

Mechanism of metastasis by membrane type 1-matrix metalloproteinase in hepatocellular carcinoma

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Abstract

AIM: To investigate the precise role of membrane type 1-matrix metalloproteinase (MT1-MMP) in hepatocellular carcinoma (HCC) metastasis.

METHODS: Human HCC cells Hep3B with overexpression of MT1-MMP were established by stable transfection, and compared with control cells carrying the empty vector. Cells were examined *in vivo* for their differences in the metastatic ability of athymic nude mice, and analyzed *in vitro* for their differences in invasion ability by invasion chamber coated with Matrigel, adhesion towards collagen I and migration through culture chamber. Cell proliferation and apoptosis in adherent and suspension status were evaluated by MTT and flow cytometry analysis.

RESULTS: We found that overexpression of MT1-MMP could increase intrahepatic metastasis in nude mice with orthotopic implantation of HCC cells (incidence of 100% [MT1-MMP transfectants] vs 40% [vector control transfectants], $P < 0.05$). MT1-MMP could also enhance cell invasion through Matrigel (107.7 vs 39.3 cells/field, $P < 0.001$), adhesion towards matrix (0.30 vs 0.12 absorbance unit at 540 nm, $P < 0.001$), cell migration (89.3 vs 39.0 cells/field, $P < 0.001$), and cell proliferation (24.3 vs 40.5 h/doubling, $P < 0.001$). We also observed that MT1-MMP supported cell survival (71.4% vs 23.9%, $P < 0.001$) with reduced apoptosis (43.7% vs 51.0%, $P < 0.05$) in an attachment-free environment.

CONCLUSION: MT1-MMP overexpression could enhance metastasis. In addition to its active role in matrix degradation during tumor invasion, MT1-MMP enhances tumor cell survival upon challenge of detachment, which is important during metastasis when cells enter the circulation.

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Key words: MT1-MMP/MMP14; Liver cancer; Invasion; Metastasis

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INTRODUCTION

Membrane type 1-matrix metalloproteinase (MT1-MMP or MMP14) is the well-characterized transmembrane MMP. It triggers the activation cascade of other pro-MMPs via the activation of pro-MMP2^[1] and pro-MMP13^[2] resulting in the cleavage of a complex array of extracellular matrix proteins. MT1-MMP itself also aids in maintaining the homeostasis of extracellular matrix by regulating the turnover of types I-III collagens, fibronectin, vitronectin, laminin, fibrin, and proteoglycan^[3,4] for tissue remodeling and development. MT1-MMP knock-out mice show inadequate collagen turnover resulting in dwarfism, osteopenia, arthritis, connective tissue abnormalities, and early postnatal death^[5]. In addition, MT1-MMP deficiency causes impaired endochondral ossification and angiogenesis^[6].

MT1-MMP is involved in the progression of different malignancies^[7], and localizes in invadopodia of invading tumor cells tethering extracellular matrix breakdown at invasive front^[8,9]. Numerous studies support its role in cancer dissemination by demonstrating that MT1-MMP alters cell behaviors through proteolysis^[10-13]. All these point out that MT1-MMP is a versatile enzyme that contributes to invasion and metastasis through numerous mechanisms^[14].

Our recent study has revealed upregulation of MT1-MMP specifically in hepatocellular carcinoma (HCC) tissues with venous invasion ($P < 0.001$)^[15]. HCC is a hypervascular tumor, in which venous invasion is common and an important risk factor for tumor metastasis. Earlier studies of MT1-MMP focused on its increased expression in invasive HCC^[16-20], association with extracellular matrix remodeling^[21], poor prognosis^[19], and induction of its expression by HBV X protein^[22]. In this study, we demonstrated a novel role of MT1-MMP in metastasis, where cell survival in an attachment-deprived condition was maintained with reduced apoptosis. The current data indicate that MT1-MMP plays an important role in supporting cell survival when cells enter the circulation during metastasis, in addition to its prominent role in matrix degradation during tumor invasion.

