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Original Article

Membrane type 1 matrix metalloproteinase expression correlated with the clinicopathological feature and invasion and metastasis in oral squamous cell carcinoma

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Abstract: In the current study, we intended to analyze the expression of membrane type 1 matrix metalloproteinase (MT1-MMP) in oral squamous cell carcinoma (OSCC) and its correlations with clinicopathologic characteristic, and to further identify the function of MT1-MMP in promoting cancer invasion from the point of view of the extracellular matrix degradation. We collected 122 cases of OSCC tissue specimens from patients. Test results were correlated with clinical and pathological parameters. The effects of MT1-MMP in oral cancer cells on the regulation of associated MMPs expression and the invasive and migratory abilities were analyzed. Our results demonstrated that the high expression of MT1-MMP in tumors significantly correlated with the lymph node metastasis, pathological grade, recurrence and follow-up, and high expression of MT1-MMP at the invasive front of tumor significantly correlated with the lymph node metastasis. Furthermore, the patients with higher expression of MT1-MMP had shorter survival time in OSCC. Multivariate analysis indicated that MT1-MMP expression could serve as an independent prognostic indicator for the survival of patients with OSCC. In addition, MT1-MMP, through regulating associated MMPs expression, contributes to promoting the invasion and metastasis in oral cancer cells. In conclusion, MT1-MMP may play an important role in OSCC invasion and metastasis and could act as a promising target for prognostic prediction.

Keywords: Membrane type 1 matrix metalloproteinase, oral squamous cell carcinoma, prognosis

Introduction

Much effort has been devoted to suggest a key determinant for cellular components in the development and progression of carcinoma [1-3]. However, recent studies have also highlighted the importance of noncellular components during cancer progression, especially the extracellular matrix (ECM) [4-7]. ECM has been known as a major component of the local microenvironment in which cancer cell plays important roles in cancer development, invasion and metastases [8]. ECM can directly or indirectly influence almost all cellular behaviors so that affects cancer progression, thus promotes formation of a tumor microenvironment [8, 9]. The mechanism of ECM function for this regulation of cellular behaviors is dependent on the remolding of ECM structure. Matrix metalloproteinases (MMPs), as key components of the ECM, are the most significant enzymes in ECM degradation and remodeling [9-11].

Membrane type 1-MMP (MT1-MMP), a member of the MMPs, has a potential transmembrane domain and a short cytoplasmic tail [12, 13]. The expression and activity of MT1-MMP in tumors implicated that MT1-MMP as a membrane-bound proteinase was associated with the ECM-induced cancer invasion and metastasis [11, 14-16]. MT1-MMP can directly degrade a number of ECM molecules such as collagen types I, III, IV, fibronectin, laminin and vitronectin as well as active MMP-2 [12], MMP-13 to their active form [12, 17, 18], which in turn activate pro-MMP-9. MMP-2 (gelatinase-A) is secreted proteinase produced by tissues as inactive form which plays a significant role in con-

tributing to the invasion and metastases of malignant tumor by degrading the elements of ECM and basement membrane (BM) [18-24]. Previous findings have revealed that MT1-MMP expression associated with clinical pathological parameters in various cancer, including breast cancer, lung cancer, cervical cancer, colon cancer and prostate cancer [19-23]. MT1-MMP has been reported to serve as a clinical prognostic factor in cervical carcinoma, breast cancer, non-small cell lung cancer and gastric cancer [20, 23-25]. However, the expression of MT1-MMP correlating with the clinicopathological characteristics and prognoses of OSCC patients has not been clarified clearly. Our previous research demonstrated that MT1-MMP plays a crucial role in inducing an epithelial to mesenchymal transition (EMT) in OSCC, and thus promoting cancer cell invasion and metastasis [26]. However, the role of MT1-MMP in mediating oral cancer cell invasion through ECM degradation has not been reported yet.

In the current study, we intended to analyze the expression of MT1-MMP in OSCC and its correlation with clinicopathologic characteristics, and then to further identify the function of MT1-MMP in promoting cancer invasion from the point of view of the ECM degradation. These aspects of MT1-MMP function in cancer invasion and metastasis are giving our approaches to a better understanding of cancer therapy and prognosis strategies to OSCC.

Materials and methods

Cell culture

Human oral squamous cell carcinoma SCC9 and SCC25 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's medium and Ham's F12 medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 400 ng/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Invitrogen).

Transient and stable cell transfections

The cDNA expression vector for GFP-tagged MT1-MMP was constructed. Expression plasmids were transfected into cells using lipofectamine 2000 (Invitrogen). For transfection experiments, cells were cultured in six-well plates (Corning, Lowell, MA, USA) to 80% confluence. The stable overexpression of MT1-MMP in SCC9 cells were obtained from the experiment before [26]. Four lentivirus-miRNA interference vectors and one negative control vector were constructed and we performed real-time RT-PCR to detect and select the interfere efficiency (data not shown). The most effective and specific lentivirus-miRNA interference vector was transfected to SCC25 cells (SCC25-miM), the same vector with a scrambled miRNA sequences was transfected to SCC25 cells (SCC25-mock) as experimental control.

Western blot

To investigate the proteins activities, the cells were grown to 90% confluence in 25 cm^2 culture-flasks (Corning) and washed with phosphate-buffered saline (PBS), lysed with a RIPA lysis buffer (Beyotime, Shanghai, China), and then subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were blocked for 2 h at room temperature with 5% nonfat milk in PBST. The membranes were incubated with the primary antibodies: anti-β-actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MT1-MMP (1:500, Abcam Cambridge MA, USA), anti-MMP-9 (1:200, Bioworld technology, USA), anti-MMP-13 (1:500, Bioworld technology), anti-MMP-2 (1:200, Proteinetch Group, Chicago IL, USA). β-actin was used as internal control.

