide gel and processed for immunoblotting with anti-Fas (for SW480, HT-29 and HCT-15 colon carcinoma cells) or anti-FasL (for Jurkat cells) antibodies and developed using enhanced chemiluminescence (NEN Life Science, Boston, USA). Each blot was subsequently reprobed with an anti-β-tubulin monoclonal antibody to confirm equal sample loading.

Flow cytometric apoptosis analysis

Cells were trypsinized, washed with PBS, resuspended in 0.5 ml of lysis buffer (0.5% Triton X-l00, 5 mM EDTA, l% BSA in PBS) and placed on ice for 15 min before being fixed by methanol for 5 min at −20°C. After washing twice with ice-cold PBS, cells were suspended in DNA-staining solution (5 μg/ml propidium iodide, 10 mg/ml RNaseA) and incubated overnight at 4°C. Flow cytometry was carried out with FACS Caliburs for relative DNA content based on red fluorescence levels. The percentage of cells in different phases of the cell cycle as well as those that underwent apoptosis (represented by the sub-G_1 peak) was calculated using Modfit software (Verity Software House, Topsham, ME, USA).

Cell viability assay

For analyzing the viability of these cells under the influence of MMP-7, FasL or fixed Jurkat cells treatments, 1×10^4 cells/well were seeded in 96-well plates containing 0.2 ml of medium. After treatment, medium was replaced by 0.1 ml of fresh medium containing 10 μl of 0.5% MTT, and 4 h later 0.1 ml lysis buffer (20% SDS in 50% DMF) was added. Cell viability, represented by the absorbance at a wavelength of 570 nm, was measured using a Model 550 Microplate Reader (BioRad, UK).

Patients

From October 2002 to April 2003, a total of 54 patients with histologically confirmed Stage II or Stage III colorectal adenocarcinoma treated at Taipei Veterans General Hospital were enrolled. Paraffin-embedded tissue samples were available for this study. They all received curative tumor resection followed by 6 months of adjuvant chemotherapy with 5-fluorouracil and leucovorin on a weekly bolus schedule. The Tumor-Node-Metastasis (TNM) status was classified according to the international TNM staging system for CRC from the American Joint Committee on Cancer Manual for staging of cancer. Briefly, T1-2 was defined as tumors confined to submucosa or muscularis propria of the colon, whereas T3 represented tumors that invade through muscularis propria into subserosa, or into non-peritonealized pericolic

Table I. MMP-7 IHC staining status in different subgroups of CRC patients

Characteristics	MMP-7(+) (*n*)	MMP-7(−) (*n*)	*P*
All patients	31	23	
Age (years)			
<50	13	7	0.503
≥50		18	16
Gender			
Male	21	14	0.814
Female	10	9	
Performance status			
0	18	12	0.878
1, 2	13	11	
Primary tumor			
Colon	22	16	1.000
Rectum	9	7	
Histological differentiation			
Well-/moderately	22	16	1.000
Poorly-/Unknown	9	7	
Invasive extent			
T1-2	8	15	0.009
T3-4	23	8	
Nodal status			
N0	11	10	0.754
N1-3	20	13	
Distant metastasis			
No	6	17	<0.001
Yes	25	6	
Fas IHC status			
Positive	5	20	<0.001
Negative	25	6	
Serum CEA level (ng/ml)			
≤6	14	11	1.000
>6	17	12	

According to international TNM staging system for CRC, colorectal carcinoma; IHC: immunohistochemical staining; CEA: carcinoembryonic antigen.

or perirectal tissues. T4 was defined as tumors that perforate visceral peritoneum or directly invade other organs or structures. N0 was defined as patients without lymph node metastasis, whereas N1-3 represented at least one lymph node metastasis. M0 and M1 indicated patients without and with distant metastasis, respectively. These patients were followed up every 3 months to assess loco-regional recurrence as well as distant metastasis. Their characteristics are shown in Table I.

