

# Unique activation of matrix metalloproteinase-9 within human liver metastasis from colorectal cancer

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**Summary** Experimental in vitro and animal data support an important role for matrix metalloproteinases (MMPs) in cancer invasion and metastasis via proteolytic degradation of the extracellular matrix (ECM). Our previous data have shown that MMP-9 mRNA is localized to the interface between liver metastasis and normal liver tissue, indicating that MMP-9 may play an important role in liver metastasis formation. In the present study, we analysed the cellular enzymatic expression of MMP-9 in 18 human colorectal cancer (CRC) liver metastasis specimens by enzyme-linked immunosorbent assay (ELISA) and zymography. ELISA analysis reveals that the latent form of MMP-9 is present in both liver metastasis and paired adjacent normal liver tissue. The mean level of the latent form of MMP-9 is  $580 \pm 270$  ng per mg total tissue protein (mean  $\pm$  s.e.) in liver metastasis vs  $220 \pm 90$  in normal liver tissue. However, this difference is not significantly different ( $P = 0.26$ ). Using gelatin zymography, the 92-kDa band representative of the latent form is present in both liver metastasis and normal liver tissue. However, the 82 kDa band, representative of the active form of MMP-9, was seen only in liver metastasis. This was confirmed by Western blot analysis. Our observation of the unique presence of the active form of MMP-9 within liver metastasis suggests that proMMP-9 activation may be a pivotal event during CRC liver metastasis formation.

**Keywords:** matrix metalloproteinase: 92 kDa type IV collagenase (MMP-9); activation: colorectal cancer: liver metastasis

Colorectal cancer (CRC) is the third most common malignancy in the United States, with an estimated 131 200 new cases and 54 900 deaths in 1997 (Parker et al. 1997). The liver is the most common site for blood-borne metastasis, with 15% of CRC patients harbouring liver metastasis at diagnosis and 60% of these patients having liver-only or liver-predominant disease (Kemeny and Seiter, 1993).

Liver metastasis formation is a complex, multifactorial, multi-stage process, requiring degradation of the extracellular matrix (ECM), breach of basement membrane, tumour cell intravasation and extravasation of the vascular system and colonization of the liver. This process is likely to involve numerous proteolytic enzymes, including the matrix metalloproteinases (MMPs), a family of Zn<sup>2+</sup>- endopeptidases capable of degrading ECM (Liotta and Stetler-Stevenson, 1991; Matrisian, 1992).

Increased metastatic capacity following transfection of MMP-9 into Ha-ras + E1A-transformed cells supports a causal role for MMP-9 in metastasis formation (Bernhard et al. 1994). In addition, elevated MMP-9 activity in experimental colon tumours transplanted into mice has been shown to correlate with metastasizing (Nakajima et al. 1990). Furthermore, overexpression of MMP-9 has been observed in various human cancers, including CRC (Pyke et al. 1993; Liabakk et al. 1996; Nielsen et al. 1996; Zeng and Guillem, 1996), breast (Davies et al. 1993a), bladder (Davies et al. 1993b), prostate (Hamdy et al. 1994), lung (Brown et al. 1993a), squamous cell carcinoma (Pyke et al. 1992) and brain tumours (Rao et al. 1993). Our recently published data have shown that tumour tissue MMP-9 RNA level can predict colorectal cancer relapse and

survival (Zeng et al. 1996), emphasizing the clinical importance of elevated MMP-9 expression in primary CRC.

We have previously demonstrated that MMP-9 mRNA is localized in the interface between liver metastasis and normal liver tissue, indicating that MMP-9 may play an important role in liver metastasis formation. To investigate further the significance of elevated MMP-9 RNA in liver metastasis formation, the present study examined MMP-9 enzymatic expression in CRC liver metastasis.