Real-time RT-PCR

Total RNA was extracted from cancer cells with the Trizol reagent (Invitrogen). The purified total RNA was then reverse-transcribed into cDNA using the PrimeScript™ RT Master Mix (Takara, Otsu, Shiga, Japan) at 37°C for 15 min for reverse-transcription and 85°C for 5 s for heat inactivation of reverse transcriptase. The expression levels of human MT1-MMP, MMP-2, MMP-9, MMP-13 were detected by real-time quantitative PCR using SYBR Premix Ex Taq (Takara) with the 7300 ABI Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used to the real time RT-PCR. GAPDH (5'-GAAGGTGAAGGTCGGAGTC-3', 5'-GAGATGGTGATGGGATTTC-3'), MT1-MMP (5'-GGAACCCTGTAGCTTTGTGTCTGT-

C-3', 5'-TGAGGGTCCTGCCTTCAAGTG-3'), MMP-2 (5'-CTCATCGCAGATGCCTGGAA-3', 5'-CAGCCTAGCCAGTCGGATTTG-3'), MMP-9 (5'-ACGCACGACGTCTTCCAGTA-3', 5'-CCACCTGGTTCAACTCACTCC-3'), MMP-13 (5'-CCCTTGATGCCATTACCAGTC-3', 5'-TCCGCATCAACCTGCTGAG-3'). The reaction conditions were followed by the manufacture's protocol: initial denaturation at 95°C for 30 s, and then run for 40 cycles of 95°C for 5 s, 60°C for 31 s. The data were analyzed using the 2 (-ΔΔCT) methods. The expression levels of GAPDH mRNA in each sample were used as controls.

Immunofluorescence assay

Cells grown on glass coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature, and blocked with goat serum albumin for 30 min 37°C, followed by reacted with indicated antibodies overnight at 4°C: fibronectin (1:50) (Sigma-Aldrich), MT1-MMP (1:50) (Abcam). The appropriate secondary antibodies (diluted 1:25) were then used, and the nuclei were stained by 4, 6-diamidino-2-phenylindole (DAPI, 1:1000, Beyotime) for 20 s. Negative controls without primary antibodies were also included; no obvious fluorescence was observed. Samples were observed by Zeiss LSM-710 laser-scanning confocal microscopy and image-captured.

Transwell assay

The polycarbonate filters (8 μm pore size, costar, Lowell, MA, USA) were pre-coated with Matrigel Basement Membrane Matrix (BD Biosciences, MA, USA) diluted with serum-free medium (1:7) for 30 min at 37°C. The cells (3×10^4) were resuspended with 200 μl serum-free medium inoculated in the upper chamber and then 500 μl medium containing 10% FBS was placed in the lower chamber. The plates were placed at 37°C in 5% CO_2 for 16 h. Then the chambers were fixed with 4% PFA and stained with 0.1% crystal violet (Beyotime) for 20 min. Cotton tips were used to remove the cells remained in the matrigel or attached to the upper side of the filter. The non-migratory cells were removed, and the migratory cells were counted as those presenting on the lower surface of the upper chamber. Images of randomly ten fields per chamber were viewed at 100× magnification (Olympus).

Wound healing migration assay

The wound healing migration assay was used to evaluate the effect of MT1-MMP on cell migration. The cells were allowed to grow to 90% confluence and then the cell wounded by scratching with a pipette tip in the suitable area, and then washed by PBS to remove the floating cells and debris. The cells were allowed to grow and close the wound. Photographs were taken at the same position of the wound at the indicated time points.

Flow cytometry

The effect of MT1-MMP on the ability of OSCC to proliferation was demonstrated by flow cytometry. The cells were synchronized with serum-free medium for 12 h. Then cells were treated with trypsin and resuspended as single-cell, washed three times with PBS, and then resuspended in 75% alcohol. For proliferation assay, the prepared cells were stained with 100 mg/ml of propidium iodide (BD Pharmingen, San Jose, CA, USA) and analysed using a BD FACS Calibur (BD Biosciences) and Cell Quest Pro software (BD Biosciences).

Patients and tissue specimens

A total of 122 cases of OSCC tissue specimens from patients (August 2007-March 2013) diagnosed and treated at the Department of Oral Pathology and the Department of Oral Maxillofacial Surgery, Affiliated Hospital of Stomatology, Nanjing Medical University. A total of 72 patients were men and 50 patients were women. The mean age was 61 years (range 34-88 years). None of the patient underwent chemotherapy or radiotherapy before surgery. As a result, the primary tumors were located in the tongue (n=49), gingiva (n=24), buccal mucosa (n=31), palate (n=5), lower lip (n=4), jaw (n=4) and others (n=5). The Tumor-Nodes-Metastasis classification and clinical staging were classified according to the International Union Against Cancer (UICC). The pathological grade was classified into groups according to the WHO classification. The patients were followed up for 2 to 91 months with a median of 39 months. The study was approved by the

Table 1. Relationship between MT1-MMP expression levels of tumors and clinical variables

Variable	No.	MT1-MMP			MT1-MMP (invasive front)		
		N+L	H	P	N+L	H	P
Sex				0.814			0.502
Male	72	39	33		15	57	
Female	50	26	24		8	42	
Age (years)				0.203			0.140
≤50	23	15	8		7	16	
>50	99	50	49		16	83	
Tumor location				0.256			0.300
Tongue	49	27	22		11	38	
Gingiva	24	13	11		2	22	
Buccal mucosa	31	12	19		5	26	
Palate	5	3	2		1	4	
Lower Lip	4	4	0		2	2	
Jaw	4	2	2		0	4	
Others	5	4	1		2	3	
Tumor size				0.318			0.656
T1	21	8	13		3	18	
T2	66	37	29		13	53	
T3	25	16	9		5	20	
T4	10	4	6		2	8	
Lymph node metastasis				**0.014**			**0.001**
N0	72	45	27		21	51	
N (+)	50	20	30		2	48	
Metastasis				0.216			1.000
M0	120	65	55		23	97	
M (+)	2	0	2		0	2	
Clinical stage				0.087			0.134
I	12	6	6		3	9	
II	42	26	16		10	32	
III	32	20	12		6	26	
IV	36	13	23		4	32	
Pathological grade				**0.048**			0.280
I	75	44	31		16	59	
II	39	21	18		7	32	
III	8	0	8		0	8	
Local invasion				0.446			0.467
No	88	45	43		18	70	
Yes	34	20	14		5	29	
Recurrence				**0.005**			0.056
No	80	50	30		19	61	
Yes	42	15	27		4	38	
Follow-up				**0.002**			0.093
Live	89	55	34		20	69	
Dead	33	10	23		3	30	

No., Number of patients; N, Negative; L, Low expression; H, High expression. N0, No lymph node metastasis; N (+), Node metastasis. M0, No metastasis; M (+), Metastasis. Bold values signify P<0.05.

Ethics Committee of the Nanjing Medical University.

Immunohistochemical (IHC) staining

The formalin-fixed, Paraffin-embedded specimens were cut at 4 μm interval, heated for 3 h at 70°C, deparaffinized in xylene, and rehydrated in ethanol series. Antigen retrieval was carried out with microwave heating in citrate retrieval buffer (PH 6.0) for 20 min, and then washed three times with PBS for 3 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature followed by washing with PBS plus 0.1% Tween 20. The slides were incubated with primary monoclonal anti-human MT1-MMP (1:200, Abcam) antibodies at 4°C overnight. After washing with PBS buffer, sections were incubated with the streptavidin-conjugated horseradish peroxidase (HRP) (MaxVision, Fuzhou, China) for 15 min. Finally, the reaction complexes were viewed using a diaminobenzidine kit (Maixin Biotechnology, Fuzhou, China). PBS was used in all negative controls of IHC.

