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Original Article

MT2-MMP expression associates with tumor progression and angiogenesis in human lung cancer

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Abstract: Matrix metalloproteinases (MMPs) are a family of important proteolytic enzymes that play an important role in the remodeling of the tumor microenvironment and associate with tumorigenesis and metastasis. We previously reported that membrane type-2 MMP (MT2-MMP) is highly expressed in human esophageal cancer tissues, and its expression level is positively correlated to tumor size and intratumoral angiogenesis. In order to reveal whether MT2-MMP expression is operative in human lung cancer and its underlying physio-pathological role, in the present study, we examined both mRNA and protein expression levels of MT2-MMP in non-small cell lung caner (NSCLC) tissues and in adjacent normal tissues by using real-time RT-PCR and immunohistochemistry respectively, which showed that both MT2-MMP mRNA (P=0.0359) and protein (P<0.0001) expression levels were significantly increased in cancer tissues in contrast to adjacent normal tissues. Moreover, we also found that the MT2-MMP protein level in cancer tissues positively correlated to lymph node metastasis (P=0.0483), tumor stage (P=0.0483), intra-tumoral microvessel density (MVD) (P=0.0445). We have not found statistically significant correlation between MT2-MMP expression and patients' prognoses, but we found that the patients with both higher MT2-MMP protein expression and higher intra-tumoral microvessel density showed better prognoses than that of the patients with either higher MT2-MMP protein expression or higher intra-tumoral microvessel density (P=0.0311). Thus, our data suggest that MT2-MMP expression positively involves in NSCLC, and might play an important role in promoting the tumor progression and intra-tumoral angiogenesis in NSCLC.

Keywords: MT2-MMP, NSCLC, intratumoral angiogenesis, prognosis

Introduction

Lung cancer, a serious hazard to human health and life, has been shown to be a significant rising trend in terms of morbidity and mortality worldwide in recent years [1]. Lung cancer can be histologically divided into two major subtypes, i.e., non-small cell lung cancer (NSCLC) and small cell lung cancer, and the NSCLC accounts for approximately 80% of all lung cancers [2]. Because of the difficulty in early detection, the majority of NSCLC patients usually present with advanced stage at diagnosis. The conventional platinum-based chemotherapy and radiation have low efficacy against this malignancy due to its frequent recurrence and distant metastasis, and the overall 5-year survival rate of NSCLC patients still remains at 10~15% [2, 3]. Therefore, considerable efforts are needed to explore novel biomarkers to benefit the early diagnosis and the targeted therapeutic interventions of NSCLC.

MMPs are a family of important proteolytic enzymes that contribute importantly in the remodeling of the tumor microenvironment and associate with tumorigenesis and metastasis [4]. MMPs consist more than 25 well-characterized members of secreted or trans-membranous proteins, which could be predominantly divided into five sub-groups: collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and other MMPs [5, 6]. MT2-MMP, an important member of membrane-type MMPs, was originally isolated from a human lung cDNA library, and it consists of a 76-kDa product with 73.9% overall similarity to MMP-14 [7]. As yet, the clinical implication of MT2-MMP expression in human cancers still remains elusive. Some studies indicated that MT2-MMP

Table 1. Sequences of primers and probes

Genes	Sequences (5'→3')
MT2-MMP	
Forward primer	AGGTACTGGCGCTTCAACGAG
Reverse primer	CTTGTAGAAGTAGGTGTAGGCTGCGT
Probe	FAM-AGACACAGCGTGGAGACCCTGGGTAC-TAMRA
β-actin	
Forward primer	GACTTAGTTGCGTTACACCCTTTC
Reverse primer	GCTGTCACCTTCACCGTTCC
Probe	FAM-TGACAAAACCTAACTTGCGCAGAAAACA-TAMRA

might involve in the progression of certain types of human cancer, such as that of the breast, cervix, ovary and colorectum [8-11]. Recent report also indicated MMP-15 as an anti-apoptotic factor in cancer cells [12]. Our previous study showed that MT2-MMP is highly expressed in human esophageal cancer tissues, and its expression level is positively correlated to tumor size and intratumoral angiogenesis [13].

