Matrix Metalloproteinase-7 Expression in Colorectal Cancer Liver Metastases: Evidence for Involvement of MMP-7 Activation in Human Cancer Metastases

Zhao-Shi Zeng,[1] Wei-Ping Shu, Alfred M. Cohen,[2] and José G. Guillem

Colorectal Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Purpose: **Matrix metalloproteinase-7 (MMP-7) is a member of the MMP family, which is overexpressed by some tumor cells and is thought to enhance the tumor metastatic potential. The aim of this study is to examine the MMP-7 expression in the human colorectal cancer (CRC) liver metastases and normal liver tissue using multiple techniques and to determine its association with liver metastases formation.**

Experimental Design: **MMP-7 mRNA, protein, and enzymatic levels were determined by reverse transcription-PCR, Western blot analysis, and casein zymography in the specimens of human CRC liver metastases and paired normal liver tissue from 44 patients. The cellular localization of MMP-7 was analyzed by immunohistochemistry.**

Results: **Our data reveal that all of the investigated liver metastases samples overexpressed MMP-7 mRNA and protein compared with the normal liver tissue. By zymogram, higher levels of the latent form of MMP-7 were found in 88.6% (39 of 44) liver metastases samples, whereas normal liver tissue exhibited only trace amounts. The activated form of MMP-7 was only found in those in which the pro-MMP-7 was present ($n = 39$); in contrast, it was not detected in the normal liver tissues. Immunohistochemically, MMP-7 is localized to the cytoplasm of tumor cells, and the strong signal is concentrated in the tumor front areas.**

Conclusions: **Our observations emphasize the important role of MMP-7 production and activation in human CRC liver metastases formation.**

INTRODUCTION

Dissemination of malignant tumor cells is the major cause of mortality in cancer patients. The liver is the most common site for blood-borne metastases in CRC,[3] and it is estimated that liver metastases will develop in 75% of patients who die of CRC (1). In recent years, our understanding of the biology of the metastases has improved rapidly. Several mechanisms are implicated in metastases formation, including release of ECM-degrading proteinases.

MMPs are a family of structurally related zinc-dependent proteinases that are capable of degrading the components of the ECM. MMPs are thought to be responsible for normal matrix remodeling and pathological tissue destruction by virtue of their ability to catabolize ECM components. Currently, at least 18 distinct MMPs have been identified (2). According to their structures, substrate specificities, and cellular localization, MMPs are classified into the following groups: collagenases, gelatinases, stromelysins, membrane-type MMPs, and others, including MMP-7 (3). The MMPs are frequently overexpressed in various human cancers (2, 4, 5). In addition, enhanced expressions of MMPs have been associated with an aggressive malignant phenotype and adverse prognosis in cancer patients (2, 4–6).

MMP-7 (Matrilysin, pump-1) is the smallest known member of the MMP family. The cDNA of MMP-7 was originally cloned from a human carcinoma cDNA library (7), and the protein product was first identified in the culture medium of the human rectal carcinoma cell line (8). MMP-7 is capable of degrading various ECM proteins including proteoglycans, fibronectin, entactin, laminin, gelatin, and elastin (9).

Because of the strong ECM-degradative activity, much evidence supports the role of MMP-7 in tumorigenesis and progression *in vitro,* and in the animal model (10–12). MMP-7 is elevated in several human primary cancers, including breast (13), lung (14), prostate (15), esophagus (16), stomach (17, 18), endometrium (19), and ovarian carcinomas (20), as well as esophageal squamous cell carcinomas (21).

It has been reported that MMP-7 was overexpressed both in benign and malignant colorectal tumors (8, 10, 17, 22–24). In addition, the levels of MMP-7 mRNA expression were correlated with the stage of CRC progression (23, 25). In CRC metastases, several studies have shown MMP-7 overexpression to variable degrees in cell lines (26, 27), animal models (11, 27), and in human tumor samples (25, 27). For an additional understanding of the role of MMP-7 in the metastases, we compared the mRNA, protein, and enzymatic expression of MMP-7 in human CRC liver metastases and the corresponding normal liver tissue to determine whether changes might play a role in CRC liver metastases formation.

