MMP-7 is upregulated by COX-2 and promotes proliferation and invasion of lung adenocarcinoma cells

J. Zhang,1 J. Luo,2 J. Ni,2 L. Tang,1 H.P. Zhang,3 L. Zhang,1 J.F. Xu,2 D. Zheng2
1Central Lab, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai
2Department of Medical Oncology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China

Abstract

Matrix metalloproteinases (MMPs) have been implicated in a variety of pathophysiological conditions, of which MMP-7 is expressed by tumor cells of epithelial and mesenchymal origin. However, the function of MMP-7 in human lung adenocarcinoma (LAC) is unclear. In the present study the expression of MMP-7 in LAC was examined by immunohistochemical assay using a tissue microarray procedure. A loss-of-function experiment was performed to explore the effects and molecular mechanisms of lentiviral vector-mediated MMP-7 siRNA (siMMP-7) on cell proliferation and invasive potential in LAC A549 cells, measured by MTT and Transwell assays, respectively. It was found that, the expression of MMP-7 protein in LAC was significantly increased compared with that in adjacent non-cancerous tissues (ANCT) (76.0% vs 44.0%, P<0.001), and positively correlated with lymph node metastases of the tumor (P=0.014). Furthermore, targeted inhibition of cyclooxygenase-2 (COX-2) by siRNA downregulated the expression of MMP-7 and inhibited invasion of LAC cells, and knockdown of MMP-7 suppressed tumor proliferation and invasion in LAC cells. Taken together, our findings indicate that increased expression of MMP-7 is associated with lymph node metastasis and upregulated by COX-2, and promotes the tumorigenesis of LAC, suggesting that MMP-7 may be a potential therapeutic target for the treatment of cancer.

Introduction

Lung cancer is the most commonly diagnosed type of cancer and the primary cause of cancer-related deaths worldwide.1 The current best approach for treatment of cancer is complete surgical removal of the tumor and adjacent lymph nodes. However, the efficacy of this therapeutic approach alongside hormone, radio, and chemo-therapy are very limited.2 Tumor is also a genetic disease that develops from a multi-step process. Single or multiple mutations in genes related to growth control, invasion, and metastasis form the molecular genetic basis of malignant transformation and tumor progression.3 Therefore, identification of key genes or targets related to tumorigenesis is critical for prevention and treatment of cancer.

Matrix metalloproteinases (MMPs), produced by stromal fibroblast-like cells in the vicinity of various malignancies, have been shown to have a significant role in determining cancer cell behaviors, of which MMP-7 is identified to be overexpressed in non-small cell lung cancer (NSCLC).4,14 The expression of MMP-7 is higher in adenocarcinoma than in the epidermoid form of NSCLC,7 but not in normal epithelia.6 MMP-7 is also expressed in bronchiolization of alveoli (BCA), a precursor of lung cancer, promoting proliferation, migration, and attenuation of apoptosis.8 Overexpression of MMP-7 is associated with tumor proliferation and chemoresistance, and constitutes a prognostic factor in several solid tumors,9,10 suggesting an independent positive prognostic factor in NSCLC patients.11 However, some studies have shown that MMP-2, but not MMP-7 and MMP-9, may be implicated in early-stage tumor invasion, metastasis, and apoptosis inhibition in human lung adenocarcinoma (LAC).12-14 MMP-7 expression does not significantly correlate with the clinicopathological factors and unfavorable prognosis in NSCLC.11,15 Thus, in the present study, the expression of MMP-7 in LAC was examined by immunohistochemical assay using a tissue microarray procedure. A loss-of-function experiment was performed to explore the function of MMP-7 in LAC A549 cells. We hypothesized that MMP-7 might function as an oncogen in human LAC, and serve as a potential therapeutic target for the treatment of cancer.

In addition, COX-2 is highly expressed in LAC and represents an independent prognostic factor.16 COX-2 can mediate the tumor metastasis of breast cancer cells through EGFR-activated PI3K/Akt and MAPK pathways.17 Targeting COX-2 controls tumor growth, angiogenesis, lymphangiogenesis and lung metastasis in breast cancer, and selective COX-2 inhibitor celecoxib induces epithelial-mesenchymal transition in lung cancer via activating MEK-ERK signaling.18,19 But, whether COX-2 is involved in the tumorigenesis of LAC through regulation of MMP-7 expression is unknown. Our present study will clarify the regulation of COX-2 on MMP-7 as well as the function of MMP-7 in LAC cells.