Our present study is aimed to determine whether MT2-MMP is operative in human NSCLC. We examined both mRNA and protein levels of MT2-MMP in cancer tissues and adjacent normal tissues from NSCLC patients by using real-time RT-PCR and immunohistochemistry methods respectively. MT2-MMP protein levels in cancer tissues in relation to patients' clinical parameters, intratumoral microvessel density (MVD) and post-operative prognoses were also analyzed.

Patients and methods

Patients and tissue samples

NSCLC tissue samples were collected from 85 patients who underwent surgical pulmonary resection between Oct 2005 and Sep 2011 in our hospital (aged from 33 to 81 years old, median age 62 year old). No patient received pre-operative chemotherapy or radiotherapy. All cases were histologically classified as NSCLC and the cancer stages were classified according to the American Joint Committee on Cancer Criteria [14]. Immediately after resection, both caner tissues and adjacent normal tissues were fixed in 10% (v/v) formalin and embedded in paraffin until use. In addition, a part of cancer tissues and adjacent normal tissues from 21 patients were also snap-frozen and stored in liquid nitrogen for further investigation of RNA level. The detailed clinical parameters of patients are listed in **Table 3**. Of all the 85 patients, 71 patients' survival intervals were available and dated to the end of Jan 2014. The present study was approved by the Ethics Committee of the hospital.

Total RNA extraction, reverse transcription and real-time PCR

The mRNA levels of *MT2-MMP* and the reference gene *β-actin* were measured under real-time RT-PCR by using TaqMan technology. In brief, total RNA in tissues was extracted according to the manufacturer's instructions by using a total RNA purification kit (Biocolor BioScience and Technology Company, Shanghai, China). The quality of the RNA samples was determined by the absorbance measurements at 260/280 nm. Using the first strand cDNA synthetic kit (Fermantas, Vilnius, Lithuania) according to the manufacturer's instructions, 2 μg total RNA was reverse transcribed to cDNA. The primers and TaqMan probes of *MT2-MMP* and the reference gene *β-actin* were designed according to the National Center for Biotechnology Information (NCBI) database NM_002428.2 and NM_001101.3, respectively, using Primer Primier 5.0 software (Palo Alto, CA, USA). The sequences of all primers and TaqMan probes used in the present study were listed in **Table 1**. The cycling conditions for *MT2-MMP* was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 5 sec and 60°C for 20 sec, collecting the fluorescence signal at 60°C. The cycling conditions for reference gene *β-actin* was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 5 sec, 56°C for 5 sec and 60°C for 15 sec, collecting the fluorescence signal at 60°C. All PCRs were performed on the LightCycler (Roche, Switzerland) real-time PCR system.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into 3-μm-thick consecutive sections, and were dewaxed in xylene, rehydrated and graded ethanol solutions. Polyclonal rabbit against human MT2-MMP antibody (AB855,