MATERIALS AND METHODS

Construction of plasmid

Full-length human MT1-MMP cDNA was PCR-amplified

using the first-stranded cDNA reverse-transcribed from total RNA of normal liver according to the published sequence (GenBank accession number NM 004995) using specific primers (forward primer: 5'-GAGAT ATCAT GTCTC CCGCC CCAAG A-3'; reverse primer: 5'-CCTCT AGACA CCTGC TGCCA CTG-3') under the conditions: at 94 °C for 10 min; at 94 °C for 1 min, at 60 °C for 1 min, at 68 °C for 1 min for 30 cycles by Platinum® *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, CA, USA). Expression construct was made by cloning the PCR product into a mammalian expression vector, pcDNA3.0 (Invitrogen).

Cell culture and transfections

Human HCC cell line Hep3B purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in DMEM supplemented with 100 mL/L fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Constructs were transfected using the LipofectAMINE reagent (Invitrogen) according to the protocol suggested by the manufacturer. Stable transfectants were selected in DMEM complete medium with 400 µg/mL Geneticin (USB, Cleveland, OH, USA) for 3 wk. Hep3B cells stably transfected with empty pcDNA3.0 vector were used as control for all the experiments, in comparison with the MT1-MMP transfectants. Expression levels of MT1-MMP in transfectants were compared by semi-quantitative RT-PCR and Western blotting.

Intrahepatic tumor model

Male BALB/c *nu/nu* (nude) mice were obtained from the Charles River Laboratories (Wilmington, MA, USA) and the study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Hepatic tumor was generated by slow intrahepatic injection of 2×10^6 cells into the left liver lobe of a 7-wk-old mouse during laparotomy. Ten mice were injected for each transfectant and were killed 60 d after the operation. After macroscopic examination and measurement, livers, hearts, spleens, lungs, kidneys, and pancreas were harvested. Intrahepatic metastasis was defined macroscopically as nodules that were clearly distinguishable from primary tumors by naked eyes or microscopically as clusters of tumor cells with α -fetoprotein (AFP) expression confirmed by immunohistochemistry and hematoxylin and eosin staining on paralleled sections.

MT1-MMP RNA expression quantitation

MT1-MMP RNA expression level was assessed by semi-quantitative RT-PCR using MT1-MMP specific primers (forward primer as described above; reverse primer: 5'-AATGC TCGAG CCCC GGGCA-3') and control 18S rRNA (forward primer: 5'-CTCTT AGCTG AGTGT CCCGC-3' and reverse primer: 5'-CTGAT CGTCT TCGAA CCTCC-3'). PCR for MT1-MMP and 18S rRNA was first performed for 20-32 and 10-32 cycles, respectively. The PCR product was electrophoresed in agarose gel and the intensity was quantified by densitometric scanning using a gel-imaging system and UVP GelWorks ID Intermediate version 3.01 (Ultra Violet Products Ltd, Cambridge, UK). The number of cycles at linear increase of PCR product

was chosen for comparison. The relative MT1-MMP RNA level was determined as the MT1-MMP intensity normalized by the 18S rRNA intensity of respective samples.

Immunohistochemistry

For immunohistochemical staining, AFP polyclonal antibody (DAKO, Carpinteria, CA, USA) and Ki67 mAb (BD PharMingen, San Diego, CA, USA) were used at 1:3 000 and 1:100, respectively. Paraffin-embedded tissues were cut into 4-µm-thick sections, dewaxed, rehydrated in graded ethanol and antigen retrieved by boiling for 10 min in a citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by DAKO peroxidase block, followed by 30-min incubation with primary antibody. After being washed with PBS, localization of protein was detected using the DAKO Envision™+System. The tissue sections were counterstained with hematoxylin.

Protein extraction and Western blotting

Total protein was extracted with urea buffer (9 mol/L urea, 50 mmol/L Tris-Cl, pH 7.5) in the presence of Complete™ protease inhibitor cocktail (Roche, Mannheim, Germany). Twenty micrograms of total protein was analyzed with 2 µg/mL MT1-MMP polyclonal antibody (AGI, Sunnyvale, CA, USA).

Immunoreactive bands were visualized with the Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham Biosciences, Buckinghamshire, UK). β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) served as loading control. The reactive bands in the exposed films were quantified by densitometric scanning as described above. The relative amount of MT1-MMP level for the active form (62 ku) and the total MT1-MMP (summation of pro-form, 64 ku; active form, 62 ku; and cleaved forms, 20-43 ku) were calculated, respectively, after normalization by the actin reactive band.