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[1] To whom requests for reprints should be addressed, at Colorectal Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-7783; Fax: (212) 717-3053; E-mail: zengz@mskcc.org.
[2] Present address: Lucille P. Markey Cancer Center, University of Kentucky, 800 Rose Street MCC 140, Lexington, KY 40536.

[3] The abbreviations used are: CRC, colorectal cancer; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; ECM, extracellular matrix; mAb, monoclonal antibody.

MATERIALS AND METHODS

Patients and Tissue Samples Process. The liver metastases and corresponding normal liver specimens were obtained from 44 CRC liver metastases patients with the approval of the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center. The tissue samples were quick frozen in liquid nitrogen. Frozen-embedded samples were embedded in Tissue-TEC O.C.T. Compound (Miles, Elkhart, IN) and frozen in 2-methylbutane cooled with liquid nitrogen. Both frozen tissues and OCT blocks were stored at −80°C until processed.

RT-PCR Analysis of MMP-7. Total RNA was isolated from tissue samples by guanidium isothiocyanate-phenol-chloroform extraction as described previously (28). The thermal cycler, GeneAmp PCR Systems 9700 (Perkin-Elmer, Foster City, CA), was used for RT-PCR. Reverse transcription of RNA and PCR amplification of cDNA was carried out according to the manufacturers' protocol. Briefly, strand cDNA synthesis was performed in a 20-μl reaction volume containing 20 units of RNasin RNase inhibitor, 2.5 μM of random hexamers, 2.5 mM of each deoxynucleotide triphosphate, 50 units of Molony murine leukemia virus reverse transcriptase, and 2 μg of total RNA. The reaction mix was incubated for 10 min at room temperature. The program for the reverse transcription step was 15 min at 42°C and terminated by heating to 99°C for 5 min. For cDNA amplification, the human MMP-7 primers were 5′-AAACTCCCGCGTCATAGAAAT-3′ (sense, position 249–269) and 5′-TCCCTAGACTGCTACCATCCG-3′ (antisense, position 623–643). The β-actin primers used for loading control were 5′-GCACTCTTCCAGCCTTCCTCC-3′ (sense, position 819–839) and 5′-GGAGTACTTGCGCTCAGGAGGAGC-3′ (antisense, position 1035–1055).

cDNA (1.5 μl) was amplified by PCR in a final volume of 50 μl with 1 × TaqMan buffer, 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 200 μM of each primer, and 1.25 units of Ampli Taq Gold DNA Polymerase (Perkin-Elmer, Branchburg, NJ). PCR thermocycling conditions for MMP-7 and β-actin cDNA detection were set up as follows: 1 cycle of denaturing at 95°C for 10 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min 50 s followed by a final primer sequence extension incubation at 72°C for 5 min. Ten μl of each amplification reaction were analyzed electrophoretically using 1.2% agarose gels in the presence of 5 ng/ml ethidium bromide, and DNA was visualized under UV light. Contamination was routinely checked by RT-PCR assay of RNA template-free samples (water control).