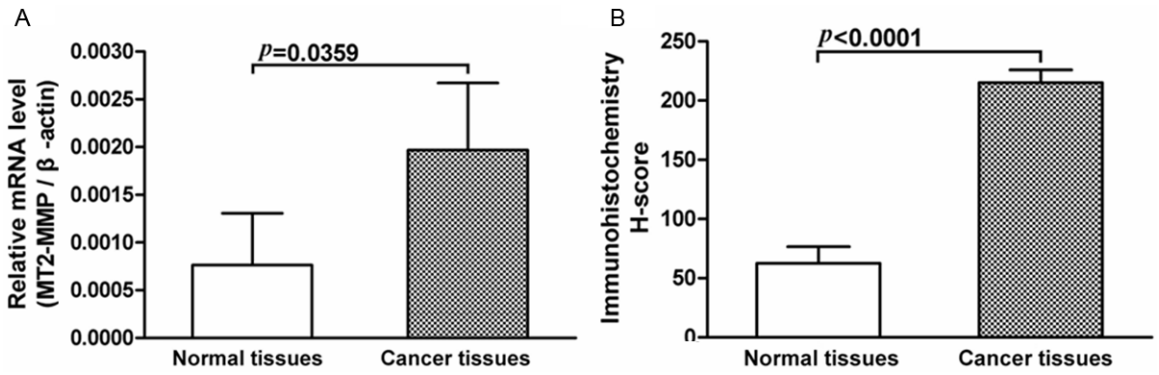

Figure 1. *MT2-MMP mRNA and protein levels in cancer tissues and adjacent normal tissues from NSCLC patients*. We detected and compared both mRNA and protein levels of MT2-MMP in cancer tissues and adjacent normal tissues by using real-time RT-PCR and immunohistochemistry, respectively. A: MT2-MMP mRNA level in cancer tissues was significantly higher than that in adjacent normal tissues in 21 cases of NSCLC patients (P=0.0359). B: MT2-MMP protein level in cancer tissues was significantly higher than that in adjacent normal tissues in 24 cases of NSCLC patients (P<0.0001).

diluted in 1:600, Chemicon, USA) and monoclonal mouse against human CD34 antibody (MAB-0034, ready to use, Maixin Biotechnology Limited Corporation, Fuzhou, P. R. China) were used in the present study. The CD34 antigen was retrieved by heating the slides in the citrate solution (10 mmol/L, pH 6.0) for 30 min, while no special pretreatment is required for MT2-MMP antigen according to the manufacture's instruction. Then, the sections were immersed in a 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase activity, rinsed in PBS for 5 min, and then incubated with primary antibodies at 4°C overnight. A negative control was performed without the primary antibodies. The sections were then incubated with horseradish peroxidase-labeled goat against mouse/rabbit secondary antibody (ready to use, Maixin Biotechnology Limited Corporation, Fuzhou, P. R. China). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain.

Evaluation of MT2-MMP immunostaining and intratumoral MVD

The MT2-MMP immunostaining intensities were assessed according to the H-score method described by our previous study [13, 15]: H-score=(% tumor cells unstained ×0) + (% tumor cells stained weak ×1) + (% tumor cells stained moderate ×2) + (% tumor cells stained strong ×3). The H-scores ranged from 0 (100% negative tumor cells) to 300 (100% strong staining tumor cells). Immunopositive staining of endothelial cell marker, CD34, was chosen for the evaluation of intratumoral MVD as described in our previous studies [13, 16]. In brief, tumor sections were first examined at low magnification (×40), and five intratumoral areas with the most intense neo-vascularization were selected. The positive CD34 immunostaining micro-vessels, as well as single endothelial cells, were counted per high power field (HPF, ×200 magnification).

Statistical analyses

Statistical analyses were performed using the GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, USA). Wilcoxon matched pairs test, Mann-Whitney test, Chi-square test or log-rank test was used where appropriate. A p-value of <0.05 was considered as significant.

Results

MT2-MMP mRNA expression levels in lung cancer tissues and adjacent normal tissues

In the present study, we detected MT2-MMP mRNA expression levels in both cancer tissues and adjacent normal tissues from 21 NSCLC patients by using real-time RT-PCR. As shown in **Figure 1A**, we found that MT2-MMP mRNA expression level in cancer tissues is significantly higher that in adjacent normal tissues (P=0.0359). As shown in **Table 2**, we didn't find that MT2-MMP mRNA expression level in can-

Table 2. Correlation between clinical parameters and MT2-MMP mRNA levels

Clinical parameters	Cases	Relative mRNA level (MT2-MMP/β-actin)	*P*
Gender			
Male	17	0.0023 ± 0.0035	0.3027
Female	4	0.0008 ± 0.0011	
Age (years)			
≤60	10	0.0033 ± 0.0043	0.0908
>60	11	0.0008 ± 0.0007	
Tumor size			
pT_1	5	0.0014 ± 0.0012	0.7102
pT_2	16	0.0021 ± 0.0036	
Lymph node metastasis			
With	10	0.0026 ± 0.0046	0.2595
Without	11	0.0014 ± 0.0010	
Tumor stage			
I	11	0.0014 ± 0.0010	0.2595
II+III	10	0.0026 ± 0.0046	
Histological type			
Adenocarcinoma	10	0.0030 ± 0.0045	0.6219
Squamous cell carcinoma	11	0.0010 ± 0.0008	

cer tissues was significantly correlated to any clinical parameters of NSCLC patients.