IHC staining

Paraffin-embedded sections from primary tumors of patients with CRC were stained using the streptavidin-biotin-immunoperoxidase technique according to manufacturer's instructions. Briefly, formalin-fixed paraffin-embedded tumor samples were sectioned at 4 μm, placed on slides, deparaffinized and hydrated. After depleting the endogenous peroxidase activity by 3% hydrogen peroxide treatment, tissue samples were probed, respectively, by a polyclonal anti-human MMP-7 antibody and a monoclonal anti-Fas antibody (ZB4) as primary antibodies, followed by the biotin-conjugated secondary antibodies and then a peroxidase-conjugated streptavidin system. Finally, these slides were examined microscopically by an experienced pathologist at Taipei Veterans General Hospital. In this study, both the intensity and distribution of IHC staining signals were analyzed. A positive MMP-7 staining was defined as a strong cytoplasmic signal, whereas a positive Fas staining was indicated by a strong membranous signal.

Statistical analysis and survival curve plotting

Patients were divided into two groups (positive or negative) for survival analysis according to MMP-7 as well as Fas IHC levels in tumor samples. The cause-specific survival curves were plotted using the Kaplan–Meier product limit method, and the statistical differences in survival among subgroups were compared by log-rank test. The correlations of primary anatomical site, histological grade, Tumor-Node-Metastasis (TNM) classification and serum carcinoembryonic antigen (CEA) levels were analyzed separately according to the expression of MMP-7 in carcinoma cells. The statistical difference of these correlations was determined with the χ^2-test. Similar analysis was applied to examine the correlation between Fas and MMP-7 IHC levels in these tumor samples. To assess the independent prognostic values of MMP-7 as well as Fas expression, we used Cox's proportional hazards regression analysis (multivariate analysis) that included MMP-7, Fas, histological grade, TNM classification and serum CEA levels. Each data point in the figures represents the mean ± standard deviation for three individual determinations. Statistically significant differences ($P< 0.05$) between differently treated groups were determined by one-way ANOVA and the Dunnet *t*-test with repeated measures. All statistical analyses were performed using the SPSS software system (SPSS for Windows, version 10.0, Chicago, IL, USA).

Results

MMP-7 decreases the susceptibility to FasL-triggered apoptosis in colon carcinoma cells

Since specific cleavage of Fas by MMP-7 has been found in hepatoma and colon carcinoma cells (24), we examined whether the susceptibility of SW480 colon cancer cells to FasL-triggered apoptosis was altered by MMP-7 pretreatment. Unlike the untreated ones, MMP-7-pretreated cells were highly resistant to the cytotoxic effects of an agonistic Fas antibody (clone CH11, data not shown), indicating a reduction of Fas-mediated death signaling in these cells after they were incubated with MMP-7. We next asked whether MMP-7 treatment also altered the sensitivity of SW480 cells to the cytolytic effect of T cells. To ascertain that FasL is expressed in Jurkat T cells, the ones chosen by us for the following experiments, western blotting was performed. As shown in Figure 1A, FasL expression was clearly detected in these T cells. We then co-cultured SW480 cells pretreated with or without MMP-7 with Jurkat cells for 48 h. MMP-7 pretreatment indeed resulted in a substantial decrease in apoptosis of SW480 cells upon being co-cultured with Jurkat cells (Figure 1B). To examine whether these findings were cell type-specific, similar experiments were then conducted in two other colon carcinoma cell lines, HCT-15 and HT-29, whose Fas levels were much higher than those of SW480 (Figure 2A). Accordingly, both HCT-15 and HT-29 cells were more sensitive to the toxic effects of the FasL-bearing Jurkat cells (Figure 2B). On the other hand, similar to SW480 cells, the susceptibilities of HCT-15 and HT-29 cells to Jurkat cell-induced apoptosis were also dramatically reduced by the preincubation of a FasL neutralizing antibody or MMP-7 (Figure 2B). As expected, apoptosis resistance of all three CRC lines that resulted from MMP-7 pretreatment could be reversed by GM6001, a broad-spectrum MMP inhibitor (26).

