Effect of cisplatin on expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 and their correlations in Lewis lung cancer in mice

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Abstract Background and Objective: Metastasis of lung cancer is the leading cause of disease progression and treatment failure. Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are related to the metastasis of lung cancer via regulating the degradation of extracellular matrix. This study was to observe the impacts of cisplatin (DDP) on the expression of MMP-9 and TIMP-1 in Lewis lung cancer, and explore their correlations and roles in metastasis. Methods: Lewis lung cancer model was established in C57BL/6 mice. DDP group was given intraperitoneal DDP injection, and compared with normal control and tumor-bearing groups. The expression of MMP-9 and TIMP-1 were determined by ELISA in serum and detected by immunohistochemistry in tumor tissues. Results: The inhibition rates of tumor growth and metastasis were 41.2% and 39.0% in DDP group, respectively. The positive rates of MMP-9 and TIMP-1 were 100% in tumor-bearing group, and their serum concentrations were significantly higher in tumor-bearing group than in normal control group (P<0.05). Serum concentrations of MMP-9 and TIMP-1 and positive rate of MMP-9 were all significantly lower in DDP group than in tumor-bearing group (P<0.05). Serum concentration of MMP-9 and positive rates of MMP-9 and TIMP-1 were positively correlated to tumor weight (r=0.665, 0.749 and 0.615, all P<0.05) and lung metastasis (r=0.668, 0.545 and 0.664, all P<0.05). MMP-9 expression was positively correlated to TIMP-1 expression both in serum and tumor (r=0.617 and 0.695, all P<0.05). The ratio of sMMP-9/TIMP-1 became a constant in normal distribution, with a mean of 1.72. Conclusions: Both MMP-9 and TIMP-1 are highly expressed in Lewis lung cancer, correlated to tumor invasion and metastasis. DDP may suppress tumor metastasis via down-regulating the expression of MMP-9 and TIMP-1 in serum and tumor. Key words: Lewis lung cancer, cisplatin, metastasis, MMP-9, TIMP-1, mouse.
14% \textsuperscript{1}, which may attribute to the uncontrollable distant metastasis resulting in disease progression and therapeutic failure. Current studies show that the metastasis of lung cancer is associated with the abnormality in the expression of matrix metalloproteinase (MMP) \textsuperscript{2,3} and its inhibitor tissue inhibitor of metalloproteinase (TIMP). MMP-9 is an important member of gelatinases in MMPs family whereby the highly expressed MMP-9 and its inhibitor TIMP-1 in lung cancer tissue is associated with the poor prognosis of lung cancer as a predictive factor;\textsuperscript{2,3} and the dynamic balance between MMP-9 and TIMP-1 is closely associated with the metastasis of cancer cells. The anti-metastatic effect of cisplatin on Lewis lung cancer transplanted tumor was examined in C57BL/6 mice and the expression of MMP-9 and TIMP-1 and their serum levels were also measured in treated tumors, to analyze the expression of MMP-9 and TIMP-1 and their interactions in experimental lung cancer and investigate the role of MMP-9 and TIMP-1 in the process of lung cancer invasion and metastasis. And the role of the dynamic balance in lung cancer and the mechanism of action in which cisplatin inhibited the metastatic lung cancer were hereby discussed.

**Materials and methods**

**Materials.** Reagents. Frozen dry cisplatin (DDP) powder was purchased from Qilu Pharmaceutical Co., Ltd., Shandong (20mg each vial, batch number 20023461). Cisplatin powder 20 mg was diluted in 200 mL normal saline prior to the use, and prepared to 10% solution for further use.

Animals and tumor cell line. A total of 24 C57BL/6 purebred mice (12 male and 12 female) weighing (17 ± 1) g were purchased from China Science Academy Shanghai Slac Laboratory Animal Co., Ltd. (Certificate No. SCXK (SH): 2007-0005) and housed in Longhua Hospital Laboratory Animal Center. And C57BL/6 Lewis lung cancer mice were provided by Shanghai Institute of Pharmaceutical Industry.