Immunohistochemical staining of MT2-MMP in lung tissues

As shown in **Figure 2**, positive MT2-MMP immunochemical staining was predominantly observed on the membrane and in cytoplasm of cancer cells (Panel A, adenocarcinoma and Panel C, squamous cell carcinoma), while none or weak staining was found in normal lung tissues (Panel E). As shown in **Figure 1B**, we compared the intensities of MT2-MMP immunostaining in both cancer tissues and adjacent normal tissues from 24 cases of NSCLC patients, which showed that MT2-MMP protein level was also significantly increased in cancer tissues ($P<0.0001$).

MT2-MMP protein levels in NSCLC tissues in relation to patients' clinical parameters and prognoses

In order to reveal the clinical importance of MT2-MMP protein levels in the NSCLC tissues, we further sub-grouped 85 patients into two groups according to the intensity of MT2-MMP immunochemical staining with the following results: H-score <225, 46 cases and H-score ≥225, 39 cases. As shown in **Table 3**, we found that MT2-MMP protein level in lung cancer tissues was significantly correlated to nodal metastasis and tumor stage (both $P=0.0483$). In order to reveal the prognostic value of MT2-MMP expression in human lung cancer, we selected log rank test to study the prognostic value of MT2-MMP expression (both mRNA and protein levels) as well as intra-tumoral microvessels density in human lung cancer tissues, and the minimum *P* value seek was conducted according to the method from the literatures and our previous studies [17, 18], using different values as cut-off respectively. As shown in **Figure 3**, we have not found statistically significant correlation between MT2-MMP expression and patients' prognoses, but we found that the patients with both higher MT2-MMP protein expression and higher intra-tumoral microvessel density showed better prognoses than that of the patients with either higher MT2-MMP protein expression or higher intra-tumoral microvessel density ($P=0.0311$).

MT2-MMP protein levels in NSCLC tissues in relation to intratumoral MVD

In order to reveal the underlying physio-pathological role of MT2-MMP expression in NSCLC tissues, we used consecutive sections and included CD34 marker to further investigate the relationship between MT2-MMP expression and intratumoral angiogenesis as reported in our previous study [13]. As shown in **Table 3**, we found that the ratio of higher MT2-MMP protein level in the subgroup of high intratumoral MVD (>22.4/HPF) was significantly higher than that in the subgroup of low intratumoral MVD (≤22.4/HPF) ($P=0.0445$), which suggests that MT2-MMP could be positively involved in the intratumoral angiogenesis of NSCLC progression.

Discussion

Cancer metastasis is a multi-step process involves and requires cancer cell migration and