Increased MMP-7 expression is correlated with tumor invasion, distant metastasis and decreased Fas expression in colorectal cancer patients

We noticed that in MMP-7 positive cases, prominent signals seemed to diffuse in the cytoplasm of cancer cells (Figure 3A). In contrast, in tissue samples stained positively by the anti-Fas antibody, this protein seemed to localize mainly on the plasma membrane (Figure 3A). Extensive positive staining for MMP-7 (>50% of the cells were positive) was found in 57% (31 of 54), whereas strong positive staining for Fas was found in 46% (25 of 54) of the cases. The correlation between MMP-7

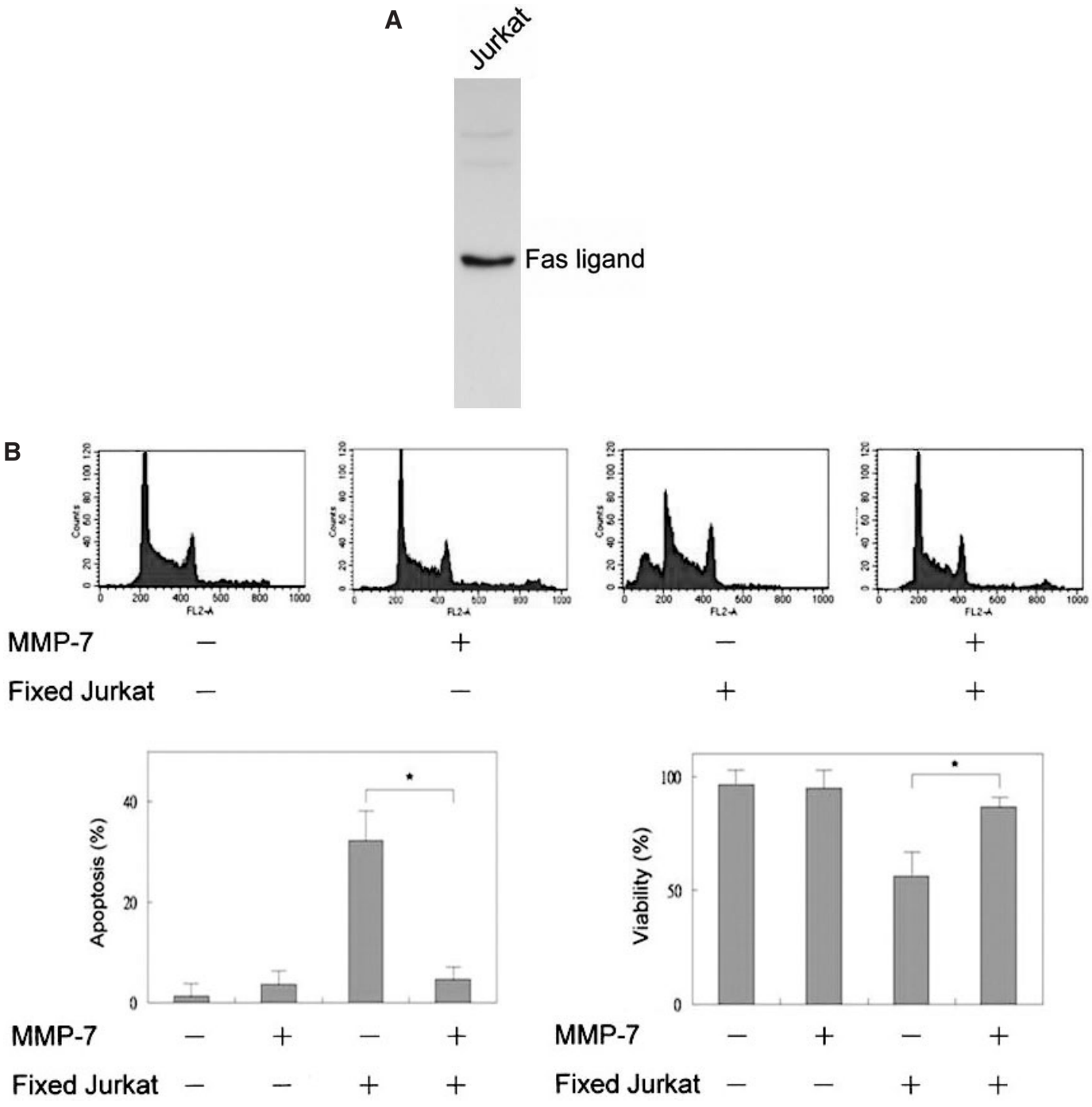

Fig. 1. MMP-7 decreases the susceptibility of SW480 colon carcinoma cells to the apoptosis induced by Jurkat T cells. (**A**) Fifty micrograms of total lysate prepared from Jurkat T cells was subjected to western blot analysis by probing with an anti-FasL antibody. (**B**) SW480 cells seeded at a density of 1×10^6/well in 6-well plates were treated with or without MMP-7 (250 ng/ml) for 24 h before being co-cultured with 1×10^6 paraformaldehyde-fixed Jurkat cells for another 48 h. Apoptosis and viability of SW480 cells were measured by flow cytometric and MTT assays, respectively. Each data represents the mean ± standard deviation for three individual determinations. *$P < 0.05$ when groups pretreated with and without MMP-7 were compared by one-way ANOVA with repeated measures.

and clinical parameters were then analyzed by χ^2-test and positive correlations between MMP-7 IHC levels and tumor invasion ($P = 0.009$) as well as distant metastasis ($P < 0.001$) were clearly detected (Table I and Figure 3B). Since MMP-7 is capable of cleaving Fas, an inverse correlation between the expression levels (i.e. IHC signal intensities) of MMP-7 and Fas in patients' tumor tissues was postulated. Such a correlation was indeed demonstrated herein (Table I and Figure 3B).

MMP-7 overexpression and Fas downregulation are associated, respectively, with poor prognosis of colorectal cancer patients

Since upregulation of MMP-7 was found to correlate with enhanced tumor invasion and distant metastasis, as well as reduced Fas expression in CRC patients, a shortened survival (i.e. poor prognosis) was postulated in patients with higher MMP-7 and lower Fas expression levels. As can be seen in