Evaluation of IHC

The results of the IHC stained sections were calculated by two independent pathologists blinded to the clinical information of the patients. Each slice was randomly selected from 10 high visual field, the positive cells were observed and counted the cell percentage, as well as the cells staining intensity. The immunoreactive score of the cells

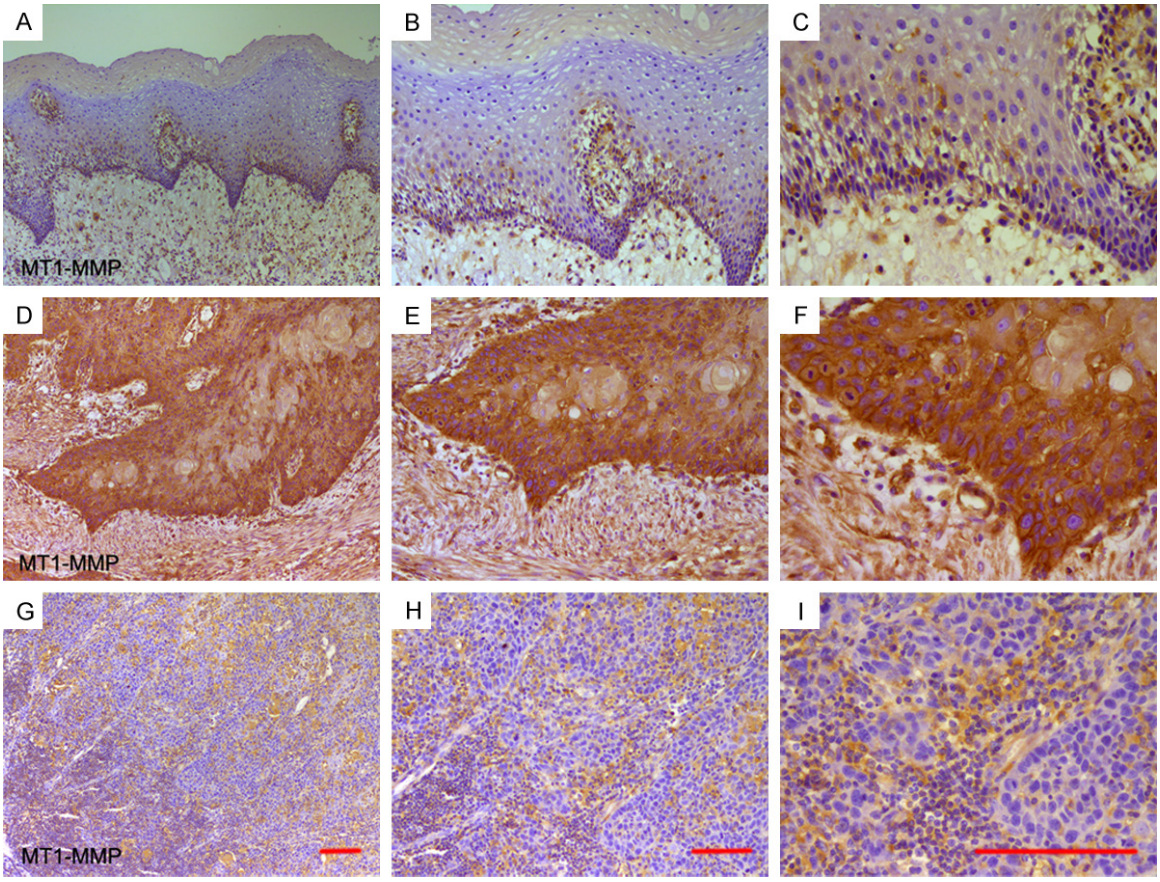

Figure 1. MT1-MMP expression was examined in the normal oral mucosa and in oral squamous cell carcinoma (OSCC) specimens by immunohistochemistry. (A-C) Negative staining of MT1-MMP was observed in the normal mucosa. The strong positive expression of MT1-MMP (D-F) was observed in tumor cells. The low expression of MT1-MMP (G-I) was found in tumor cells. Bar, 100 μm.

were calculated as intensity score × proportion score as we reported previously. Intensity score was defined as: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion score was defined as: 0, negative; 1, <10%; 2, 11-50%; 3, 51-80%; and 4, >80% positive cells. Immunoreactivity was divided into two expression groups based on the final score: 0-4, low expression; and 4-12, high expression (The final score: 0, negative only 2 cases <5).

Statistical analysis

Statistical analyses were performed using the SPSS statistical software (for Windows, version 16.0). The various clinical characteristics and the expression of MT1-MMP were assessed with the chi-square test, Fisher's exact test, Cochran-Mantel-Haenszel chi-squared test. The overall survival rate was analyzed using Kaplan-Meier method and compared with the log-rank test. The univariate and multivariate Cox proportional hazard regression model were used to analyze the independent prognostic factors. *P*-value (two sided) less than 0.05 were considered statistically significant.

Results

MT1-MMP expression in OSCC patients and the correlation of the clinicopathological parameters with the expression levels of MT1-MMP

In order to understand the clinical significance of MT1-MMP in OSCC patients' prognosis, the correlations between MT1-MMP and clinical pathological parameters were evaluated. The clinical pathological characteristic (age, gender, tumor location, tumor size, lymph node metastasis, metastasis, clinical stage, pathological grade, recurrence, follow-up) of these patients are analyzed and summarized in **Table 1**. In the tumor nest, 57 out of 122 (47%) tissue samples