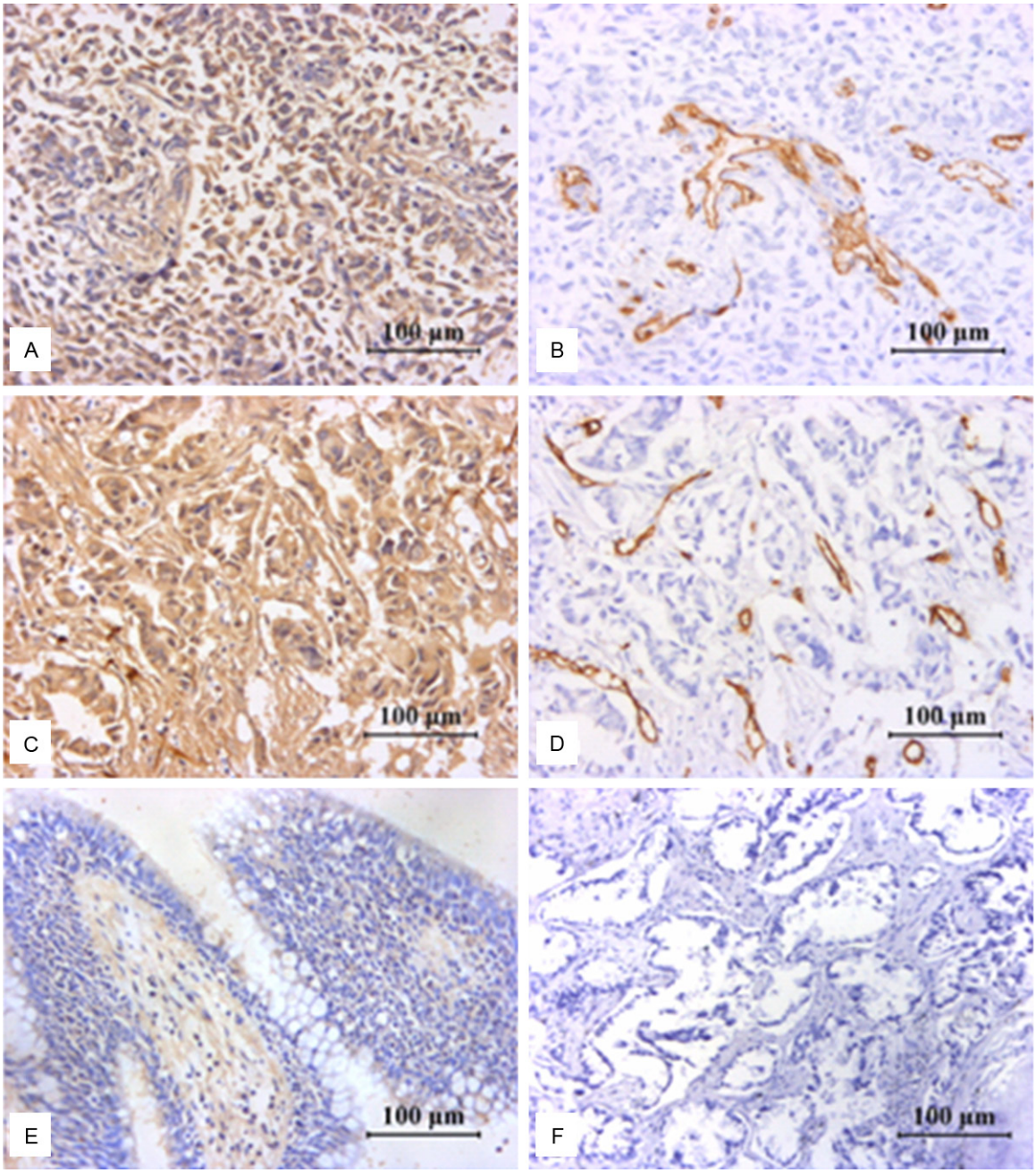

Figure 2. *Immunostaining of MT2-MMP and CD34 in lung tissues*. Panel (A) to (B) represent one of the consecutive sections of an adenocarcinoma NSCLC specimen with high MT2-MMP and CD34 immunostaining, (A) MT2-MMP, (B) CD34. Panel (C) to (D) represent one of the consecutive sections of a squamous cell carcinoma NSCLC specimen with high MT2-MMP and CD34 immunostaining, (C) MT2-MMP, (D) CD34. (E) Week MT2-MMP immunostaining in normal lung tissue. (F) Negative control.

invasion, during which the degradation of various components of the extracellular matrix is an initial step. MMPs have been suggested to be closely correlated with cancer-cell invasion and metastasis, and many studies have focused on MMPs in the tumorigenesis, invasion and metastasis of lung cancer [19]. As a result, some members from MMPs family, i.e. MMP-1, -7 and -9 have become important candidate biomarkers for lung cancer, and some inhibitors targeted MMPs have currently been used in clinical trials of lung cancer treatment [20,