Western Blot Analysis. Whole protein extracts from tissue samples were obtained by our methods described previously (29). Briefly, tissue was homogenized in Tris buffer [50 mM Tris-HCI (pH 7.5), containing 75 mM NaCl, 1% Triton, and 0.1 × SDS] and centrifuged at 5000 × *g* for 20 min at 4°C. The supernatants were used for Western blot and zymogram analysis. The total tissue proteins (125 μg) were electrophoresed on a 12% SDS-PAGE gel using a MINIGEL apparatus (Bio-Rad, Richmond CA). Protein (20 μg) of SW620 cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (100 ng/ml) was loaded as a positive control. Separated proteins were transferred to nitrocellulose membranes in Tris/glycine buffer [50 mM Tris base, 0.4 M glycine, 2 mM EDTA (pH 8.5)] at 4°C and 100 V for 1 h using a MINI system. Nonspecific binding sites were blocked for 1 h at room temperature with Tris–buffered saline–Tween 20 [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 4% BSA. The blots were incubated overnight at 4°C in a solution containing MMP-7 (Ab-1) mAb (Oncogene Research Products, Cambridge, MA). The blots were washed three times with TBS-T followed by an incubation step with horseradish peroxidase-labeled antimouse antibody (1:5000 in TBS-T for 30 min in room temperature). Reactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Casein Zymography. NOVEX 4–16% zymogram blue casein gels (Invitrogen, Carlsbad, CA) were used to detect MMP-7 enzymatic levels. Total tissue protein (125 μg/lane) was loaded on a gel. The gel was run in Tris/glycine SDS running buffer under nondenaturing conditions. The gels were washed twice in 2.5% (v/v) Triton X-100 (TX-100) for 30 min at room temperature to remove SDS. Zymograms were subsequently developed by incubation 72 h at 37°C in zymogram developing buffer [0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100 and 0.02% NaN_3 in 50 mM Tris-HCl (pH 7.4)]. Enzymatic activity was visualized as a clear band against a dark background of stained gelatin. The SW620 protein was used as a positive control.

Immunohistochemistry. Immunocytochemistry was performed in 44 patients. Immunoperoxidase staining by the avidin-biotin-peroxidase complex method was performed with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) as our methods described previously (29). Frozen sections were fixed in 4°C acetone then treated with 1% hydrogen peroxide for 15 min. Slides were incubated with diluted normal blocking serum for 20 min at room temperature. Sections were incubated at 4°C overnight with 5 μg/ml MMP-7 (Ab-1) mAb, the same antibody that was used for Western blot. A negative control reaction with no primary antibody was always performed alongside the reaction-containing sample by replacing the primary antibodies with 1% BSA-PBS. After washing, the slides were incubated with diluted biotinylated secondary antibody solution for 30 min and rinsed with PBS. Alkaline phosphatase was detected using reagents of the alkaline phosphatase substrate Kit III (Vector Laboratories) in the presence of levamisole (Vector Laboratories). Nuclei were counterstained with nuclear Fast Red (Vector Laboratories), and slides were then observed by conventional light microscopy.

Statistical Analysis. MMP-7 protein levels were quantitated by measuring the intensities of the appropriate autoradiographic bands using ChemiImager 4000 Low Light Imaging System (Alpha Innotech Corporation, San Leandro, CA). The results were expressed as tumor (liver metastases) to normal (liver tissue) fold increase. Student's t test was used to determine the P.

RESULTS

Expression of MMP-7 RNA in Liver Metastases and Normal Liver. The expression of MMP-7 mRNA was evaluated by RT-PCR. The oligonucleotide primers in this study were used to amplify a 394-base cDNA for human MMP-7. All 44 of the liver metastases samples showed increased levels of MMP-7 gene expression; in contrast, the normal liver tissue specimens showed no apparent or significantly low MMP-7

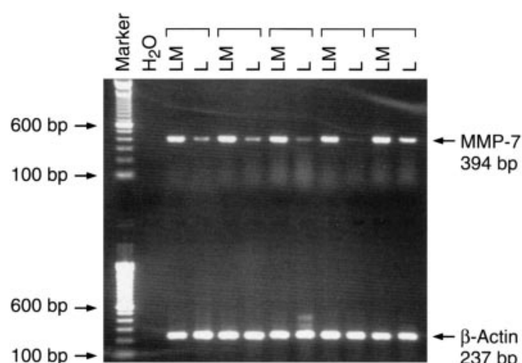

Fig. 1 Representative RT-PCR analysis from 5 CRC liver metastases patients. The total RNA isolated from each tissue was reverse transcribed and PCR-amplified using a specific primer described in "Materials and Methods." *Arrows* indicate the MMP-7 (*top*) or β-actin (loading control; *bottom*). *Lane 1,* 100-bp ladder as the size marker; *Lane 2*, water control; *LM,* liver metastases; *L,* corresponding normal liver tissue.