Materials and Methods

Materials

The LAC A549 cell line used for experiments was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Adenovirus-mediated small interference COX-2 (siCOX-2) and lentivirus-mediated MMP-7 siRNA (siMMP-7) vectors, negative control vectors, and virion-packaging elements were purchased from Genechem (Shanghai, China); COX-2 and MMP-7 primers were synthesized by ABI (Framingham, MA, USA). The tissue microarray of human LAC was purchased from the branch of Biomax (Xi’an, China). All antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Drugs and reagents

Dulbecco’s Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA); TRizol Reagent and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was purchased from Promega (Madison, WI, USA); SYBR Green Master Mix was obtained from Takara (Otsu, Japan).
Antibodies were used at a dilution of 1:200. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked with 0.5% casein and 5% normal serum for 30 min at room temperature. Finally, staining was developed using diaminobenzidine substrate, and sections were counterstained with hematoxylin. Normal serum or PBS was used to replace anti-MMP-7 and COX-2 antibodies in negative controls.

Quantification of MMP-7 protein expression

MMP-7 expression was semiquantitatively estimated as the total MMP-7 immunostaining score, which was calculated as the product of a proportion score and an intensity score. The proportion score reflected the fraction of positively stained cells (score 0, <5%; score 1, 5%–10%; score 2, 10%–50%; score 3, 50%–75%; score 4, >75%). The intensity score represented the staining intensity (score 0, no staining signal; score 1, weak positive signal; score 2, moderate positive signal; score 3, strong positive signal). Finally, a total expression score was given, ranging from 0 to 12. The score 0 was regarded as negative, score 1-3 was regarded as +, score 4-6 was regarded as ++, score 7-9 was regarded as ++++, and score 10-12 was regarded as ++++. Two observers estimated the total immunostaining score, independently and blindly. The total score reported was the average of two observers.

Cell culture and transfection

LAC A549 cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 µM of penicillin, and 100 µg/mL of streptomycin. Cells in this medium were plated in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were subcultured at a 1:5 dilution of medium containing 300 µg/mL G418 (an aminoglycoside antibiotic, commonly used stable transfection reagent in molecular genetic testing). On the day of transduction, LAC cells were reseeded at 5×10⁴ cells/well in 24-well plates containing serum-free growth medium with polybrene (5 µg/mL). When reached 50% confluence, cells were transfected with recombinant experimental virus or control virus at the optimal MOI (multiplicity of infection) of 50, and cultured at 37°C and 5% CO₂ for 4 h. Then supernatant was discarded and serum containing growth medium was added. At 2 days of post-transduction, transduction efficiency was measured by the frequency of green fluorescent protein (GFP)-positive cells under a fluorescence microscope. The transduction efficiency of lentivirus-mediated shMMP-7 was calculated according to the ratio of fluorescent cells and non-fluorescent cells, arriving at more than 70%. Positive and stable transfectants were selected and expanded for further study. The MMP-7 siRNA virus vector-infected clone, the negative control vector-infected cells, and LAC cells were named as siMMP-7 group, negative control (NC) group, and LAC control (CON) group, respectively.
[\textsuperscript{3}H]-thymidine incorporation

DNA synthesis was determined by measuring \textsuperscript{[3}H\textsuperscript{-}]\textsuperscript{-}thymidine incorporation. Cells were plated onto 24-well plates at a density of 1.0×10\textsuperscript{5} cells/well in quadruplets. Cells were serum deprived for 24 h, and serum stimulated in culture media containing 1.5 \(\mu\text{Ci/mL}\) tritiated thymidine (\textsuperscript{[3}H\textsuperscript{-}]dT) (specific activity of 740 GBq/mmol) (Perkin-Elmer, Waltham, MA, USA) for 4 h. Cells were fixed and washed in ice-cold 10\% trichloroacetic acid. DNA was solubilized in 0.1 mol/L NaOH for 1h at 37°C. \textsuperscript{[3}H\textsuperscript{-}]dT incorporated into the DNA was measured using liquid scintillation counting.