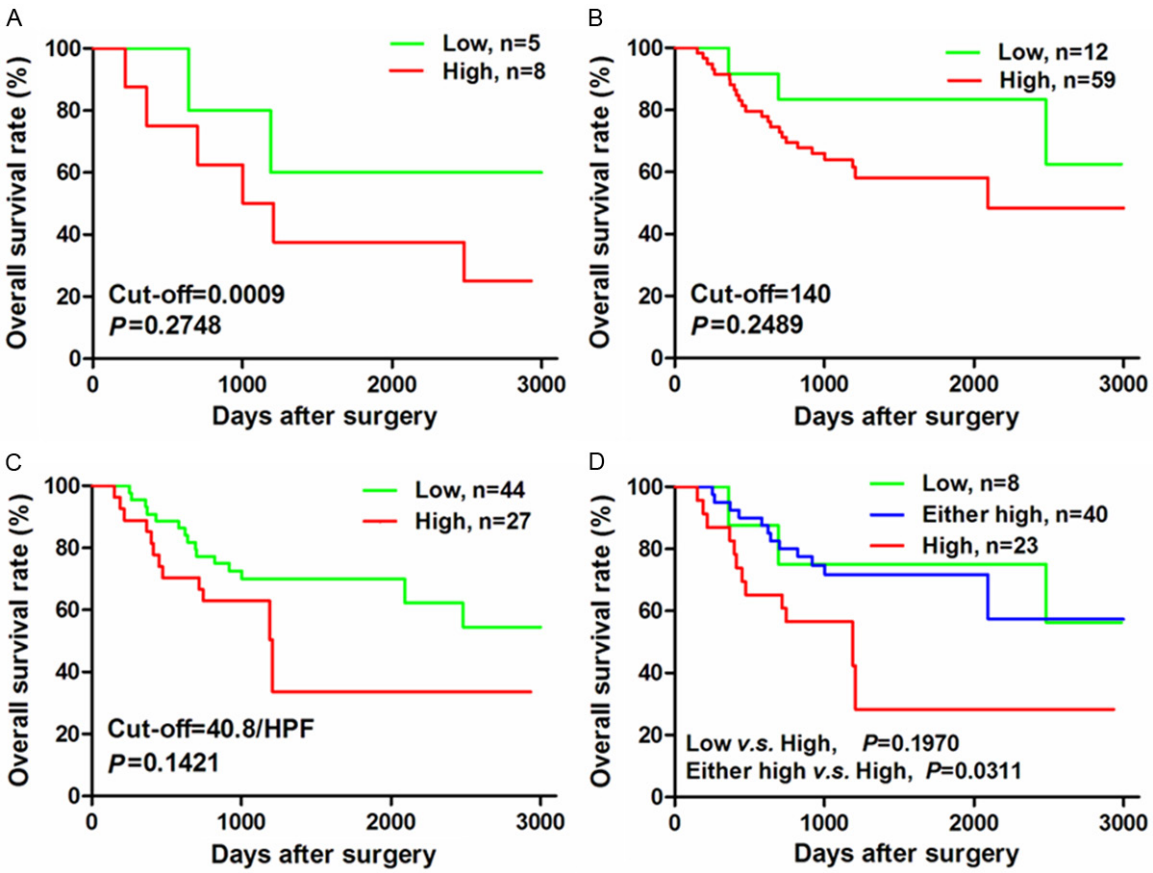

Figure 3. Prognostic values of MT2-MMP and intra-tumoral microvessel density in lung cancer tissues. The survival data of 71 of all the 85 patients involving in the present study were collected and used in the survival analysis. Panel (A) to (C), MT2-MMP mRNA expression level (P=0.2748), MT2-MMP protein expression level (P=0.2489) as well as the intra-tumoral microvessel density (P=0.1421) in relation to patients' prognoses was analyzed, and we have not found any statistically significant correlations between these parameters and patients' prognoses. Panel (D) We combined the data of MT2-MMP protein expression level and the intra-tumoral microvessel density, to analyze its relation to patients' prognoses, and we found that the patients with both higher MT2-MMP protein expression and higher intra-tumoral microvessel density showed better prognoses than that of the patients with either higher MT2-MMP protein expression or higher intra-tumoral microvessel density (P=0.0311).

21]. The aim of the present study was to address the current lack of information regarding the physio-pathological role of MT2-MMP expression in lung cancer tissues.