Cell proliferation

Three thousand cells per well were seeded at the start of the experiment. Cell proliferation was assessed using conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) to formazan product at 24-h intervals. Briefly, 0.5 mg/mL MTT was added to the well for 4 h and terminated by adding 100 µL of 0.1 mol/L HCl/isopropanol. Absorbance was measured at 540 nm. Cell proliferation was also assessed by counting the total cell number. Briefly, 30 000 cells/well were seeded at the start of the experiment. The cells were trypsinized and counted at 24-h intervals for 5 d by the trypan blue exclusion method. The doubling time of the cells was assessed at the linear phase of the growth curve.

In vitro invasion assay

Fifty thousand cells were seeded in 500 µL DMEM in the upper compartment of the BD BioCoat™ Matrigel™ Invasion Chamber (Becton Dickinson, Bedford, MA, USA). The lower compartment was filled with 500 µL of DMEM/10% FBS. After 24 h, cells on the undersurface of the filters were fixed, stained with hematoxylin and eosin, and counted under a microscope at a magnification of $\times 200$

for 10 random fields.

Cell attachment assay

Fifty thousand cells were seeded in 100 μ L DMEM/10% FBS in collagen I-coated plates for 1 h. After extensive washes, adherent cells were fixed and stained with crystal violet in 10% ethanol. Following washing with PBS, the incorporated dye was extracted with a 1:1 mixture of 100 mmol/L sodium phosphate, pH 4.5 and 50% ethanol. Absorbance was read at 540 nm.

Cell migration assay

Fifty thousand cells were re-suspended in 0.5 mL DMEM and loaded into cell culture insert. The lower chamber was filled with 0.5 mL DMEM/10% FBS. After 24 h, cells at the membrane's undersurface were fixed, stained, and counted at a magnification of $\times 200$ for four microscopic fields.

Anoikis assay

Three million cells were held in suspension in poly-HEMA (Sigma-Aldrich)-coated petri dishes. At indicated time points, the cells were collected, centrifuged, and washed once in PBS. Cell clumps were dispersed by a 5-min treatment of 500 μ L 0.5 mmol/L EDTA, followed by an addition of 9.5 mL DMEM/10% FBS. Then, 100 μ L cells was seeded and the ability to convert MTT to formazan product was examined as described above for 3 h. The percentage of absorbance value divided by the absorbance value at 0 h after background correction was presented. Cells at indicated time points were also stained with propidium iodide for 15 min followed by an analysis using the flow cytometer (Becton Dickinson).

Statistical analysis

Each data point represented results from at least three independent experiments performed in duplicate and was presented as mean \pm SD. Comparison between groups was made with the Student's *t*-test and considered statistically significant if the *P* value was less than 0.05. Statistical analysis was carried out using the statistical software (SPSS version 9.0 for Windows; SPSS, Chicago, IL, USA).

RESULTS

Establishment of stable MT1-MMP overexpression transfectant

To examine the biological function of MT1-MMP, we first established a stable Hep3B transfectant that overexpressed MT1-MMP. Semi-quantitative RT-PCR (Figure 1A) confirmed that the MT1-MMP RNA transcript level in overexpressing transfectants increased by 2.9-fold compared to vector control transfectants, whereas the Western blot analysis (Figure 1B) showed that in MT1-MMP transfectants there was an 1.5-fold increase of active MT1-MMP and 2.0-fold increase of total reactive forms of MT1-MMP protein compared to the vector control. The higher levels of the 43 and 20 ku MT1-MMP, which are cleavage products generated by active MMP2, indirectly evidenced the increased active MMP2 generated by MT1-MMP in overexpression transfectants compared to the vector control^[23,24].

MT1-MMP increased metastasis and tumorigenicity

The stable transfectants were inoculated into the left liver lobe of the athymic nude mice to study the effect on metastasis (Figure 2). The intrahepatic tumors grew within the liver and the tumor nodules were physically close to each other. Physical separation of tumor and non-tumor liver tissues by detailed dissection for weight and volume measurement became infeasible, because this would ruin the subsequent histological analysis for tumor margin assessment of these nodules. Hence, the size was presented as the greatest dimension measured reflecting the weight and volume of the tumor mass. In 60 d, MT1-MMP overexpression resulted in a higher incidence of xenograft formation and a larger primary tumor (incidence of 80% and size of 2.0 ± 1.3 cm), as compared to the control group (incidence of 50% and size of 0.8 ± 0.5 cm; Table 1), supporting its role in enhancing tumorigenicity and

Table 1 Summary of *in vivo* tumorigenicity assay

	Primary tumor		Metastasis tumor
	Incidence	Size (cm)	
MT1-MMP	8/10	2.0 ± 1.3	8/8
Vector control	5/10	0.8 ± 0.5	2/5

MT1-MMP overexpression resulted in significantly larger primary tumor and frequent metastasis.