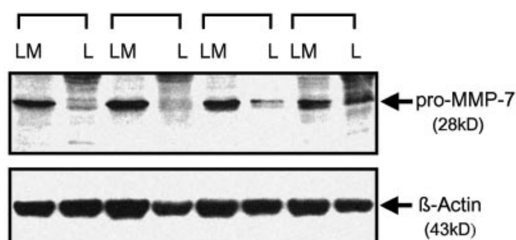

Fig. 2 Western blot analysis for determining the specificity of antibody to MMP-7 under nonreducing conditions. *LM,* CRC liver metastases; *L,* normal liver tissue. *Top panel,* Western blot analysis of MMP-7 under nonreducing conditions. The antibody reacts with protein band corresponding to the molecular weight of pro-MMP-7 (M_r 28,000). *Bottom panel,* Western blot analysis of β-actin.

mRNA expression. The representative finding is presented in Fig. 1. This difference cannot be related to the loading of RNA, because the β-actin mRNA (housekeeping gene) showed little difference between the tumor and normal tissues.

Pro-MMP-7 Protein in Liver Metastases. To examine whether mRNA expression resulted in production of MMP-7 protein, whole protein extracts from tumor and normal tissue were analyzed by Western blot. MMP-7 protein is expressed at low levels in the normal liver tissue but is markedly up-regulated in all 44 of the CRC liver metastases. Fig. 2 shows a representative Western blot of MMP-7 from the liver metastases and adjacent normal liver tissues. Blots were retreated with a mAb; β-actin are shown as internal controls. ChemiImager analyses of the M_r 28,000 bands indicated that the pro-MMP-7 is significantly higher in the liver metastases (10.7 ± 0.7) than in the normal liver (4.6 ± 0.37; $P < 0.001$).

Zymographic Detection of Activated Form of MMP-7. To additionally examine whether increased levels of MMP-7 mRNA and protein are correspond to augmented enzymatic activity, we performed the casein zymography. With this method, one can distinguish between the latent and the activated forms of MMP-7. The respective pro-MMP-7 and proteolytically activated species with a molecular weight of M_r 28,000 and M_r 19,000 are indicated in Fig. 3. The zymography demonstrated that higher levels of the latent form of MMP-7 were found in 88.6% (39 of 44) liver metastases samples, whereas normal liver tissue exhibited only trace or nondetectable amounts of the pro-MMP-7. Furthermore, as shown in Fig. 3, a significant enhancement of the M_r 19,000 caseinolytic activity attributable to the activated form of MMP-7 was clearly observed in the liver metastases when compared with the pro-MMP-7 form. The activated form of MMP-7 was only found in those in which the pro-MMP-7 was present ($n = 39$). In contrast, the MMP-7 activated form was not detected in the normal liver tissues.