In the present study, we found that MT2-MMP mRNA expression level in lung cancer tissues is significantly higher than that in adjacent normal tissues. Similarly, we also found that the MT2-MMP protein level in lung cancer tissues was also significantly higher than that in adjacent normal tissues, which suggests that MT2-MMP expression could be up-regulated in the tumorigenesis and progression of human NSCLC. MT2-MMP has been studied for its role in cancer progression and metastasis, it can cleave gelatin, degrade the extracellular matrix molecules including fibronectin and aggrecan, and can activate MMP-2 [22]. Sena *et al.* [23] reported that the MT2-MMP protein is highly expressed in the early stages of colorectal carcinoma. And MT2-MMP has also been suggested to be a possible prognostic factor in human colorectal carcinoma [11]. Tao *et al.* [24] reported that MT2-MMP is a direct target of Snai1, which is an important regulator in epithelial to mesenchymal transition, suggesting that MT2-MMP might also play an important role in cancer invasion and metastasis.

The underlying physio-pathological role of MT2-MMP in human NSCLC still remains unknown. In the present study, we didn't find that the mRNA expression level of MT2-MMP in cancer tissues were significantly correlated to any clinical parameters of NSCLC patients, but we

Table 3. Clinical parameters, MT2-MMP protein levels and Intratumoral MVD

Clinical parameters	MT2-MMP immunostaining (*H-score*)		*P*-value
	Low (%) ≤225	High (%) >225	
Gender			
Male	37 (59.7)	25 (40.3)	0.0912
Female	9 (39.1)	14 (60.9)	
Age (years)			
≤60	20 (54.1)	17 (45.9)	0.9918
>60	26 (54.2)	22 (45.8)	
Tumor size			
pT_1	15 (57.7)	11 (42.3)	0.6606
pT_2	31 (52.5)	28 (47.5)	
Nadal metastasis			
Without	31 (63.3)	18 (36.7)	0.0483
With	15 (55.6)	21 (44.4)	
Stage			
I	31 (63.3)	18 (36.7)	0.0483
II+III	15 (55.6)	21 (44.4)	
Histological type			
Adenocarcinoma	21 (44.7)	26 (55.3)	0.0522
Squamous cell carcinoma	25 (65.8)	13 (34.2)	
Intratumoral MVD			
Low (≤22.4/HPF)	11 (78.6)	3 (21.4)	0.0445
High (>22.4/HPF)	35 (49.3)	36 (50.7)	

found that the protein expression level of MT2-MMP in cancer tissues was significantly correlated with lymph node metastasis and TNM stage, which suggests a potential value of MT2-MMP protein expression level evaluation in predicting lymph node metastasis and cancer progression of NSCLC patients. Moreover, our and others' studies demonstrated that MT-MMPs could have an important contribution on intratumoral angiogenesis [13, 25-27], and it has been demonstrated that the membrane type matrix metalloproteinases, such as MT1-, MT2-, MT3-MMP were able to induce capillary-like tube formation [28, 29]. Our present results showed that the ratio of higher MT2-MMP protein level in the subgroup with high intratumoral MVD was significantly higher than that in the subgroup with low intratumoral MVD, and confirmed a positive role of MT2-MMP expression in promoting intratumoral angiogenesis of NSCLC.

Moreover, in the present study, we have not found statistically significant correlation between MT2-MMP expression (at both mRNA and protein levels) and patients' prognoses, but we found that the patients with both higher MT2-MMP protein expression and higher intra-tumoral microvessel density showed better prognoses than that of the patients with either higher MT2-MMP protein expression or higher intra-tumoral microvessel density. We also didn't find a significant correlation between MT2-MMP expression and patients' prognoses in human esophageal cancer tissues, although the overall survival rate of patients with higher MT2-MMP protein expression trended better than that of patients with lower MT2-MMP protein expression [17]. Thus, in future, the prognostic value of abnormal MT2-MMP expression in cancer tissues, the detailed mechanism of the up-regulation of MT2-MMP expression in tumorigenesis and tumor micro-environment, and its contribution to promote intratumoral angiogenesis in human cancers merit further investigation.

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Disclosure of conflict of interest

None.

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