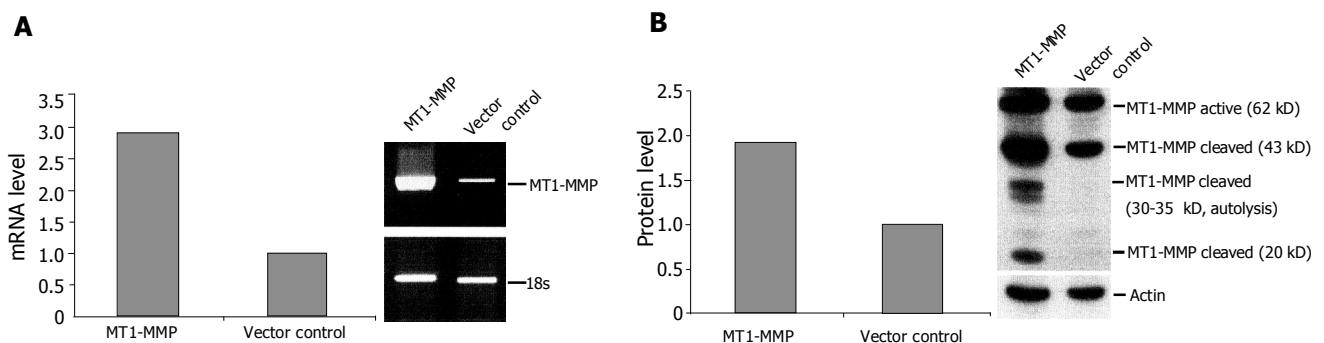


Figure 1 Establishment of stable MT1-MMP transfectants. **A:** RNA transcript levels were compared in linear phases by semi-quantitative RT-PCR after

normalization with 18S level; **B:** protein expression levels were compared by Western blotting analysis after normalization with actin level.

growth. The possibility of tumor implantation into the portal vein during the experiment was excluded, because portal vein injection resulted in multiple liver tumors (data not shown).

In the non-injection liver lobes, a higher incidence of variably sized tumor nodules was found in the MT1-MMP transfected group (Figure 2). Microscopic metastasis was further confirmed by AFP staining, as Hep3B cells expressed AFP but not the normal mouse hepatocytes. The microscopic metastases were counted by the presence

of clusters of AFP-positive cells, as the clinical significance of single solitary AFP-positive tumor cells was unknown. In general, more intrahepatic metastases were found in the MT1-MMP transfected group than in the vector control group (100% and 40%, respectively; Table 1). No AFP-producing cell was found in kidneys, lungs, spleens, hearts, and pancreas.