ELISA and immunohistochemical reagents, MMP-9 and TIMP-1 ELISA kits were purchased from Bionewtrans Pharmaceutical Bioengineering Co., Ltd. (BBP). Rabbit-anti-mouse MMP-9 and TIMP-1 antibodies and Envision secondary antibody were purchased from Santa Cruz Co. And DAB kit was purchased from Wuhan Boster Co.

**Establishment of animal model and grouping.** Tumor mice were killed by cervical dislocation and subaxillary tumor was dissected. And the superficial vessels, connective tissues and internal necrotic tissues were removed and rinsed with normal saline. The tumor tissues were further divided into pieces and homogenized into cell suspension, resuspended in normal saline at the density of $1 \times 10^7$/mL.

C57BL/6 mice (n=24) were randomized into normal control, tumor-bearing control and DDP groups, with eight mice in each group (four male and four female). Mice from tumor bearing control and DDP groups were injected with 0.2 mL cell suspension (containing $2 \times 10^6$ cells) in their right subaxillary regions. Drug intervention was initiated at day 2: mice in DDP group were daily intraperitoneally injected with 1 mL 10% cisplatin solution i.p. for three days and tumor-bearing group was lavaged with 0.4 mL normal saline, twice daily. All the mice were killed at day 20 (post-dose) and indices were appropriately examined.

**Observational indices and methods.** Inhibition rate and lung metastasis inhibition rate. Tumor-bearing mice were killed to retrieve the tumor, which was weighed on an electronic scale. The inhibition rate was calculated with the following formula:
Inhibition rate = (average tumor weight of tumor-bearing control group average tumor weight of experimental group)/ average tumor weight of tumor-bearing control group × 100%.

The thoracic cavities of mice were opened to dissect the tracheas. And 1 mL syringe containing Bouins solution was inserted through the cervical trachea into carina. Appropriate amount of Bouins solution was injected into left and right bronchi to inflate lungs, which were stained yellowish. The trachea below the needle was ligated with a silk suture and the needle was
subsequently withdrawn. And the trachea was dissected from below the ligature to take out the lungs, which was immediately fixed in Bouins solution. Lung metastasis was counted after 24 hours of fixation.

Lung metastasis inhibition rate = (average metastasis number of tumor-bearing control group average metastasis number of experimental group)/ average metastasis number of tumor-bearing control group × 100%.

ELISA assays of serum MMP-9 and TIMP-1. Retro-orbital fresh blood was sampled and centrifuged with Eppendorf Centrifuge 5415R centrifuge (7347 × g) at 4°C for 12 minutes. And the supernatant was aspirated to obtain the serum. MMP-9 and TIMP-1 ELISA assays were strictly performed based on the manufacturers instructions. Absorbance (A) of each sample were read with Thermo Labsystem Multiskan MK3 enzyme-labeling instrument at the wavelength of 450 nm; the standard curve was plotted with CurveExpert V1.38 to export the curve function, showing MMP-9 and TIMP-1 concentrations of each sample.

Immunohistochemical examination of MMP-9 and TIMP-1. Tumors were routinely dehydrated, vitrified and embedded in paraffin. Paraffin sections were routinely dewaxed and rehydrated. Sections were treated with 0.3% H2O2 at room temperature for 20 minutes and rinsed with distilled water for three times. The sections were retrieved with 0.01 M citrate buffer (pH = 6.0) and microwaved until the boiling at the interval of five minutes for three times, followed by the rinse with PBS (pH = 7.3) for twice after cooling. Sections were blocked with 5% BSA at room temperature for 20 min and the excess serum was removed. The primary antibodies (rabbit-anti-mouse MMP-9 and TIMP-1 IgG) were added at 37°C for one hour and rinsed with PBS for two minutes for three times. EnVision secondary antibodies were added at 37°C for 30 minutes and rinsed with PBS for two minutes for three times. DAB was added at room temperature for colorization and the reaction duration was microscopically controlled around ten minutes, followed by the rinse with distilled water. Sections were mildly counterstained with hematoxylin, dehydrated, vitrified, mounted and examined by microscopy.