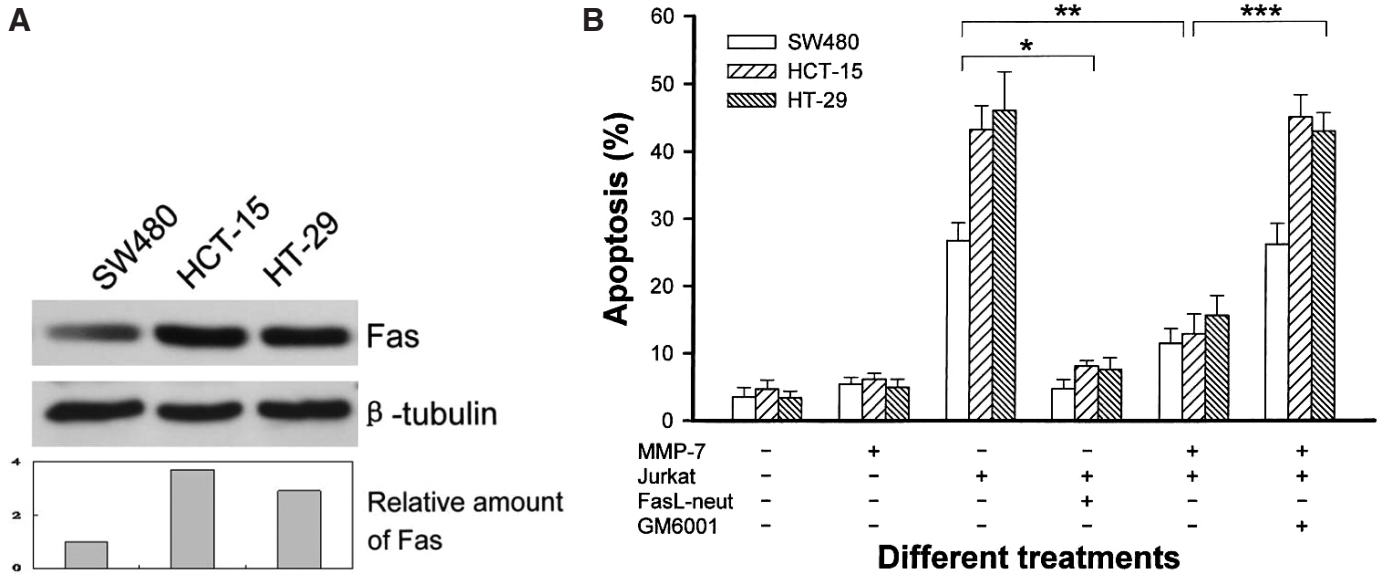

Fig. 2. Apoptosis in three different lines of colon cancer cells triggered by Jurkat T cells can be suppressed by the pretreatment of a FasL neutralizing antibody or MMP-7. (**A**) Total lysates (50 μg) prepared from SW480, HCT-15 and HT-29 colon cancer cells were subjected to western blot analysis using an anti-Fas antibody as a probe. (**B**) Experiments similar to those described in Figure 1 were performed in SW480, HCT-15 and HT-29 cells except that some of these cells were pretreated with a FasL neutralizing antibody (FasL-neut) or 25 μM of GM6001, a broad-spectrum MMP inhibitor, before being co-cultured with Jurkat cells. *$P < 0.05$ between groups pretreated with or without the FasL neutralizing antibody by one-way ANOVA with repeated measures. **$P < 0.05$ between groups pretreated with or without MMP-7. ***$P < 0.05$ between groups pretreated with or without GM6001 before the addition of MMP-7.

Figure 3C, the Kaplan–Meier analysis showed that patients with positive MMP-7 staining in their tumors survived significantly shorter than those with negative staining ($P < 0.0001$). By multivariate analysis, MMP-7 expression level was found to be an independent prognostic factor since a stronger MMP-7 IHC signal was accompanied by a shortened survival ($P = 0.001$; Table II). Meanwhile, a poor prognosis was proposed in the patients with negative Fas IHC staining in their tumors because reduced Fas might confer apoptosis resistance to tumor cells, resulting in their immune evasion. As shown in Figure 3D, patients whose tumors stained positively by an anti-Fas antibody survived significantly longer than those with a negative staining ($P < 0.0001$). Additionally, Fas expression level was also identified as an independent prognostic factor for CRC patients since longer survival was found in the ones with a stronger Fas IHC signal in their tumors ($P = 0.036$; Table II).

Discussion

MMPs regulate the turnover of ECM components and play important roles in embryo development, morphogenesis, tissue remodeling, as well as tumor invasion and metastasis (2,3). For tumor to metastasize, its invasive capability must be enhanced, and the extracellular proteases, especially MMPs, appear to be crucial for this process. In fact, the invasion of cancer cells into nearby stroma, across blood vessel walls and through normal epithelial cell layers has been shown to be promoted by various MMPs (1–3). Interestingly, roles beyond matrix degradation played by MMPs during cancer progression have been identified. For example, cell surface-localized MMP-9 might proteolytically activate TGF-β, thus providing a physiological mechanism of tissue remodeling that can be adopted by cancer cells to promote their growth and invasion (6). MMP-2, by cleaving laminin-5 γ2 subunit and exposing a putative cryptic promigratory site on this molecule, might enhance tumor cell migration (4).