## MATERIALS AND METHODS

### Human tissue process

Eighteen liver metastases from CRC and paired normal liver tissue were obtained from the operating room immediately after resection with the approval of the Institutional Review Board of the Memorial Sloan-Kettering Cancer Center. They were quick frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processed. The presence of liver metastasis was confirmed by pathological assessment on haematoxylin- and eosin-stained paraffin sections. Tissue was homogenized in Tris buffer [50 mM Tris-HCl, pH 7.5, containing 75 mM sodium chloride, 1% Triton, 0.1% sodium dodecyl sulphate (SDS)] and centrifuged at 5000 g for 20 min at  $4^{\circ}\text{C}$  as previously described (Zeng and Guillem, 1996). Protein concentration of the supernatant was determined with protein assay reagent according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant of liver metastasis and paired normal liver tissue were used for ELISA and zymography.

### ELISA plate assay

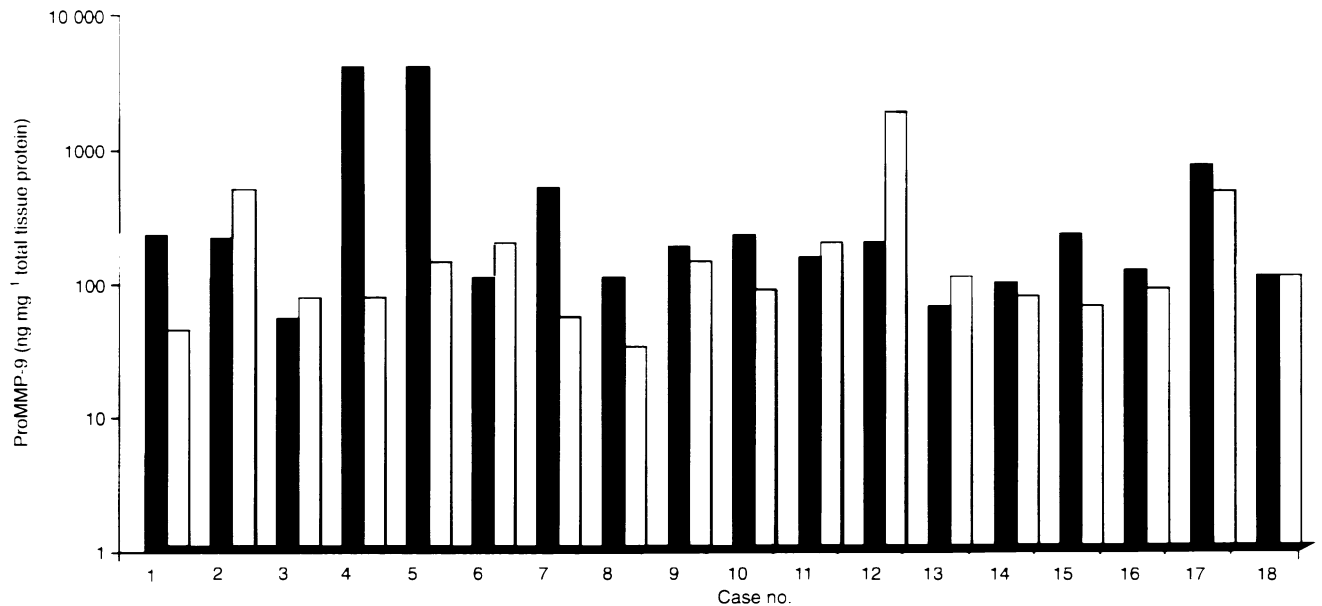
Quantification of MMP-9 levels in liver metastasis and normal liver tissue was performed by the Biotrak MMP-9 ELISA kit

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**Figure 1** Comparative distribution of ProMMP-9 levels in liver metastasis and normal liver tissue from 18 colorectal cancer patients with liver metastasis determined by ELISA analysis. ■, Liver metastasis; □, normal liver

(Amersham International, UK). Two monoclonal antibodies, both recognizing pro-MMP-9, were used for this assay. The methods used were those described in the protocol accompanying the ELISA kit. Briefly, 60 µg of total protein was added to each well in duplicate. The plates were incubated at room temperature for exactly 1 h. After washing four times with wash buffer (0.01 M phosphate buffer, pH 7.0, containing 0.05% Tween 20), a lyophilized anti-MMP-9 antibody conjugated to horseradish peroxidase was added and incubated at room temperature for 2 h. After another wash, TMB substrate was added into each well and followed with constant shaking at room temperature for exactly 30 min. The optical density (OD) was determined at 450 nm.