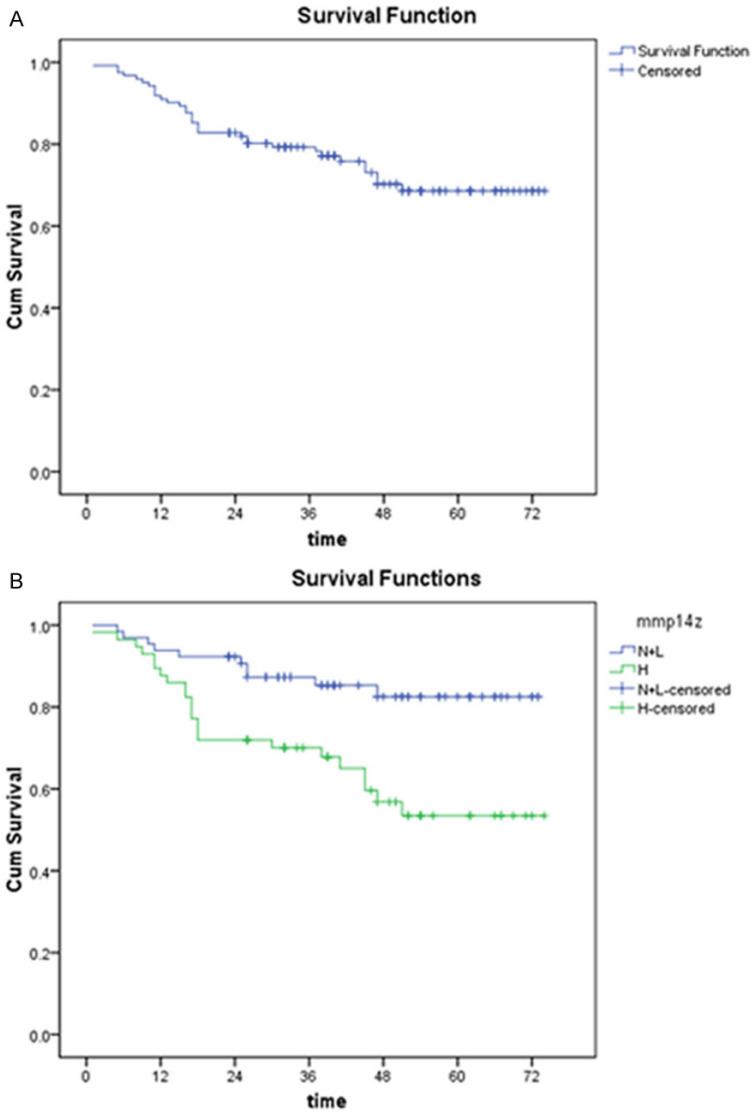

Figure 2. Survival curves of 122 OSCC patients. A. Overall survival curves of patients for 122 OSCC patients. B. Patients with high expression of MT1-MMP have a significantly poorer survival rate than patients with negative and low MT1-MMP expression (log-rank test, P<0.05). N: negative; L: low expression; H: high expression.

showed high expression of MT1-MMP, which significantly correlated with the lymph node metastasis (P=0.014), pathological grade (P=0.048), recurrence (P=0.005) and follow-up (P=0.002). Of the 122 cases, 99 (81%) showed high expression of MT1-MMP at the invasive front of tumor, which significantly correlated with the lymph node metastasis (P=0.001). Representative labeling of MT1-MMP in normal mucosa and OSCC is shown in **Figure 1**. The IHC results exhibited that negative staining of MT1-MMP was observed in the normal mucosa. However, MT1-MMP exhibited strong positive expression and localized in the membrane and the cytoplasm of tumor cells in OSCC, otherwise MT1-MMP exhibited low expression in tumor cells.

Correlation between the expression of MT1-MMP and survival analysis

At the end of the follow-up period, 89 of the 122 patients (73%) were alive and disease-free, 33 (27%) had died due to metastases, recurrence or other unrelated diseases; 42 (34.4%) had recurrence, and 50 (41%) lymph nodal metastases. The Kaplan-Meier survival analysis by log-rank test indicated that patients with high expression of MT1-MMP in OSCC was significantly associated with shorter overall survival (P=0.005). And the 3-year survival rate was 77.95% and 5-year survival rate was 70.96% (**Figure 2**). The univariate and multivariate survival analyses (Cox proportional hazards regression model) were performed to further determine the independent prognostic factors for OSCC patients. In the univariate survival analysis, MT1-MMP expression was significantly associated with overall survival [hazard ratio (HR)= 2.924, P=0.005 for MT1-MMP] (**Table 2**). Furthermore, the multivariate survival analysis was employed to estimate the independent prognostic factors, whereas MT1-MMP was identified as an independent prognostic markers for the overall survival of OSCC patients (HR=2.575, P=0.038) (**Table 3**).

Cancer cell MT1-MMP expression was associated with the expression of MMP-2, MMP-9 and MMP-13

To further determine the role of MT1-MMP in oral cancer cells, the most effective lentivirus-

Table 2. Univariate Cox regression analysis of overall survival

Covariate	*P*-value	Risk ratio	95% CI
Sex (male, female)	0.274	1.464	(0.780, 2.148)
Age (≤50 or younger, >50)	0.131	2.498	(1.310, 3.686)
Tumor location Post1	0.802	1.125	(0.206, 2.044)
Tumor location Post2	0.517	1.314	(0.489, 2.139)
Tumor location Post3	0.885	0.861	(-1.173, 2.895)
Tumor location Post4	0.983	0.000	(-1235.653, 1235.653)
Tumor location Post5	0.340	2.068	(0.576, 3.560)
Tumor location Post6	0.981	0.000	(-1081.610, 1081.610)
Tumor size (T1-T4)	0.243	1.273	(0.867, 1.679)
Nodal metastasis (N0, N (+))	0.307	1.428	(0.746, 2.110)
metastasis (M0, M (+))	0.561	0.048	(-10.201, 10.297)
Clinical stage (I, II, III, IV)	0.400	1.162	(0.813, 1.511)
Pathological grade (I, II, III)	0.736	0.888	(0.200, 1.576)
MT1-MMP in tumor expression (Low, High)	**0.005**	2.924	(2.181, 3.667)

Bold values signify P<0.05.

Table 3. Multivariate Cox regression analysis of overall survival

Covariate	*P*-value	Risk ratio	95% CI
Sex (male, female)	0.057	2.210	(1.393, 3.027)
Age (≤50 or younger, >50)	0.293	2.068	(0.716, 3.420)
Tumor location Post1	0.644	0.763	(-0.388, 1.914)
Tumor location Post2	0.213	1.779	(0.873, 2.685)
Tumor location Post3	0.935	0.916	(-1.189, 3.021)
Tumor location Post4	0.985	0.000	(-1391.818, 1391.818)
Tumor location Post5	0.672	1.571	(-0.522, 3.664)
Tumor location Post6	0.985	0.000	(-1310.411, 1310.411)
Tumor size (T1-T4)	0.161	1.523	(0.935, 2.111)
Nodal metastasis (N0, N (+))	0.962	1.030	(-0.160, 2.220)
metastasis (M0, M (+))	0.984	0.000	(-1490.292, 1490.292)
Clinical stage (I, II, III, IV)	0.430	0.756	(0.062, 1.450)
Pathological grade (I, II, III)	0.025	1.755	(1.261, 2.249)
MT1-MMP in tumor expression (Low, High)	**0.038**	2.575	(1.679, 3.470)