MMP-7, the smallest member of the MMP family, is almost always overexpressed in CRC cells (12). By degrading ECM components such as type-IV collagen, fibronectin and laminin, MMP-7 plays important roles in the invasion and metastasis of carcinoma cells (27). Interestingly, cleavage of cell surface proteins other than ECM components by this metalloproteinase may also contribute to its tumorigenic effects. For example, MMP-7 activates the epidermal growth factor (EGF) receptor by releasing an EGF ligand, tumor growth factor-α (28). Additionally, proteolysis of the insulin-like growth factor binding protein 3 (IGFBP-3) by this proteinase has been shown to promote the survival of colon cancer cells by regulating IGF-I bioavailability (29). By cleaving osteopontin (OPN), a secreted phosphoprotein critical in wound healing, inflammation and tumor progression, MMP-7 enhances the function of OPN as an adhesive and migratory stimulus through its interaction with integrins (5). Moreover, by processing E-cadherin and thereby inducing loose and then tight aggregation of tumor cells, this proteinase induces homotypic adhesion of human CRC cells and enhances their *in vivo* metastatic potential (30). By disrupting tight junction structure and a consequent induction of cell dissociation, MMP-7 might enhance invasion as well as metastasis of pancreatic cancer cells (31). Interestingly, epithelial expression of MMP-7 as well as MMP-13 is associated with malignant progression in chronic wounds, and may provide a diagnostic clue for distinguishing squamous cell carcinoma from non-malignant wounds (32).

In addition to barrier clearance, resistance to host defense mechanisms, especially the immune surveillance, is also critical for the survival and metastasis of tumor cells. Engagement of FasL with its receptor that activates caspase-8 and subsequent apoptosis in tumor cells represents an important immune surveillance mechanism (33). In this regard, constitutive