MT1-MMP enhanced tumor dissemination and growth

Enhanced metastasis *in vivo* further prompted us to look

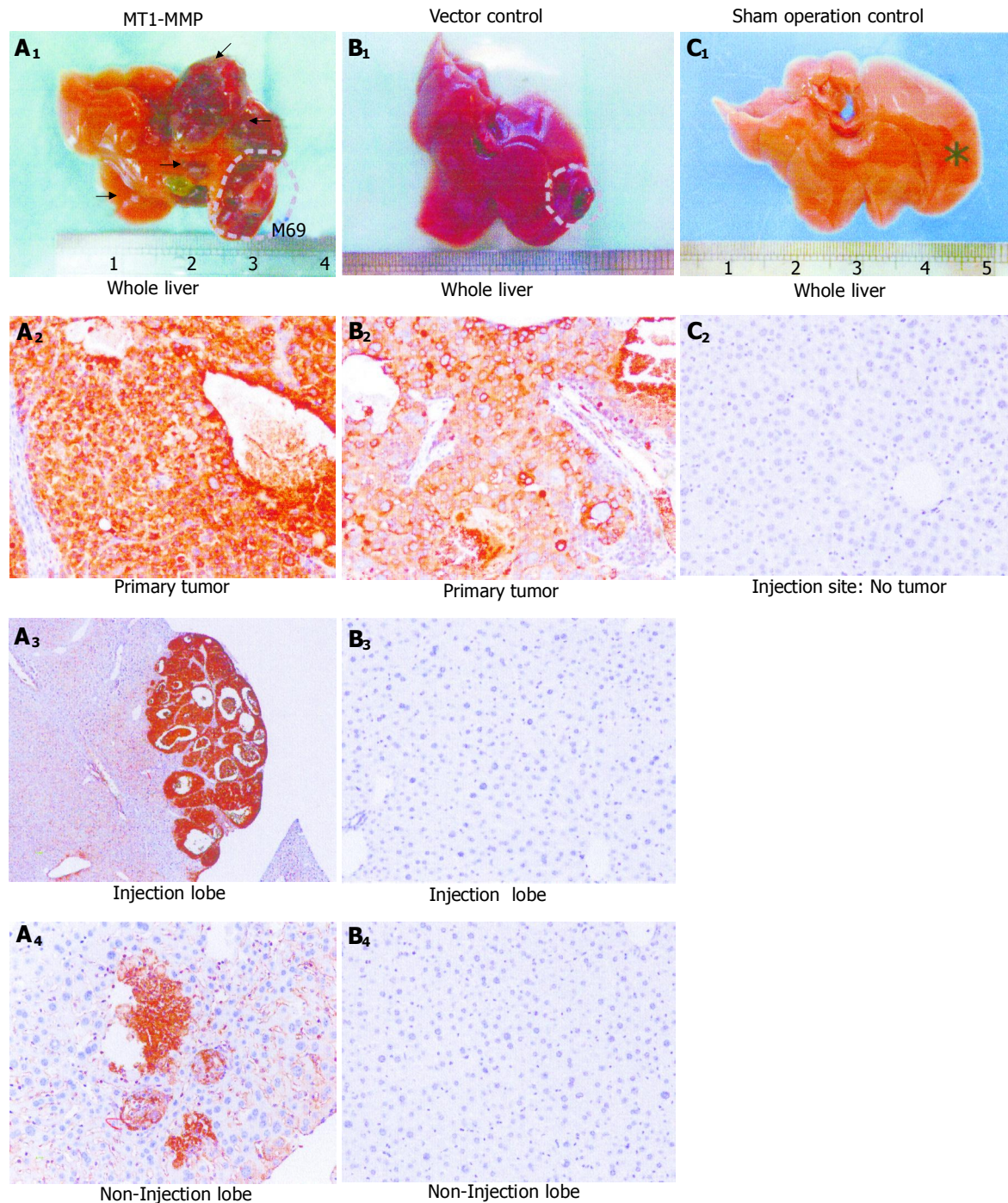


Figure 2 Tumorigenicity and metastasis *in vivo* enhanced by MT1-MMP. **A₁₋₄**: Large primary tumor nodule with prominent metastases produced by MT1-MMP overexpression; **B₁₋₄**: Small primary tumor nodule and rare metastasis produced by vector control transfectants; **C₁₋₂**: Tumor-free control liver injected with saline. Primary tumors, metastases, and injection site were indicated by dotted lines, arrows, and an asterisk, respectively. Sections from all tissues collected were then subjected to AFP staining.