Bold values signify P<0.05.

miRNA interference vector was transfected to down-regulate MT1-MMP expression in SCC25 cells. Human oral squamous cell carcinoma SCC25 cells are more aggressive, which correlate with a higher expression of endogenous MT1-MMP than observed in less aggressive SCC9 cells (**Figure 3A**). We used the same vector with a scrambled miRNA sequences to transfect SCC25 cells (SCC25-mock) as an experiment control. The transfected SCC25 cells expressed green fluorescent protein (GFP) can be viewed under fluorescence microscope (**Figure 3B**). Furthermore, the down-regulation of MT1-MMP in SCC25 cells was also confirmed by real-time RT-PCR (**Figure 3C**) and western blot (**Figure 3D**). The immunofluorescence staining exhibited the down-regulation of MT1-MMP in SCC25 cells by miRNA interference vector (**Figure 3E**).

MT1-MMP was recognized as an activator of pro-MMP-2 and pro-MMP-13, which in turn activated pro-MMP-9 [27]. To identify whether MT1-MMP was correlated with activating of MMPs in oral cancer cells, the expressions of genes were detected using real-time RT-PCR. The quantitative determination of the mRNA expression in SCC25 cells revealed that down-

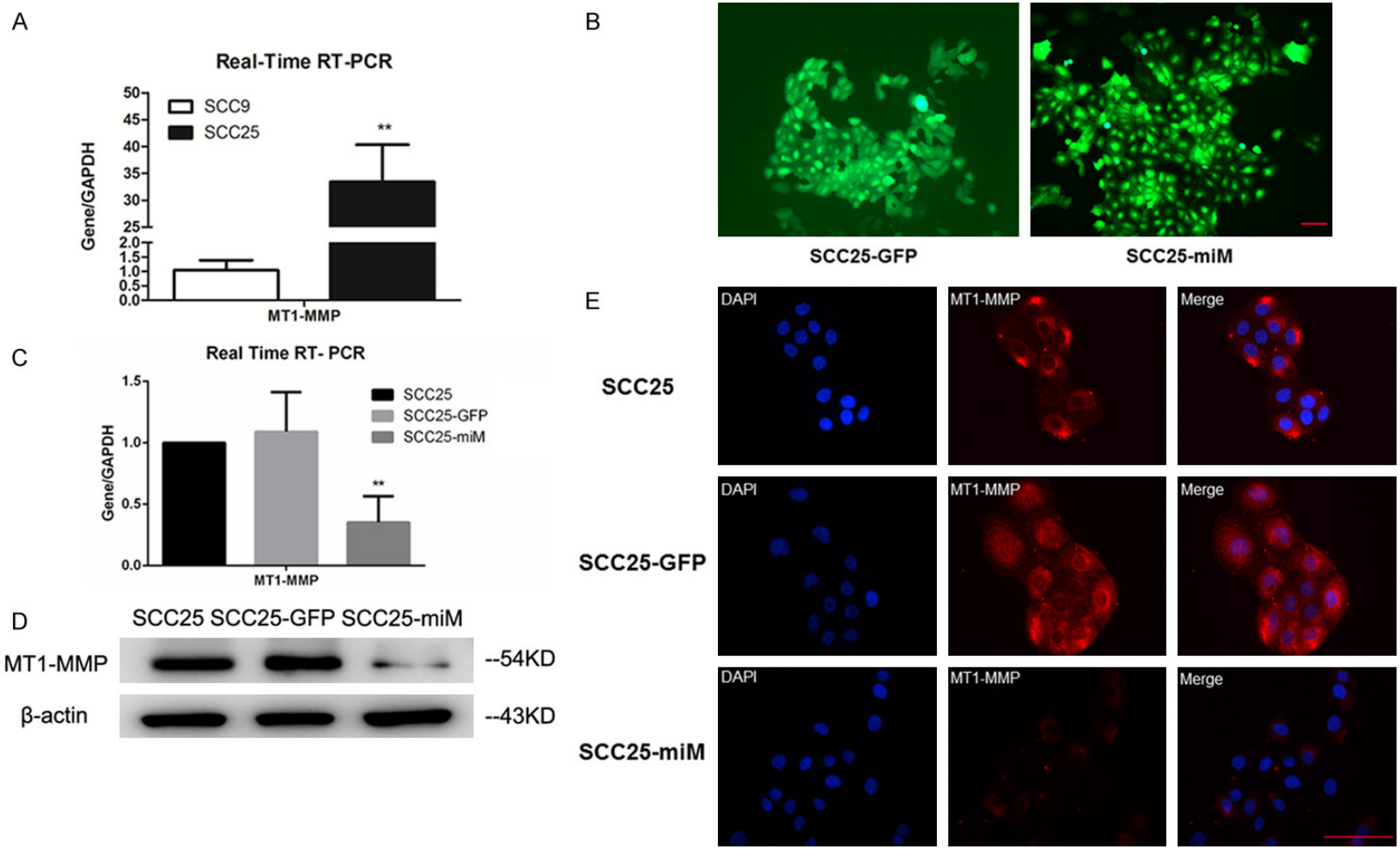

Figure 3. Downregulation of MT1-MMP in SCC25 cells. A. The Real-Time RT-PCR results showed that the SCC25 cells have a higher expression of endogenous MT1-MMP than observed in SCC9 cells. GAPDH was used as a control. **P<0.01. B. The transfected SCC25 cells expressed green fluorescent protein (GFP) can be viewed under fluorescence microscope. Bar, 100 μm. C. Quantitative determination of miRNA expression of MT1-MMP in SCC25, transfected SCC25 cells expressing GFP (SCC25-GFP) and MT1-MMP (SCC25-miM). GAPDH was used as a control. **P<0.01. D. MT1-MMP protein level in SCC25, SCC25-GFP and SCC25-miM was analyzed by Western blot. β-actin was employed as a loading control. E. Immunofluorescence staining of MT1-MMP (red) in SCC25, SCC25-GFP and SCC25-miM. The nuclei were stained with DAPI (blue). Images were taken at ×400 magnification. Bar, 100 μm.