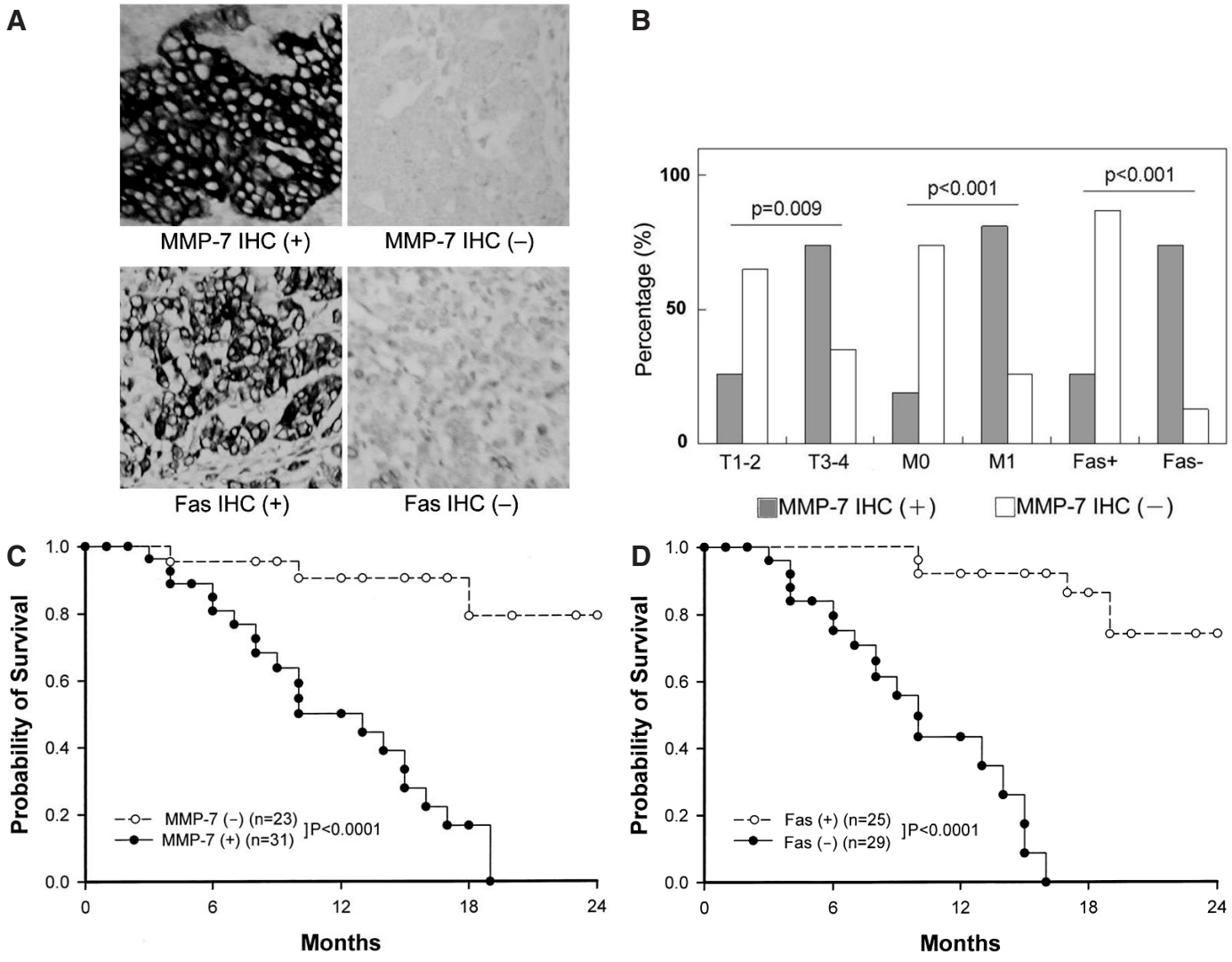

Fig. 3. Increased MMP-7 expression is associated with invasive tumor growth, distant metastasis and reduced Fas levels in patients with CRC. (**A**) Representative IHC staining patterns of MMP-7 and Fas of patient's tumor samples. (**B**) Correlations between MMP-7 expression level (i.e. IHC staining intensity) and invasive tumor growth, distant metastasis, as well as Fas level in CRC patients were analyzed by χ^2-test. MMP-7 IHC(+) was defined as a very strong staining for MMP-7, whereas MMP-7 IHC(−) represents weak or no staining. T1-2 was defined as tumors confined to submucosa or muscularis propria of the colon, whereas T3 represented tumors that invade through muscularis propria into subserosa, or into non-peritonealized pericolic or perirectal tissues. T4 was defined as tumors that perforate visceral peritoneum or directly invade other organs or structures. M0 and M1 represented patients without or with distant metastasis, respectively. Fas+ represented tissue samples that were stained strongly by an anti-Fas antibody, whereas Fas− represented those with little or no signal. (**C**) Survival curves of CRC patients with positive (filled circle) or negative (open circle) MMP-7 IHC staining in their tissue samples plotted by Kaplan–Meier method ($P < 0.0001$; log-rank test). (**D**) Similar method was used to plot the survival curves of CRC patients whose tissue samples were stained positively (open circle) or negatively (filled circle) by an anti-Fas antibody ($P < 0.0001$).