Transwell invasion assay

Transwell filters were coated with Matrigel (3.9 mg/m\textsuperscript{2}L; 60-80 \mu\text{L}) on the upper surface of a polycarbonate membrane (diameter, 6.5 mm; pore size, 8 \mum). After incubating at 37°C for 30 min, the Matrigel solidified and served as the extracellular matrix for analysis of tumor cell invasion. Harvested cells (1×10\textsuperscript{5}) in 100 \mu\text{L} of serum-free DMEM were added into the upper compartment of the chamber. A total of 200 \mu\text{L} of conditioned medium derived from NIH3T3 cells was used as a source of chemoattractant, which was placed in the bottom compartment of the chamber. After 24 h of incubation at 37°C with 5% CO\textsubscript{2}, the medium was removed from the upper chamber. The non-invaded cells on the upper side of the chamber were scraped off with a cotton swab. Cells that had migrated through the Matrigel into the pores of the inserted filter were fixed with 100\% methanol, stained with hematoxylin, then mounted and dried at 80°C for 30 min. The number of cells invading through the Matrigel was counted in 3 randomly selected visual fields from the central and peripheral portion of the filter by using an inverted microscope (200× magnification). Each assay was repeated 3 times.

Statistical analysis

SPSS 20.0 was used for statistical analyses. The Kruskal-Wallis H test, \(\chi^2\) test, and one-way analysis of variance (ANOVA) were employed to analyze the expression rate in all groups. The LSD method of multiple comparisons was used when the probability for ANOVA was statistically significant. Significance was defined as \(P<0.05\).

Results

The expression of MMP-7 and COX-2 in LAC tissues

The expression of MMP-7 and COX-2 protein was evaluated using IHC staining in LAC tissues. As shown in Figure 1, different levels of positive expression of MMP-7 and COX-2 protein were examined in LAC tissues. Positive MMP-7 and COX-2 immunostaining was localized in the cytoplasm of cancer tissue cells. According to the MMP-7 immunoreactive intensity, the positive expression of MMP-7 and COX-2 was significantly increased in LAC tissues compared with those in ANCT (\(P<0.001\)) (Table 1).

Association between MMP-7 expression and clinicopathologic characteristics

The relationship between MMP-7 expression and various clinical and pathologic characteristics of LAC patients was analyzed. As indicated in Table 2, no significant correlation was found between MMP-7 expression and age or gender. According to the pathological TNM staging, the cases were divided into two groups: stage I+II and stage III+IV. The group with early stage showed elevated rate of MMP-7 expression, but these two groups had no significant difference (\(P=0.175\)). Neither did the group T1+T2 and group T3+T4 based on tumor size (\(P=0.943\)). The cases were then divided into two groups: those with and those without lymph node metastases. The rate of MMP-7 expression was higher in 50.0\% (25/50) of LAC with lymph node metastases than that in...
26.0% (13/50) of LAC without lymph node metastases (P=0.014).

The effect of COX-2 on the expression of MMP-7 and cell invasion

According to our previous work, adenovirus-mediated siCOX-2 was successfully constructed, and used to instantly transfect into LAC A549 cells. Then, the mRNA and protein expression levels of COX-2 and MMP-7 were detected by Real-time PCR (Figure 2 A,B) and Western blot assays (Figure 2 C-F). It was shown that the expression levels of COX-2 and MMP-7 were both decreased in siCOX-2 group compared to negative control group (CON) (**P<0.01). To determine the effect of COX-2 on the invasive potential of LAC cells, the Transwell assay was carried out. It was found that the invasive potential of LAC cells, the Transwell assay can be seen in Figure 6A. We found that the expression level of MMP-7 was significantly knocked down by siRNA in siMMP-7 group compared to NC and CON groups (**P<0.01).

Knockdown of MMP-7 expression in LAC A549 cells

After LAC A549 cells were stably transfected with lentivirus-mediated siMMP-7, the mRNA and protein expression level of MMP-7 were examined by Real-time PCR (Figure 4A) and Western blot assays (Figure 4B). We found that the expression level of MMP-7 was significantly knocked down by siRNA in siMMP-7 group compared to NC and CON groups (**P<0.01).