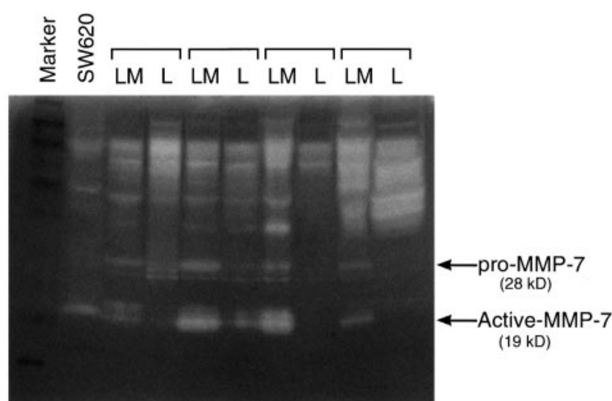

Fig. 3 Casine substrate gel electrophoresis for determining the caseinolytic activities of pro- and activated forms of MMP-7. The casine zymography shows two bands with the approximate sizes of M_r 28,000 and M_r 19,000 (noted by *arrows*), which correspond to pro- and activated form of MMP-7, respectively. Constitutive secretion of the latent and activated forms of MMP-7 appeared in every liver metastases (*LM*). In contrast, normal liver tissue (*L*) exhibited only trace or nondetectable amounts. *Lane 1*, protein molecular weight standard; *Lane 2,* colon cancer cell line SW620 as a positive control for MMP-7.

Immunohistochemical Staining of MMP-7. Immunoreactivity was seen in all 44 of the cases. Fig. 4 shows examples of immunohistochemical staining of the liver metastases with antibody to the MMP-7 protein. The relative degree of MMP-7 staining varied greatly among the different areas of liver metastases. A strong signal was observed in the front areas of the tumor (Fig. 4*A*). The cellular staining pattern in all of the tissues was mainly cytoplasmic (Fig. 4*B*). Although cell boundary staining was observed, no distinct cell surface staining was seen. In some sections, MMP-7 staining was seen in the residual normal liver cells (Fig. 4*C*), but immunoreactivity was significantly less than the cancer cells. Moreover, both normal and tumor cells showed no positive signal in negative control (Fig. 4*D*).

DISCUSSION

The probable role of MMPs *in vivo* is to degrade most components of the ECM. Although MMPs share substrates, they have different specific activities against each macromolecule. MMP-7, in particular, has the highest activity against insoluble elastin and is 11-fold more active than MMP-3 (30). Elastin is the highly cross-linked ECM component of elastic connective tissues such as blood vessels. The capability of MMP-7 to

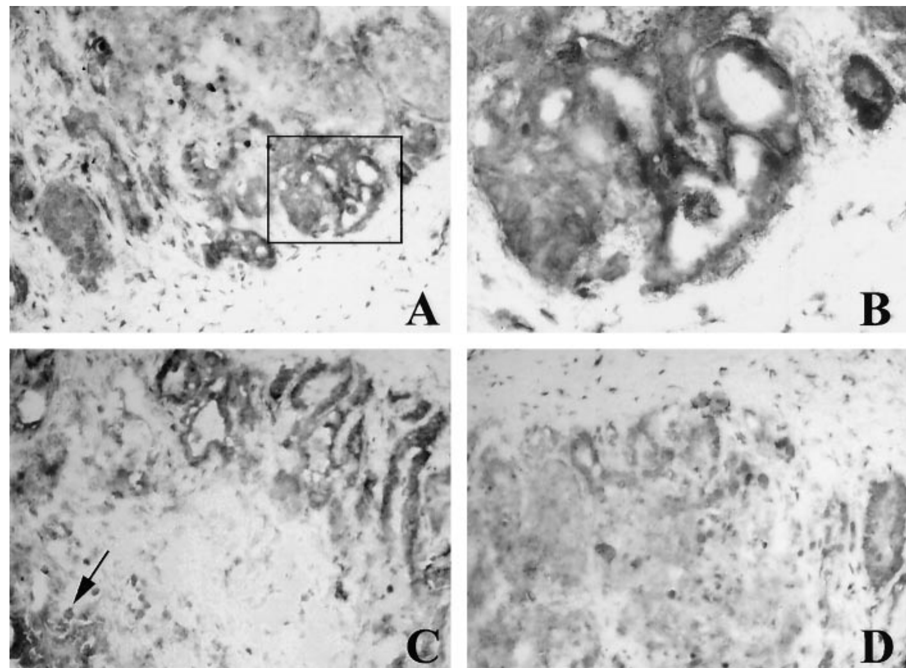

Fig. 4 Immunocytochemical staining for MMP-7 in human CRC liver metastases. In *A,* there is a different intensity of MMP-7 staining among the different areas of CRC liver metastases, strong immunoreactivity was concentrated in the invasive edge of tumor cells; ×100. *B,* higher magnification of area bordered in Fig. *A*. The staining pattern of MMP-7 was predominantly intracytoplasmic; ×400. *C,* strong immunoreactivity for MMP-7 can be seen in cancer cells, some residual normal liver cells were stained less intensely (noted by *arrow*) or were negative; ×100. *D,* negative control with 1% BSA-PBS instead of primary antibody; ×100.