### Gelatin zymography

Eight per cent SDS-polyacrylamide electrophoretic gels co-polymerized with 1 mg ml<sup>-1</sup> gelatin were used to detect both latent and activated forms of gelatinase. Equal amounts of total protein (25 µg per lane) of tumour and paired normal liver were loaded into separate lanes and separated by electrophoresis under non-denaturing conditions. The gels were washed twice in 2.5% Triton X-100 (TX-100) for 30 min at room temperature to remove SDS. Zymograms were subsequently developed by incubation overnight at 37°C in collagenase buffer (0.2 M sodium chloride, 5 mM calcium chloride, 1% (v/v) TX-100 and 0.02% sodium azide in 50 mM Tris-HCl (pH 7.4)). Zymograms were stained with 1 (w/v) Coomassie blue G-250 dissolved in 30% methanol containing 10% (v/v) glacial acetic acid at room temperature for 3 h. Gel was destained in the same solution but without the Coomassie blue stain. Gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin.

92-kDa gelatinase control consisted of a medium conditioned by RA3-1S7 cells that were derived from primary rat embryo fibroblasts by co-transfection with the Ha-ras and adenovirus *E1A* oncogenes (kindly provided by Dr RJ Muschel).

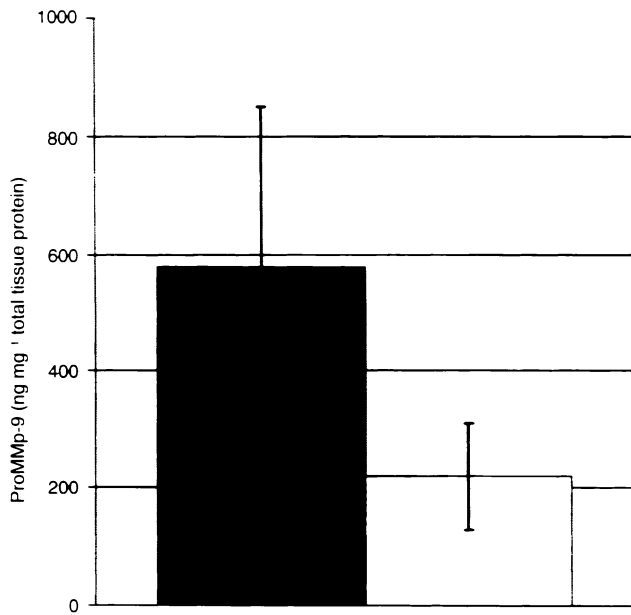
### Western blot analysis

These were carried out as previously described (Zeng et al. 1994). The supernatant of liver metastases and paired normal liver (25 µg) were electrophoresed on an 8% SDS-PAGE gel using a Minigel apparatus (Bio-Rad, Richmond, CA, USA). Separated proteins were transferred to nitrocellulose membranes (Amersham, Buckinghamshire, UK) in Tris-glycine buffer (2.5 mM Tris, 192 mM glycine and 20% methanol) at 4°C and 100 V using a Mini system. Non-specific binding sites were blocked for 1 h at room temperature with a solution containing 4% bovine serum albumin. The blots were incubated overnight at 4°C in a solution containing polyclonal rabbit antihuman MMP-9 antibody Ab110 (provided by Dr William G Stetler-Stevenson, Laboratory of Pathology, National Cancer Institute). The blot was washed several times with TBS-T, followed by an incubation step with horseradish peroxidase labelled anti-rabbit antibody (1:5000 in TBS-T for 30 min in room temperature). Reactive proteins were visualized with an enhanced chemiluminescence detection system (ECL, Amersham) as described by the manufacturer.