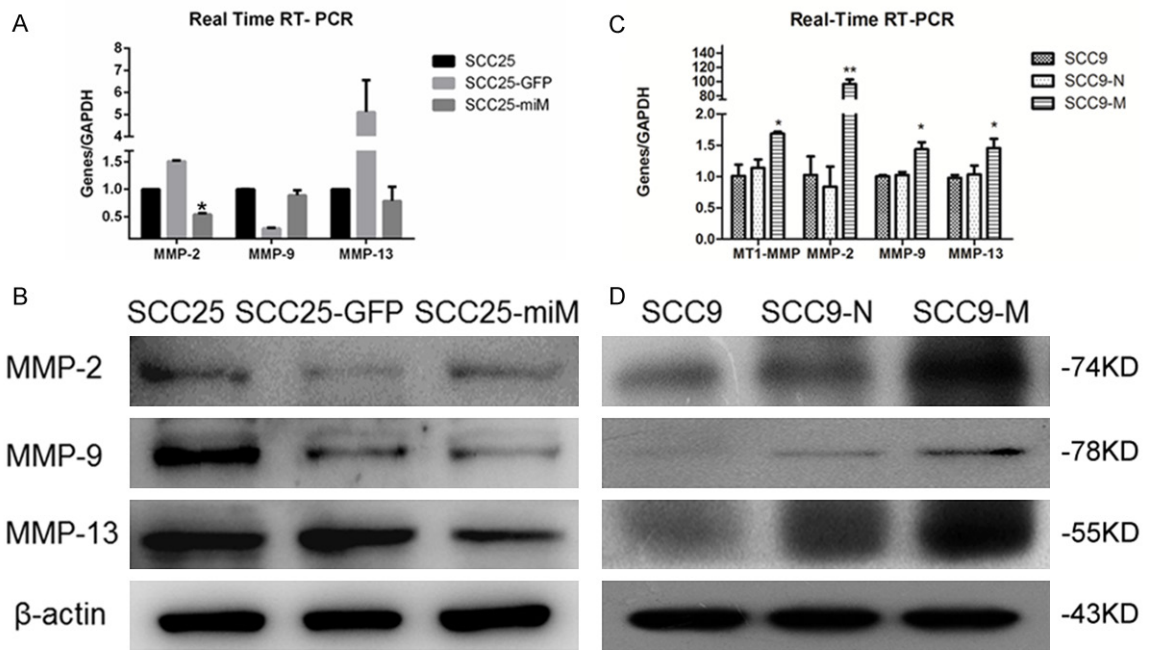

Figure 4. Cancer cell MT1-MMP expression was associated with the expression of MMP-2, MMP-9 and MMP-13. A. Quantitative determination of miRNA expression of MMP-2, MMP-9, MMP-13 in SCC25, SCC25-GFP and SCC25-miM cells. GAPDH was used as a control. *P<0.05. B. Western blot was performed to analyze the expression of MMP-2, MMP-9, MMP-13 in SCC25, SCC25-GFP and SCC25-miM cells on the protein level. β-actin was used as a control. C. The Real-Time RT-PCR was performed to detect the expression of MMP-2, MMP-9, MMP-13 in SCC9 cells, stable SCC9 cells expressing an empty vector (SCC9-N) and MT1-MMP (SCC9-M). GAPDH was used as a control. *P<0.05, **P<0.01. D. Western blot was performed to analyze the expression of MMP-2, MMP-9, MMP-13 in SCC9, SCC9-N and SCC9-M cells on the protein level. β-actin was used as a control.

regulation of MT1-MMP by miRNA interference could cause a decreased level of MMP-2, MMP-9 and MMP-13 (**Figure 4A**). The western blot analysis further confirmed the changes in the gene expression (**Figure 4B**). In our previous study, overexpression of MT1-MMP in SCC9 cells were stably constructed and were performed a series of related experiments [26]. Meanwhile, up-regulation of MT1-MMP in SCC9 cells could also activate the expression of MMP-2, MMP-9 and MMP-13. The results of real-time RT-PCR and western blot verified this point (**Figure 4C** and **4D**). All the data above demonstrated that oral cancer cell MT1-MMP expression was associated with the expression of MMP-2, MMP-9 and MMP-13.

Cancer cell MT1-MMP expression affects the invasive ability of cancer cells and promotes the invasion and metastasis by the degradation of ECM

The wound healing assay showed that the wound was closed after 48 h in SCC25 and SCC25-GFP cells, whereas, the SCC25-miM cells exhibited decreased ability to close the wound (**Figure 5A**). The flow cytometry was performed to show that there was no significant effect of MT1-MMP on the proliferation ability of OSCC cells (**Figure 5B**). All these indicated that cancer cell MT1-MMP expression could affect the migratory ability of OSCC cells. A Transwell assay was performed to investigate the role of MT1-MMP in cancer cell invasion. After cultured for 16 h, the cells of penetrating Matrigel basement membrane matrix were captured in random field. The quantity of SCC25 cells for penetrating BM was significantly decreased by MT1-MMP downregulation. A similar quantitative analysis revealed that an increase of invasive ability in SCC9 cells of MT1-MMP overexpression compared with SCC9 and SCC9-N cells (**Figure 5C**). Collectively, these data elucidated that cancer cell MT1-MMP expression affected the invasive ability of cancer cells. The immunofluorescence assay showed that FN was displayed obviously degradation in SCC9-M cells, while presented integrated expression in the cell cytoplasm and membrane of SCC25-miM cells (**Figure 5D**).