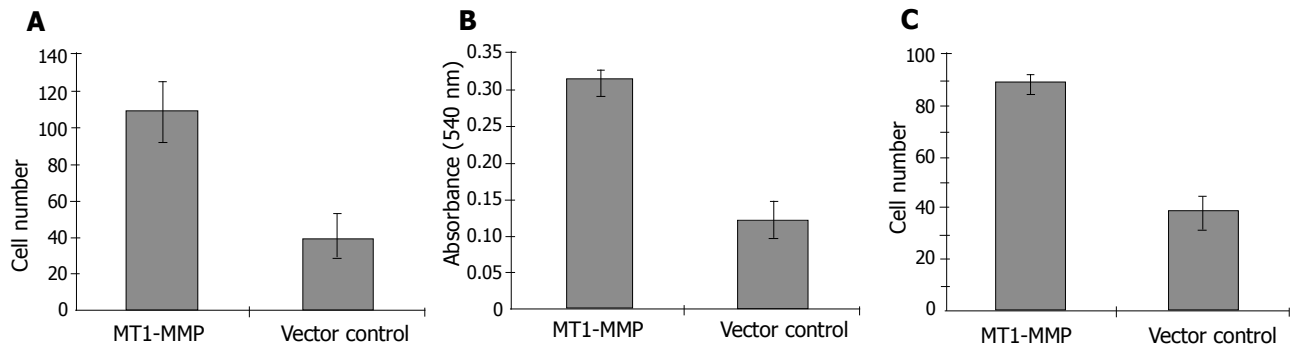


Figure 3 Cell invasion, adhesion efficiency, and cell motility *in vitro* enhanced by MT1-MMP. **A:** Cell invasive ability in standard Boyden chamber; **B:** Adhesion efficiency on collagen I; **C:** Directional migration ability by cell motility assay.

into the sequential steps of metastasis by *in vitro* assays. In a standard Boyden chamber assay, MT1-MMP transfectants significantly increased the number of cells that invaded through Matrigel-coated filters by 174.0% (107.7 ± 15.5 and 39.3 ± 12.1 cells/field, respectively; $P < 0.001$; Figure 3A). Furthermore, 150% more MT1-MMP transfectants adhered onto collagen I compared to the control within a fixed time (0.3 ± 0.02 and 0.12 ± 0.03 absorbance unit at 540 nm, respectively; $P < 0.001$; Figure 3B). When the transfectants were plated into plain transwells without a Matrigel layer, increased MT1-MMP enhanced cell migration by 129.0% (89.3 ± 6.7 and 39.0 ± 2.3 cells/field, respectively; $P < 0.001$; Figure 3C). Collectively, increased MT1-MMP facilitated cells in suspension to arrest onto collagen I (cell attachment to matrix during metastasis at the secondary site), enhanced their directional cell motility and thereby promoted invasion through matrix. This was particularly important during extravasation.

Increase in tumor size *in vivo* led us to examine the cell proliferation rate. Intense Ki67 staining was observed in MT1-MMP xenografts (Figure 4A) indicating MT1-MMP enhanced growth *in vivo*. Cell proliferation of transfectants in culture was monitored by the assessment of mitochondrial activity in MTT assay (Figure 4B) and counting the total cell number (Figure 4C) at 24-h intervals over 5 d, and both assay methods demonstrated that MT1-MMP promoted cell proliferation *in vitro*. The doubling time of MT1-MMP overexpressing transfectants and control transfectants was 24.3 and 40.5 h, respectively. Taken together, MT1-MMP increased cell proliferation *in vivo* and *in vitro*.

MT1-MMP reduced anoikis

Adherent cells undergo apoptosis shortly after loss of cell-substratum adhesion, a phenomenon known as