The effect of MMP-7 knockdown on cell invasion

To confirm the effect of MMP-7 knockdown on tumor growth in LAC A549 cells, we evaluated the proliferative activities of LAC cells by MTT assay. We found that MMP-7 knockdown markedly suppressed the proliferative activities of LAC cells in a time-dependent manner compared to NC and CON groups (Figure 5A). Thus, the effect of MMP-7 knockdown on cell proliferation was further established by measuring [3H]dT incorporation following 4 h serum stimulation of cells deprived of serum for 24 h. The increase of [3H]dT incorporation induced by serum was higher in CON and NC groups than in siMMP-7 group, and the difference was statistically significant (**P<0.01) (Figure 5B). To understand molecular mechanisms of MMP-7 on cell proliferation, we examined the expression of PCNA in Lv-siMMP-7-transfected LAC cells by Western blot assay (Figure 5C,D), indicating that the expression level of PCNA was significantly reduced in siMMP-7 group compared to NC and CON groups (**P<0.01).

The effect of MMP-7 knockdown on cell proliferation

To determine the effect of MMP-7 knockdown on the invasive potential of LAC cells, a Transwell assay was performed. The invasive potential of tumor cells in Transwell assay was determined by the ability of cells to invade a matrix barrier containing laminin and type IV collagen, the major components of the basement membrane. Representative micrographs of Transwell filters can be seen in Figure 6A. We found that the invasive potential of LAC cells was decreased in siMMP-7 group compared to NC and CON groups (**P<0.01) (Figure 6B).

Discussion

Matrix metalloproteinases (MMPs) play a crucial role in physiological and pathological matrix turnover. Some studies have shown that, the expression of MMP-1, -2, -7 and -10 is significantly increased in NSCLC, and can serve as independent prognostic factors for unfavorable outcome. Increased expression of MMP-1/-9 stimulates cell invasion and metastasis of lung cancer cells, while tissue inhibitors of MMPs inhibited cell invasion of LAC cells, suggesting that MMPs including MMP-7 may play an important role in the development of LAC. MMP-7, also known as matrilysin, is a minimal domain MMP that exhibits proteolytic activity against components of the extracellular matrix (ECM). MMP-7 is frequently overexpressed in human cancer tissues and plays an important role in cancer progression. Overexpression of MMP-7 is correlated with differentiation, lymph node metastasis and local invasiveness in some cancers. Moreover, it contributes to a poor prognosis in patients with colorectal cancer (CRC), intrahepatic cholangiocarcinoma and renal cell carcinoma, suggesting that MMP-7 can be used as a predictive marker of unfavorable prognosis in cancer patients. Considering the few studies of MMP-7 in LAC metastasis, in our present study, it was found

Table 1. The expression of MMP-7 protein in human lung adenocarcinoma.

| Target | Group | Total | Score | Positive rate (%) | \( \chi^2 \) | P |
|--------|-------|-------|-------|-------------------|----------|---|
| MMP-7  | LAC   | 50    | -     | 12                | +        | ++ | +++ | 67.6 | 16.328 | <0.001 |
| ANCT   | 50    | 28    | 14    | 1                 | 7        | 1  | 44.0 | 16.328 | <0.001 |
| COX-2  | LAC   | 50    | -     | 7                 | 19       | 15 | 9   | 86.0 | 0.039 | 0.005 |
| ANCT   | 50    | 21    | 14    | 11               | 4        | 58.0 | 0.039 | 0.005 |

LAC, lung adenocarcinoma; ANCT, adjacent non-cancerous tissues.

Table 2. Correlation of MMP-7 expression with clinicopathologic characteristics of LAC patients.

| Variables          | Cases (n) | MMP-7 | \( \chi^2 \) | P |
|--------------------|-----------|-------|------------|---|
| Total              | 50        | -     | +          | 0.034 | 0.853 |
| Age (years)        | 50        | -     | +          | 0.143 | 0.705 |
| <60                | 28        | 7     | 21         | 1.836 | 0.175 |
| ≥60                | 22        | 5     | 17         | 1.836 | 0.175 |
| Gender             |           |       |            | 0.005 | 0.943 |
| Male               | 31        | 8     | 23         | 0.005 | 0.943 |
| Female             | 19        | 4     | 15         | 0.005 | 0.943 |
| TNM staging        | 50        | -     | +          | 0.005 | 0.943 |
| I+II               | 29        | 9     | 20         | 0.005 | 0.943 |
| III+IV             | 21        | 3     | 18         | 0.005 | 0.943 |
| Tumor size         | 50        | -     | +          | 0.005 | 0.943 |
| T1+T2              | 42        | 10    | 32         | 0.005 | 0.943 |
| T3+T4              | 8         | 2     | 6          | 0.005 | 0.943 |
| Lymph node metastases | 50  | -     | +          | 0.005 | 0.943 |
| No                 | 22        | 9     | 13         | 0.005 | 0.943 |
| Yes                | 28        | 3     | 25         | 0.005 | 0.943 |