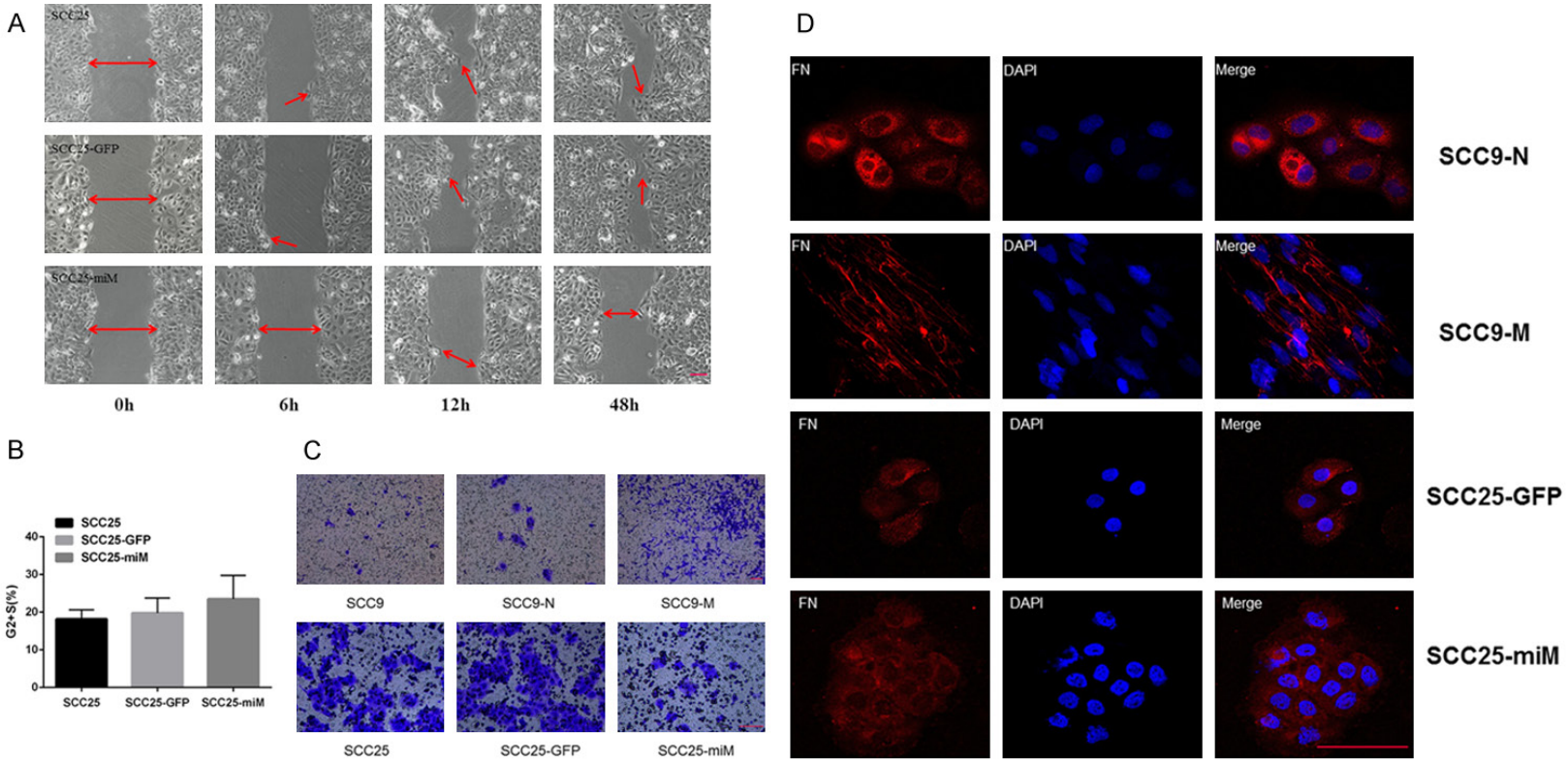

Figure 5. Cancer cell MT1-MMP expression affects the invasive and motive ability of cancer cells and functions for degrading ECM components. A. The wound healing assay indicated that SCC25-miM have the decreased ability to close the wound after 48 h. Photographs were taken at the same position of the indicated time points (×100 magnification). Bar, 100 μm. B. Cell-cycle analysis of SCC25 cells, SCC25-GFP and SCC25-miM. C. Pictures presenting the cells penetrating the Matrigel basement membrane matrix after 16 h. Bar, 100 μm. D. The immunofluorescence staining of Fibronectin (FN: red) in SCC9-N, SCC9-M, SCC25-GFP and SCC25-miM cells. The nuclei image sets were stained with DAPI (blue). Images were taken at ×400 magnification. Bar, 100 μm.

Discussion

MT1-MMP was reported to be up-regulated in almost all types of human cancer, such as breast cancer [20], lung cancer [21], colon cancer [22] and prostate cancer [28]. Previous studies have showed MT1-MMP is a key enzyme that contributes to tumor cell invasion and metastasis through direct ECM degradation and/or activation of downstream MMPs [29, 30]. However, the interaction between the MT1-MMP expression and clinicopathological features and activation of MMPs in OSCC has not been elucidated clearly.

In the current study, we first analyzed the expression of MT1-MMP in 122 OSCC samples to identify the role of MT1-MMP in the development and progression of OSCC. Consistent with previous studies [20, 24, 25], MT1-MMP expression has been demonstrated to be associated with poor clinical outcome in various human carcinoma, and our data indicated that the high expressions of MT1-MMP correlated with several important clinicopathological parameters in OSCC. The IHC staining results confirmed that high expression of MT1-MMP in tumors significantly correlated with the lymph node metastasis, pathological grade, recurrence and follow-up, and high expression of MT1-MMP at the invasive front of tumor significantly correlated with the lymph node metastasis. Collectively, these results strongly suggest that MT1-MMP was closely correlated to OSCC invasion and metastasis, which may play unfavorable roles in OSCC pathogenesis.

Advances made in tongue cancer treatment in the past decades have not improved the patient survival rate. The meta-analysis showed that MT1-MMP was a potential prognostic factor in human cancers [31]. In this study, we first presented that the patients with higher expression of MT1-MMP had shorter survival time in OSCC. According to multivariate analyses, high expression of MT1-MMP could be seen as an independent high-risk prognostic factor for OSCC patients. Our results of MT1-MMP are consistent with previous investigations on other human cancers, indicating that MT1-MMP could constitute a molecular prognostic marker for patients [20, 23, 25].

The acquisition of invasive potential by tumor cells is a complex process. Our previous research verified that overexpression of MT1-MMP induced EMT and resulted in high invasive abilities in SCC9 cells [26]. In this study, our findings suggested that MT1-MMP plays a role in activation of MMP-2, MMP-9 and MMP-13 up-regulation in oral cancer cells, and further affected the invasive and metastatic abilities of cancer cells. Our results support the previous research that the expression of MT1-MMP resulted in enhancing the associated-proteolytic activity and directly or indirectly contributed to degrading the ECM components including fibronectin [32, 33]. MT1-MMP regulates cell migration via mediating changes of cell-cell junctions and cell-matrix focal adhesions [34]. The activated MMP-2 and MMP-9 by MT1-MMP are known as the type IV collagenases, and can also degrade the major members of ECM and BM [35]. This regulation of ECM degradation affected the biological behaviors of cancer cells infiltrating in the local microenvironment. In summary, our study further clarified the function of MT1-MMP in processing MMPs expression in oral cancer cells, and the role of MT1-MMP in promoting invasion and metastasis in OSCC from the point of view of tumor microenvironment remodeling.