expression of FasL was detected in a variety of tumors (16) and FasL-mediated counterattacks by tumor were also demonstrated in a number of carcinomas (34). In the meantime, downregulation of Fas has been postulated to be another important mechanism for cancer cells, especially CRC, to escape from FasL-mediated killing (35). Recently, MMP-7-induced resistance of HepG2 hepatoma and HT-29 colon carcinoma cells to FasL-triggered apoptosis has been explained by a proteolytic degradation of Fas by this protease (24). In good agreement with this observation, we found that the apoptosis of SW480, HCT-15 and HT-29 cells induced by the FasL-bearing Jurkat cells were greatly diminished when they were preincubated with MMP-7 (Figure 2), suggesting that posttranslational mechanism(s) also plays a major role in modulating Fas levels in colon cancer cells.

To invade other tissues, tumors often generate a number of MMPs and some of them were found to have a positive correlation with CRC metastasis (36–38). Among these proteases, MMP-7 is best known for its involvement in the malignant progression of CRC (36), especially in liver metastasis (37). In fact, the worst prognosis has been reported in CRC patients with overexpressed MMP-7 and trypsin at the invasive front of tumors (38). In accordance with these earlier findings, we discovered a positive correlation between MMP-7 levels and tumor invasion as well as distant metastasis in CRC patients (Table I). More interestingly, we also found an inverse relationship between MMP-7 and Fas levels in these tumor samples.