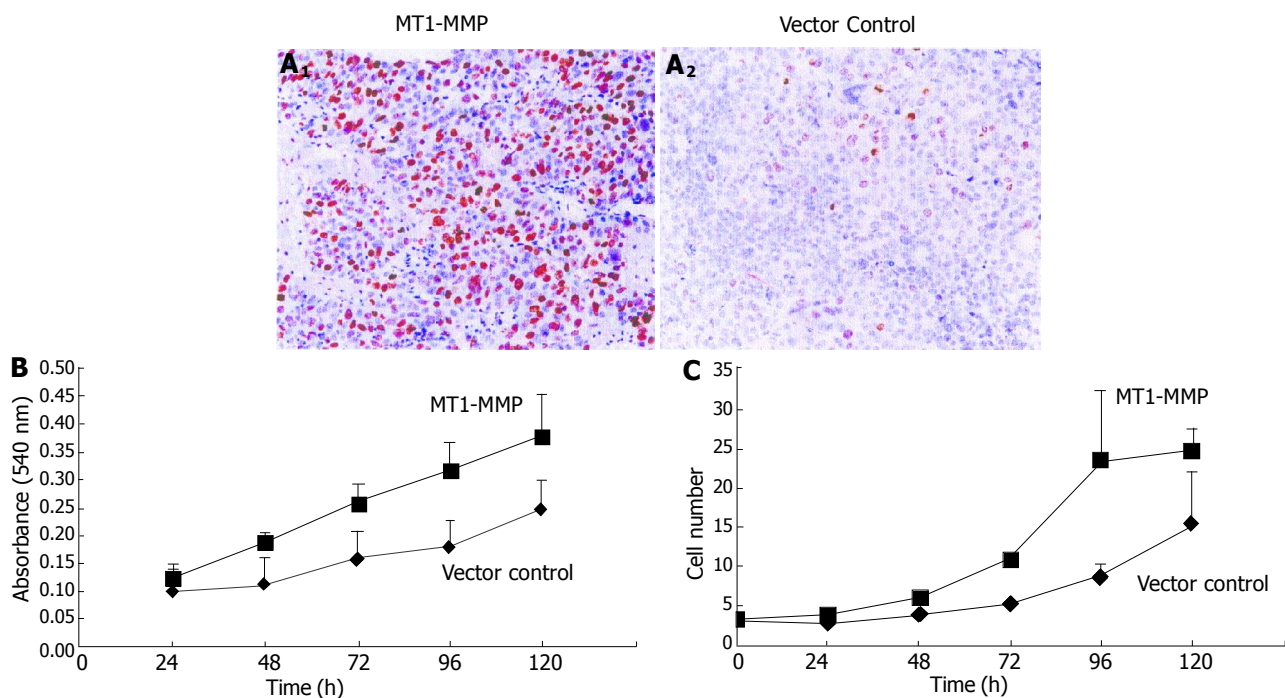


Figure 4 Cell proliferation *in vivo* and *in vitro* enhanced by MT1-MMP. **A_{1,2}:** Primary tumors in athymic nude mice examined by Ki67 staining; **B:** Cells examined by MTT assay; **C:** Cell number counted at regular time intervals.

“anoikis”. Cell viability under attachment-deprived conditions is critical after the cells enter the circulation during the process of metastasis. To examine the cell viability under attachment-deprived conditions, the transfectants were prepared as single cell suspension, challenged by anoikis by plating out in attachment-deprived dishes and examined by MTT assay (Figure 5). As reflected by the conversion of MTT to its substrate, MT1-MMP transfectants maintained a higher metabolism than the vector control during the 24 h detachment. By 24 h in an attachment-free environment, MT1-MMP transfectants maintained 71.4% metabolic activity, whereas the metabolic activity of control transfectants declined to only 23.9%.

To evaluate if the MT1-MMP level plays a role in anoikis, the two transfectants at 0 and 24 h after detachment were subjected to propidium iodide staining and then analyzed by the flow cytometer. Difference in detachment-induced death was observed between the MT1-MMP and vector control transfectants at 24 h ($43.7 \pm 2.0\%$ vs $51.0 \pm 4.1\%$, respectively; $P < 0.05$). In summary, MT1-MMP enhanced cell survival with reduced apoptosis upon detachment challenge.

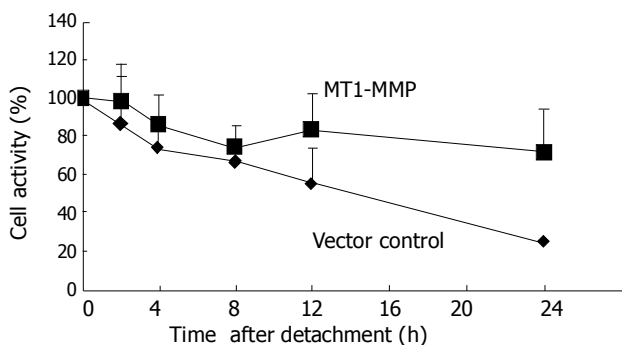


Figure 5 MT1-MMP delayed anoikis. The cell metabolic activity of two transfectants after detachment was compared.

DISCUSSION

MT1-MMP is crucial in HCC progression^[15,17,20-22]. Here, we have demonstrated that MT1-MMP protected tumor cells from anoikis, in addition to its classical roles in tumor invasion to pave way for tumor cells to migrate in circulation for distal metastasis after crossing the extracellular matrix and basement membrane. Such a finding is important because even when large number of tumor cells enter the circulation successfully, only a small percentage of tumor cells can actually metastasize due to “metastatic inefficiency”^[25-28], the failure to establish metastasis. Only if tumor cells have sustained survival in circulation, the attachment-deprived environment, there would be successful metastasis. Thus, the protection from anoikis contributed by MT1-MMP in the first 24 h upon detachment is of paramount importance during HCC invasion and metastasis.