## RESULTS

### ProMMP-9 protein in liver metastasis quantified by ELISA assay

The results of MMP-9 ELISA analysis are shown in Figure 1. ProMMP-9 was observed in all 18 CRC liver metastases and paired normal liver samples. In 8 of the 18 cases, proMMP-9 in liver metastasis was higher than in normal liver tissue (cases 2, 4, 7, 8, 9, 10, 11 and 13). In seven cases, the levels of proMMP-9 protein in liver metastasis were nearly equal to those in normal liver tissue (cases 1, 3, 5, 12, 15, 17 and 18). In three patients, proMMP-9 levels were much higher in normal liver tissue than in liver metastasis (cases 6, 14 and 16). The levels of proMMP-9 in liver metastasis varied from 20 to 3750 ng mg<sup>-1</sup> total tissue protein



**Figure 2** Comparison of ProMMP-9 levels in liver metastasis and normal liver tissue. Data are expressed as mean of MMP-9  $\pm$  s.e. ■, liver metastasis; □, normal liver.  $P = 0.26$

with a mean proMMP-9 level of  $580 \pm 270$  ng mg<sup>-1</sup> total tissue protein (mean  $\pm$  s.e.). The corresponding range for proMMP-9 in paired normal liver tissue was 30–1690 ng mg<sup>-1</sup> protein with the mean value of  $220 \pm 90$  ng mg<sup>-1</sup> protein in normal liver respectively. However, as seen in Figure 2 this difference does not reach significance ( $P = 0.26$ ).

### Zymographic detection of active and latent MMP-9 forms

Gelatin zymography detected both the pro and active forms of MMP-9 with molecular weights of 92 and 82 kDa respectively.

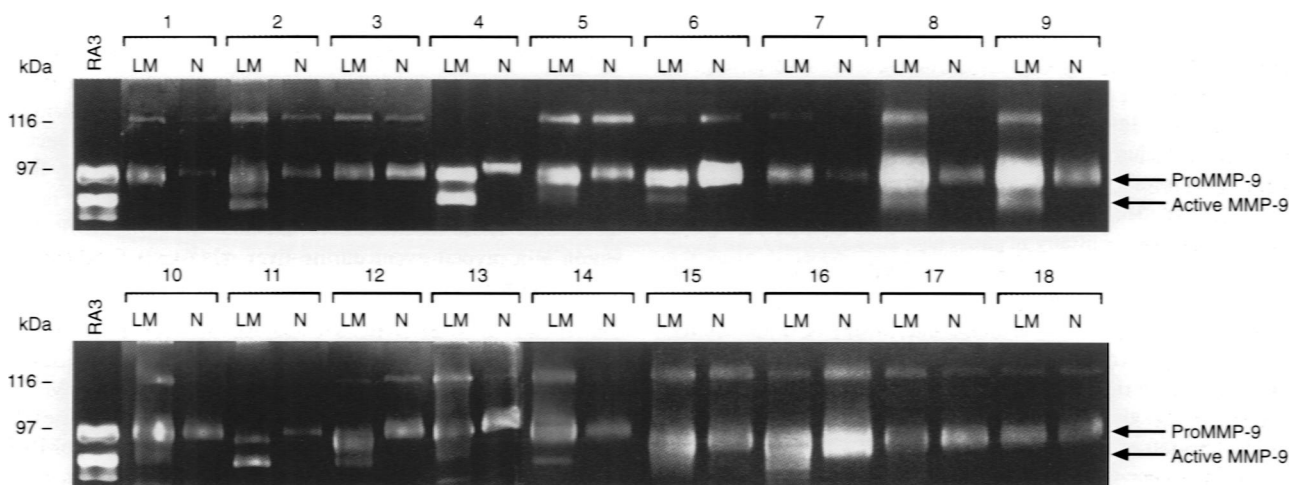
The identity of these activities was confirmed with molecular weight standards and the 92-kDa gelatinase positive control (medium conditioned by RA3-1S7 cells). The 92-kDa latent form of MMP-9 (proMMP-9) was detected in both liver metastasis and paired normal liver samples. As shown in Figure 2, the activated form of MMP-9 was seen only in liver metastasis, whereas no active MMP-9 activity could be detected by zymography in normal liver. Fourteen of 18 (77.8%) liver metastasis samples (cases 1, 2, 4, 5, 6, 8, 9, 10–16) expressed activated forms of MMP-9 (82 kDa). MMP-9 gelatinase activity in liver metastasis and normal liver was confirmed by complete inhibition of activity when duplicate gels were incubated in the presence of 10 mM EDTA (data not shown). To test the possibility of spontaneous activation of gelatinases during sample storage, we selected two paired liver metastases and corresponding normal liver samples (cases 17 and 18), both lacking the active MMP-9 form, and performed gelatin zymography 1 week, half a month and 1 month after storage at  $-80^{\circ}\text{C}$ . Despite prolonged storage, no active form was detected in both liver metastasis and liver (data not shown).