PBS in place of primary antibodies was used as the negative control while MMP-9 and TIMP-1 stained sections from breast cancer samples were used as positive control. Sections were microscopically examined at the magnification of 400 × and three visual fields were randomly selected to count the number of positive cells. Receptor positivity = number of positive tumor cells/total number of tumor cells × 100% whereby the positivity < 20% was taken as negative (−), that from 21% to 50% as weak positive (+), that from 51% to 70% as moderate positive (++), and that from 71% to 90% as strong positive (+++).

Statistical analysis. All data were processed with SPSS 16.0. Quantitative data from two groups were compared with Student-t test, those from multiple groups were tested with ANOVA and the correlation coefficient was tested with correlation analysis. P < 0.05 was considered statistically significant.

Results

Tumor weight and inhibition rate. The tumors of DDP group weighed (4.77 ± 1.59) g at average, significantly lower than that of tumor-bearing control group (8.11 ± 1.09) g (P < 0.01), and the inhibition rate was 41.2%.

Number of lung metastasis and lung metastasis inhibition rate. The mean number of lung metastasis of DDP group was 4.50 ± 1.69, significantly less than that of tumor-bearing control group (7.38 ± 2.20) (P < 0.05), and the lung metastasis inhibition rate was 39.0%.

Expression of MMP-9 and TIMP-1 in Lewis lung cancer mice serum. MMP-9 and TIMP-1 in mice serum, sMMP-9 in normal control, tumor-bearing control and DDP groups were (1.00 ± 0.14) ng/mL, (1.33 ± 0.33) ng/mL and (0.72 ± 0.26) ng/mL, respectively, as a result, tumor-bearing control group was significantly higher compared to normal control group (P < 0.05); sTIMP-1 in normal control, tumor-bearing control and DDP groups were (1.44 ± 0.46) ng/mL, (2.35 ± 0.52) ng/mL and
(1.29±0.60) ng/mL, respectively, as a result, tumor-bearing control group was significantly higher compared to normal control group (P<0.01), suggesting that both sMMP-9 and sTIMP-1 were highly expressed in tumor-bearing mice. And both sMMP-9 and sTIMP-1 were significantly lower in DDP group than both tumor-bearing and normal control groups (P<0.05).

Correlation of serum MMP-9 with tumor weight and number of lung metastasis. sMMP-9 averaged at (1.03 ± 0.42) ng/mL in tumor-bearing mice (n=16) whose tumors weighed (6.44 ± 2.17) g, showing a significant positive correlation of sMMP-9 to tumor weight (r=0.665, P=0.005). And the number of lung metastasis averaged at 5.94 ± 2.41, showing a significant positive correlation of sMMP-9 to the number of lung metastasis (r=0.668, P=0.005).

Correlation of serum TIMP-1 to tumor weight and number of lung metastasis. Tumor weight averaged at (6.44 ± 2.17) g in tumor-bearing mice (n=16), with the number of lung metastasis at 5.94 ± 2.41, and sTIMP-1 averaged at (1.82 ± 0.77) ng/mL showing no significant positive correlation of sTIMP-1 to tumor weight (r=0.411, P=0.114) but a positive correlation of sTIMP-1 to the number of lung metastasis in a statistically insignificant manner (r=0.494, P=0.052).