Increased intrahepatic metastasis by MT1-MMP was observed in the spontaneous metastasis assay performed *in vivo*. *In vitro*, MT1-MMP was also shown to enhance

invasion through matrix, adhesion to collagen I and in directional cell migration. Collagen I is the most abundant extracellular matrix component in liver, especially in cirrhotic liver. Enhanced cell attachment to collagen I was observed in the MT1-MMP transfectants in suspension status. Thus, MT1-MMP could also facilitate cell attachment at a new site for metastasis, aside from its leading role in matrix degradation and enhanced cell migration ability for cell invasion to take place to leave the primary site.

Apoptosis is the “rate-determining” step subsequent to the cells entering the circulation. Others have reported that inhibition of apoptosis in the first 24 h could already enhance lung metastasis^[25,29,30]. In the current study, MT1-MMP expression conferred a survival advantage in the attachment-deprived environment. Notably, MT1-MMP expression also contributed to evasion of apoptosis in suspension status, although the percentage difference was not enormous but statistically significant. The significant gain in survival advantage but marginal improvement in evasion of apoptosis by MT1-MMP overexpression during suspension status could be due to a number of reasons. The remaining viable control cells became dormant (or less active in converting MTT to a formazan product) in adverse conditions, whereas the viable MT1-MMP transfectant was able to withstand the stress and maintained the cell metabolism. Therefore, even when a similar proportion of cells died, a considerable difference in cell metabolism between two transfectants was observed. The higher cell metabolism of MT1-MMP transfectants in suspension culture also suggests that MT1-MMP may still underpin proliferation and, therefore, promotes the selection for a population of cells that are more tumorigenic and resistant to anoikis. Although other factors like immune attack and mechanical shear force from circulation were not taken into account in our experimental settings, cell survival upon detachment could definitely enhance metastatic potential. Further, experiments on proliferation and apoptosis are needed to confirm if proliferation occurs during suspension.

Potential mechanisms exist for the contribution of MT1-MMP to survival advantage during cell suspension status. Indirect links might be mediated through its enzymatic activity. MT1-MMP may alter processing of proteins and thus overrides the apoptotic stimuli during the challenge of detachment. For instance, MT1-MMP modulation of integrin processing and downstream regulation of FAK signaling pathways^[31] are related to apoptotic pathways. During osteoblast transdifferentiation, MT1-MMP activates latent transforming growth factor- β and maintains osteoblast survival^[32,33]. In support of this view, MMP1 degradation of type I collagen has been shown to expose new cryptic domains that bind to the $\alpha\beta3$ integrin and trigger survival signals in melanoma cells^[34]. On the other hand, MT1-MMP might directly link to various pathways^[12,31,35,36] leading to distinct biological outcome in a number of recent studies^[36-38]. The localization of MT1-MMP in clathrin-coated pits^[39,40] and caveolae^[41-44], which are rich in signaling molecules^[45], also raises the possibility of direct activation of signal transduction pathways by MT1-MMP. Therefore, it is conceivable that MT1-MMP can contribute to the activation

of intracellular pathways.

From therapeutic perspectives, total elimination of MMP protease function by inhibitors is impossible and would cause a number of side effects. Nevertheless, the MMP-related subject is promising in the field of tumor detection, prognosis, prevention, and therapeutic purposes in defined situations^[46,47]. Our findings have a direct impact on cancer therapy because molecular understanding of the biological activities mediated by proteases provides exciting opportunities for specific interventions in pathways critical for tumor progression.

In conclusion, MT1-MMP promotes HCC tumorigenicity and intrahepatic metastasis with increased tumor growth and invasion ability. More importantly, increased MT1-MMP expression reveals survival advantage and protects HCC cells from apoptosis in the first 24 h in the attachment-free condition. These results strengthen the emerging opinion that the role of MT1-MMP does not only confine to matrix degradation, but also extends to alteration of cell behaviors including survival advantage in the attachment-deprived environment.

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