### Western blot confirmation of active MMP-9

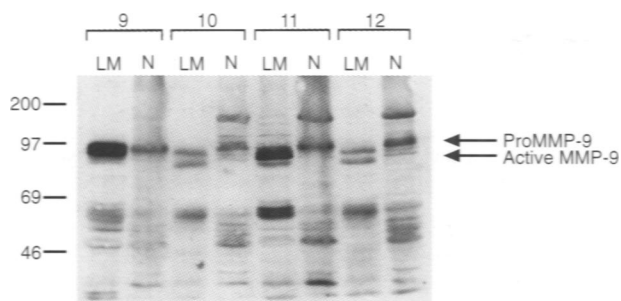
To confirm that the 82 kDa band seen in the gelatin zymography represented activated MMP-9, MMP-9 protein expression was detected by Western blot analysis using MMP-9 antibody Ab110, which recognizes both the latent and activated form of MMP-9. Figure 4 shows a representative Western blot of MMP-9 from liver metastasis and corresponding normal liver samples (cases 9–12). In agreement with zymography, the 92-kDa band of proMMP-9 was observed in both liver metastases and normal liver tissue. The MMP-9 form, which is detected in tumours, appears to correspond specifically to the activated form of MMP-9 (82 kDa). There is very little, if any, activated form of MMP-9 in normal liver tissue.

### DISCUSSION

Our previous work and that of others have demonstrated that levels of activated MMP-2 and MMP-9 correlate with progression of



**Figure 3** MMP-9 enzymatic activities in human colorectal cancer liver metastasis detected by zymography. Control lane, 25  $\mu\text{g}$  of conditional medium from RA3.1S7; LM, liver metastasis; N, normal liver tissue. Case numbers are shown above each matching tumour–normal pair. The positions of pro and active MMP-9 forms (92 kDa and 82 kDa bands) are noted by arrows. Liver metastasis (LM) and normal liver tissue (N) extracts (25  $\mu\text{g}$ ) from each patient were separated on a 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel containing 1 mg ml<sup>-1</sup> gelatin



**Figure 4** Western blot analysis of MMP-9 in CRC liver metastasis. Liver metastasis (LM) and adjacent normal liver tissue (N) extracts from each patient were separated on 8% SDS-PAGE gel and transferred to nitrocellulose membranes. The membrane was incubated with polyclonal rabbit antihuman MMP-9 antibodies Ab110 and visualized as described in Material and methods. The position of proMMP-9 and its activated form, 82 kDa collagenase, are noted by arrows

several human malignant tumours including CRC (Rajabi et al. 1990; Zeng et al. 1995), lung cancer (Brown et al. 1993b), breast cancer (Davies et al. 1993a), gastric cancer (Rajabi et al. 1990), prostate cancer (Stearns and Wang, 1993) and melanoma (MacDougall et al. 1995). The present study provides clear evidence that the activated form of MMP-9 is detected only in liver metastasis, not in adjacent normal liver tissue, suggesting that MMP-9 activation is a pivotal event during CRC liver metastasis formation.

The precise mechanism by which MMP-9 is synthesized and secreted remains unknown. However, it has become apparent that the intracellular signalling events controlling MMP-9 production are likely to involve tumour-stroma cellular interactions. As MMP-9 is secreted in a latent form, activation must occur in order to ensure extracellular matrix substrate degradation. This is accomplished by cleavage at the Glu-40-Met-41 amide bond located in the middle of the propeptide to generate an 86-kDa intermediate. Cleavage of this bond triggers a change in proMMP-9 that renders the Arg-87-Phe-88 amide bond susceptible to the second cleavage, resulting in conversion to an 82-kDa species (Ogata et al. 1992; Fridman et al. 1995). ProMMP-9 can be activated autocatalytically by organomercurial compounds or trypsin *in vitro* (Wilhelm et al. 1989; Lyons et al. 1991). In the current study, the possibility of spontaneous activation of gelatinases in liver metastasis during sample processing appears to be unlikely, as the liver metastasis and control normal liver samples were processed in an identical manner and run on the same gel. In addition, tissue samples initially negative for the active 82-kDa band remained negative despite storage and repeat analysis at different time points.