The prognostic values of MMPs in various solid tumors were well documented. For example, elevated MMP-2 levels

Table II. Analysis of factors affecting survival including MMP-7 and Fas IHC staining status of CRC patients

Characteristics	Univariate (*P*)	Multivariate (*P*)
Age (years) <50 versus ≥50	0.871	0.635
Gender Male versus female	0.926	0.883
Performance status 0 versus 1, 2	0.132	0.048
Primary tumor Colon versus rectum	0.844	0.774
Histological grade Well/moderately differentiated versus Poorly differentiated	0.372	0.645
Invasive extent T1-2 versus T3-4	0.032	0.025
Nodal status N0 versus N1-3	0.003	0.011
Distant metastasis No versus Yes	0.028	0.001
Serum CEA level (ng/ml) ≤6 versus >6	0.273	0.354
MMP-7 IHC status Negative versus Positive	0.005	0.001
Fas IHC status Positive versus Negative	0.028	0.036

According to international TNM staging system for CEA: carcinoembryonic antigen; CRC, colorectal carcinoma; IHC: immunohistochemical staining.

appear to be correlated with poor prognosis in melanoma and breast carcinoma (39,40). Shortened survival has been reported in patients with non-small cell lung cancer, renal cell carcinoma, as well as head and neck squamous cell carcinoma, whose tumors express higher levels of MMP-9 (41–43). Since a positive correlation between upregulated MMP-7 expression and enhanced tumor invasion and distant metastasis as well as reduced Fas levels in CRC patients was detected herein, a shortened survival of these patients was proposed. Indeed, a poor prognosis in patients with positive MMP-7 staining was identified by log-rank test (Figure 3C) and multivariate analysis, respectively (Table II). Since reduced Fas levels may result in apoptosis resistance of tumor cells and their escape from host immune surveillance, a poor prognosis was proposed in the patients with negative Fas IHC patterns. Unsurprisingly, patients with positive Fas signals survived significantly longer than those with negative Fas staining (Figure 3D and Table II).

In addition to cleaving Fas, MMP-7 has been shown to shed FasL and generate a truncated death ligand with anti-apoptotic activity (21,22). For instance, cleavage of FasL by MMP-7 has been shown to protect Ewing's sarcoma cells from doxorubicin-induced apoptosis, whereas inhibition of the activity of this enzyme increases their drug sensitivity (44). Doxorubicin-induced apoptosis in SW480 colon cancer cells has also been reported to be suppressed by incubating them with MMP-7; conversely, downregulation of the expression of this proteinase by an antisense strategy results in their sensitization to doxorubicin-mediated cytotoxicity (21). Since apoptosis resistance plays a crucial role in early tumorigenesis, which might be conferred at least in part by MMP-7-mediated cleavage of Fas and FasL, this protease could be an important therapeutic target for cancers, especially CRC. Even though several recent clinical trials failed to show good efficacy of MMP inhibitors in treating patients with 'late-stage' cancers, the crucial roles other than ECM degradation played by MMP-7 in 'early' tumor development should not be neglected. New thinking as well as strategies for treating patients with 'early-stage' cancers through MMP inhibition not only should not be deserted but is also worthy of further investigations.

In summary, we have shown for the first time that upregulated MMP-7 expression is associated with decreased Fas in CRC patients' tumor samples. Overexpression of this metalloproteinase, by enhancing tumor invasion and distant metastasis in CRC patients, may lead to a poor prognosis. Moreover, reduction in Fas caused by MMP-7 cleavage may result in both apoptosis resistance and immune privilege of colon cancer cells. Taken together, our data suggest that aberrant upregulation of MMP-7, by cleaving Fas and consequential decrease in the susceptibility of colon carcinoma cells to various apoptotic stimuli, a mechanism beyond extracellular matrix degradation, could be a key event for colon cancer cells to evade host immune surveillance, leading to a poor prognosis in patients with CRC.

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