LAC, lung adenocarcinoma.
Figure 2. The effect of COX-2 on the expression of MMP-7. After LAC A549 cells were transfected with siCOX-2 adenovirus for 24 h, the expression levels of COX-2 and MMP-7 were detected by Real-time PCR (A, B) and Western blot assays (C-F). The expression of COX-2 and MMP-7 was significantly decreased in siCOX-2 group compared with the CON and NC groups (each **P<0.01), suggesting that COX-2 might upregulate the expression of MMP-7 in LAC cells.

Figure 3. The effect of COX-2 knockdown on cell invasion. A,B) Transwell assay was used to determine cell invasion; cell invasive potential was markedly inhibited in siCOX-2 group compared with the CON and NC groups (**P<0.01), suggesting that targeted inhibition of COX-2 might block invasion of LAC cells. Scale bars: A) 75 µm.

Figure 4. Knockdown of MMP-7 expression in LAC A549 cells. After LAC A549 cells were transfected with siMMP-7 lentivirus for 24 h, the expression level of MMP-7 was detected by real-time PCR (A) and Western blot assays (B,C), indicating that the expression of MMP-7 could be knocked down in siMMP-7 group compared with CON and NC groups (**P<0.01).

Figure 5. The effect of MMP-7 knockdown on cell proliferation. A) MTT assay was used to evaluate cell proliferative activity for consecutive 3 days; cell proliferative activity was remarkably diminished in a time-dependent manner in siMMP-7 group compared with the CON and NC groups (**P<0.01). B) [3H] thymidine incorporation into DNA was assessed by scintillation counting. Results are expressed as percentage of increase of [3H] thymidine incorporation induced by serum over that of quiescent cells for each cell population, indicating that the increase of [3H]dT incorporation induced by serum was higher in CON and NC groups than in siMMP-7 group (**P<0.01). C,D) The expression level of PCNA protein, examined by Western blot assay, was downregulated in siMMP-7 group compared with the CON and NC groups (**P<0.01), suggesting that knockdown of MMP-7 might inhibit proliferation of LAC cells through downregulation of PCNA expression.
that MMP-7 was highly expressed in the cytoplasm of LAC tissues compared to the ANCT, and correlated with lymph node metastases in LAC patients, indicating that cytoplasmic accumulation of MMP-7 might be involved in the development and progression of LAC.

Furthermore, some studies show that overexpression of MMP-7 promotes tumorigenesis through enhancing cell migration, invasion and cellular proliferation. In addition, inhibition of MMP-7 activity by specific or non-specific inhibitor suppresses tumor metastasis and angiogenesis in lung cancer. Inversely, Liu et al. found that MMP-7 inhibits proliferation and modulates sensitivity of lung cancer cells to FasL-mediated apoptosis, indicating that MMP-7 may be multiple, multifarious, and multifaceted functions involving in tumorigenesis. Up to now, few reports have been reported about the function of MMP-7 in LAC cells. Our present study showed that knockdown of MMP-7 by RNA interference suppressed the expression of PCNA and proliferative activity of LAC cells. PCNA is a nuclear protein that is expressed in proliferating cells and may be required for maintaining cell proliferation, and used as a marker for cell proliferation of LAC. Thus, our data showed knockdown of MMP-7 might reduce cell proliferation of LAC cells. Scale bar: A) 75 µm.

Figure 6. The effect of MMP-7 knockdown on cell invasion. A-B) Transwell assay was performed to determine cell invasion; cell invasive potential was markedly weakened in siMMP-7 group compared with the CON and NC groups (**P<0.01), suggesting that knockdown of MMP-7 might inhibit invasion of LAC cells. Scale bar: A) 75 µm.

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