Several studies have demonstrated that MMPs have the ability to activate one another (Ogata et al. 1992; O'Connell et al. 1994; Cao et al. 1995; Fridman et al. 1995). Stromelysin (MMP-3) has been shown to activate proMMP-9, and ProMMP-3 can be activated by plasmin and cathepsin B. MT-MMPs, which are found on the cell membrane of tumour cells, have been shown to activate MMP-2 (Sato et al. 1994). The complex of proMMP-2 and TIMP-2 binds to activated MT-MMP and this binding ultimately results in activation of MMP-2 (Himmelstein et al. 1994; Cao et al. 1995). The active MMP-2 species may in turn activate proMMP-9

(Fridman et al. 1995). Taken together, the process of MMP-9 activation may be similar to the blood-clotting cascade. Co-expression of these enzymes in tumour tissue would facilitate their interaction for activation, leading to degradation of ECM (Fridman et al. 1995). It is interesting that our previous data demonstrated a significant increase in MMP-9 RNA expression in human liver metastasis when compared with normal liver tissue by Northern blot and *in situ* hybridization (Zeng and Guillem, 1995). However, ELISA and zymographic analysis detect pro-MMP-9 enzyme in both liver metastasis and normal liver tissue. It is difficult to explain this inconsistency, except that there may be factors that control both MMP-9 RNA up-regulation and proenzyme activation. In addition, MMP-9 activation in liver metastasis was not uniform (78% rather than 100%), supporting the notion that MMP-9 activation may not be the only mechanism involved in liver metastasis formation.

Our *in situ* and immunohistochemistry data indicate MMP-9 production by macrophages in liver metastasis (Zeng and Guillem, 1995). Because not all macrophages contain detectable amounts of MMP-9, this may be due to differences among various macrophage subpopulations (Heuff et al. 1993). It is reported that tumour-associated macrophages (TAMs) may be involved in the development of liver metastasis (Martin et al. 1989; Heuff et al. 1993). Heuff et al (1993) observed tumour-infiltrating macrophages, a peculiar type of TAM, in a rat liver metastasis model. As many tumours produce factors such as macrophage colony-stimulating factor (M-CSF) (Walter et al. 1991) and as TAM may bear receptors for M-CSF (Bottazi et al. 1990), tumour cells may stimulate the migration and growth of tumour-associated macrophages to the tumour edge, leading to increased local MMP-9 production facilitating tumour invasion. This notion is supported by the identification of agents such as lipopolysaccharide (LPS), which can stimulate macrophages to produce several MMPs including MMP-9 (Welgus et al. 1990; Xie et al. 1994), as well as our work, which demonstrates that metastatic CRC cells can stimulate THP-1 monocytes to produce MMP-9 (Swallow et al. 1996). Further support for an important role of macrophage-derived MMPs in invasion comes from studies using macrophage metalloproteinase (MMP-12 or MME)-deficient mice (MME<sup>-/-</sup>). Macrophages of MME<sup>-/-</sup> mice have a markedly diminished capacity to degrade ECM components. MME<sup>-/-</sup> macrophages are essentially unable to penetrate reconstituted basement membrane *in vitro* or *in vivo* (Shiple et al. 1996).

In conclusion, our results demonstrate the presence of proMMP-9 in both liver metastasis and normal liver tissue. However, the active form of MMP-9 is detected only in liver metastasis. These results support the notion that proMMP-9 activation is a pivotal event during liver metastasis formation. An enhanced understanding of the molecular mechanisms responsible for the activation of pro-MMP-9 may lead to the development of novel methods for the prevention and treatment of liver metastasis.

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