In conclusion, our study demonstrates that the MT1-MMP expression in OSCC patients is associated with poor overall survival and has significant clinicopathological characteristics. Furthermore, MT1-MMP expression could serve as an independent prognostic indicator for the survival of patients with OSCC. More important, MT1-MMP, through regulating MMP-2, MMP-9, MMP-13 expression, contributes to promoting the invasion and metastasis in oral cancer cells by degrading major ECM components and further influencing biological behaviors of cancer cells in tumor microenvironment. Collectively, MT1-MMP may play an important role in OSCC invasion and metastasis and could act as a promising target for prognostic prediction.

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Disclosure of conflict of interest

None.

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References

[1] Bhowmick NA, Neilson EG and Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004; 432: 332-337.

[2] Kalluri R and Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006; 6: 392-401.

[3] Liu LK, Jiang XY, Zhou XX, Wang DM, Song XL and Jiang HB. Upregulation of vimentin and aberrant expression of E-cadherin/beta-catenin complex in oral squamous cell carcinomas: correlation with the clinicopathological features and patient outcome. Mod Pathol 2010; 23: 213-224.

[4] Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, Gefen A, Reinhart-King CA, Margulies SS, Dembo M, Boettiger D, Hammer DA and Weaver VM. Tensional homeostasis and the malignant phenotype. Cancer Cell 2005; 8: 241-254.

[5] Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS and Giaccia AJ. Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 2006; 440: 1222-1226.

[6] Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL and Weaver VM. Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 2009; 139: 891-906.

[7] Cheung KJ and Ewald AJ. Illuminating breast cancer invasion: diverse roles for cell-cell interactions. Curr Opin Cell Biol 2014; 30: 99-111.

[8] Lu P, Weaver VM and Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 2012; 196: 395-406.

[9] Lu P, Takai K, Weaver VM and Werb Z. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol 2011; 3.

[10] Cawston TE and Young DA. Proteinases involved in matrix turnover during cartilage and bone breakdown. Cell Tissue Res 2010; 339: 221-235.

[11] Deryugina EI and Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 2006; 25: 9-34.

[12] Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E and Seiki M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 1994; 370: 61-65.

[13] Radisky ES and Radisky DC. Matrix metalloproteinase-induced epithelial-mesenchymal transition in breast cancer. J Mammary Gland Biol Neoplasia 2010; 15: 201-212.

[14] Sabeh F, Ota I, Holmbeck K, Birkedal-Hansen H, Soloway P, Balbin M, Lopez-Otin C, Shapiro S, Inada M, Krane S, Allen E, Chung D and Weiss SJ. Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J Cell Biol 2004; 167: 769-781.

[15] Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS and Friedl P. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat Cell Biol 2007; 9: 893-904.

[16] Friedl P and Wolf K. Tube travel: the role of proteases in individual and collective cancer cell invasion. Cancer Res 2008; 68: 7247-7249.

[17] Itoh Y and Nagase H. Matrix metalloproteinases in cancer. Essays Biochem 2002; 38: 21-36.

[18] Itoh Y and Seiki M. MT1-MMP: a potent modifier of pericellular microenvironment. J Cell Physiol 2006; 206: 1-8.

[19] Al-Raawi D, Abu-El-Zahab H, El-Shinawi M and Mohamed MM. Membrane type-1 matrix metalloproteinase (MT1-MMP) correlates with the expression and activation of matrix metalloproteinase-2 (MMP-2) in inflammatory breast cancer. Int J Clin Exp Med 2011; 4: 265-275.

[20] Li Y, Cai G, Yuan S, Jun Y, Li N, Wang L, Chen F, Ling R and Yun J. The overexpression membrane type 1 matrix metalloproteinase is associated with the progression and prognosis in breast cancer. Am J Transl Res 2015; 7: 120-127.

[21] Zhou H, Wu A, Fu W, Lv Z and Zhang Z. Significance of semaphorin-3A and MMP-14 protein expression in non-small cell lung cancer. Oncol Lett 2014; 7: 1395-1400.

[22] Okada A, Bellocq JP, Rouyer N, Chenard MP, Rio MC, Chambon P and Basset P. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. Proc Natl Acad Sci U S A 1995; 92: 2730-2734.

[23] Wang H, Zhang X, Huang L, Li J, Qu S and Pan F. Matrix metalloproteinase-14 expression and its prognostic value in cervical carcinoma. Cell Biochem Biophys 2014; 70: 729-734.

[24] Wang YZ, Wu KP, Wu AB, Yang ZC, Li JM, Mo YL, Xu M, Wu B and Yang ZX. MMP-14 overexpression correlates with poor prognosis in non-small cell lung cancer. Tumour Biol 2014; 35: 9815-9821.
[25] He L, Chu D, Li X, Zheng J, Liu S, Li J, Zhao Q and Ji G. Matrix metalloproteinase-14 is a negative prognostic marker for patients with gastric cancer. Dig Dis Sci 2013; 58: 1264-1270.
[26] Yang CC, Zhu LF, Xu XH, Ning TY, Ye JH and Liu LK. Membrane Type 1 Matrix Metalloproteinase induces an epithelial to mesenchymal transition and cancer stem cell-like properties in SCC9 cells. BMC Cancer 2013; 13: 171.
[27] Itoh Y. MT1-MMP: a key regulator of cell migration in tissue. IUBMB Life 2006; 58: 589-596.
[28] Udayakumar TS, Chen ML, Bair EL, Von Bredow DC, Cress AE, Nagle RB and Bowden GT. Membrane type-1-matrix metalloproteinase expressed by prostate carcinoma cells cleaves human laminin-5 beta3 chain and induces cell migration. Cancer Res 2003; 63: 2292-2299.
[29] Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M and Okada Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. J Biol Chem 1997; 272: 2446-2451.
[30] Seiki M and Yana I. Roles of pericellular proteolysis by membrane type-1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Sci 2003; 94: 569-574.
[31] Wu AW, Xu GW, Wang HY, Ji JF and Tang JL. Neoadjuvant chemotherapy versus none for resectable gastric cancer. Cochrane Database Syst Rev 2007; 18: CD005047.
[32] Takino T, Guo L, Domoto T and Sato H. MT1-MMP prevents growth inhibition by three dimensional fibronectin matrix. Biochem Biophys Res Commun 2013; 436: 503-508.
[33] Takino T, Nagao R, Manabe R, Domoto T, Sekiguchi K and Sato H. Membrane-type 1 matrix metalloproteinase regulates fibronectin assembly to promote cell motility. FEBS Lett 2011; 585: 3378-3384.
[34] Takino T, Saeki H, Miyamori H, Kudo T and Sato H. Inhibition of membrane-type 1 matrix metalloproteinase at cell-matrix adhesions. Cancer Res 2007; 67: 11621-11629.
[35] Kessenbrock K, Plaks V and Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010; 141: 52-67.