degrade vascular basement membranes indicates a potential to facilitate hematogenous metastases. The catalytic activity of the MMP-7 is regulated at multiple levels including transcription, secretion, activation, and inhibition. By different approaches, the present study clearly indicates that the levels of MMP-7 mRNA, protein, and enzyme are significantly increased in human CRC liver metastases compared with normal liver tissues.

Although MMP-7 are highly regulated at the gene level, it is important to emphasize that the overexpression of MMP-7 is insufficient for the promotion of invasive behavior, because most MMPs are secreted as latent precursors (zymogens). Activation is a prerequisite to its functioning *in vivo*; therefore, it is necessary to study not only their proenzymes but also their activated form. A unique feature of MMP-7 expression demonstrated by this report is that the M_r 19,000 band corresponding to the activated form of MMP-7 was expressed at high levels constitutively in the liver metastases; in contrast, it was absent in the normal liver tissue. Our results support the notion that the activation of MMP-7 is one of the critical steps that lead to liver metastases formation. The present work extends previous observations by others that elevated MMP-7 expression is associated with the invasive and aggressive behavior of CRC *in vitro* (26, 27), in the animal model (11, 27), and in the human samples (25, 27).

A large number of proteases and organomercurials have been shown to activate pro-MMP-7 *in vitro*; however, the mechanisms of MMP-7 activation are not well documented *in vivo*. A potentially important mechanism for activation of latent MMPs is the intermolecular activation. It has been demonstrated that MMP-3 and MMP-10 are good activators of pro-MMP-7 (30, 31). MMP-3 can fully activate pro-MMP-7 via the cleavage of the Glu-Tyr peptide bond and generate an active species (30). In addition, human pro-MMP-1, 2, and 9 can be activated by MMP-7 (30, 32, 33). MMP-7 is able to activate the pro-MMP-2 by bone marrow stromal cells (32). Our previous reports demonstrated that both the MMP-2 and MMP-9 activated forms are presented in human CRC and liver metastases (34); therefore, MMP-7 production by CRC cells probably participates in MMP-2 and MMP-9 activation in human CRC. The co-overexpression of activated forms of MMP-2, MMP-9, and MMP-7 in the liver metastases may function as powerful machinery for the ECM digestion, which facilitates local invasion and metastases.

It is now recognized that most MMPs, such as MMP-2, MMP-9, MMP-11, and membrane-type MMP, are expressed by stroma cells (35–38). We demonstrated previously that both MMP-9 mRNA and protein are produced by tumor-surrounding macrophages in human CRC and liver metastases (28). In the current study, we provide the evidence that unlike most MMPs, MMP-7 is abundant in the cytoplasm of tumor cells. Our data additionally demonstrated that the higher concentration of MMP-7 was produced by the invasive edge of tumor cells suggesting that MMP-7 may facilitate local ECM degradation and promotes tumor spread.

In conclusion, our results describing intense expression of MMP-7 in CRC liver metastases clearly show the implication of MMP-7 in tumor invasion and metastases. The detection of the activated form of MMP-7 in liver metastases also supports the hypothesis that the activation of pro-MMP-7 is one of the critical steps in ECM matrix breakdown, facilitating tumor invasion and metastases. An enhanced understanding of the molecular mechanisms responsible for the activation of pro-MMP-7 may lead to a new therapeutic strategy for CRC liver metastases.

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