Expression of MMP-9 and TIMP-1 in Lewis lung cancer mice tumors. 2.4 MMP-9 and TIMP-1 in tumor-bearing mice

Both MMP-9 and TIMP-1 were detected in cytoplasm as yellowish or brownish stained particles while cell membranes were sometimes yellowish or brownish stained and nuclei were occasionally stained. The positive rates of MMP-9 and TIMP-1 in tumor-bearing control group were both 100% while only one mouse in DDP group expressed MMP-9 (+), with the positive rate of 12.5% (1/8), significantly lower than that of tumor-bearing control group (Ridit test, P<0.01) (Fig 1). In DDP group, seven mice expressed TIMP-1, with the positive rate of 87.5% (7/8) in a statistically insignificant manner if compared to that of tumor-bearing control group (Ridit test, P>0.05) (Fig 2).

Correlation of MMP-9 expression to tumor weight, lung metastasis and sMMP-9. Spearman rank analysis showed that expression of MMP-9 was positively correlated to tumor weight and the number of lung metastasis in a statistically significant manner (r=0.749 and 0.545, P<0.05). And the expression of MMP-9 was also positively correlated to sMMP-9 in a statistically significant manner (r=0.768, P<0.01).

Correlation of TIMP-1 expression to tumor weight, lung metastasis and sTIMP-1. Spearman rank analysis showed that expression of TIMP-1 was positively correlated to tumor weight and the number of lung metastasis in a statistically significant manner (r=0.615 and 0.664, P<0.05). And expression of TIMP-1 was also positively correlated to sTIMP-1 in a statistically
significant manner ($r_s = 0.625, P=0.01$).

**Correlation between TIMP-1/MMP-9 in Lewis lung cancer bearing mice.** Correlation between MMP-9 and TIMP-1 in tumor. Spearman rank analysis showed that expression of MMP-9 was positively correlated to that of TIMP-1 in a statistically significant manner ($r_s=0.617, P<0.05$). Moreover, six out of seven MMP-9 negative mice (85.7%) expressed TIMP-1 positively, all the three MMP-9 weak positive mice expressed moderate or strong positive TIMP-1, two out of four MMP-9 moderate positive mice expressed strong positive TIMP-1, and both the two MMP-9 strong positive mice expressed strong positive TIMP-1, indicating that the expression of TIMP-1 was slightly stronger than that of MMP-9 in the same tumor (Table 1).

![Figure 2](image)  
Expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in Lewis lung cancer (EnVision IHC ×400)  
A: In tumor-bearing group, TIMP-1 is intensely expressed (in yellow or dark yellow) in cytoplasm, partially on cell membrane and few in nuclei of Lewis lung cancer cells.  
B: In cisplatin group, TIMP-1 is weakly expressed in cytoplasm and few in nuclei of Lewis lung cancer cells.

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<tr>
<th>Expression of MMP-9</th>
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<td>Negative</td>
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**Table 1 Relationship between expression of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1)**

Correlation of MMP-9 to TIMP-1 in serum. In C57BL/6 mice (n=24), the mean sMMP-9 and sTIMP-1 were (1.02± 0.35) ng/mL and (1.69± 0.70) ng/mL, respectively, showing a significantly linear correlation ($r=0.695, P<0.01$).

Serum TIMP-1/MMP-9 ratios among the groups and correlation to tumor weight and the number of lung metastasis. The TIMP-1/MMP-9 ratios in normal control, tumor-bearing control and DDP groups were 1.47± 0.55, 1.82± 0.42 and 1.88± 0.85, respectively, indicating that the TIMP-1/MMP-9 ratio was a constant coefficient, not interfered by tumor growth and therapeutic factors. The TIMP-1/MMP-9 ratio was not statistically correlated to tumor weight and the number of lung metastasis ($r_s=-0.325, r=-0.268, P>0.05$).

![Figure 3](image)  
Distribution of the ratio of sTIMP-1/MMP-9 ratio. In C57BL/6 mice (n=24), the mean
TIMP-1/MMP-9 ratio was 1.72 ± 0.63, with kurtosis value of 1.654 and the skewness value of 0.759, indicating that the overall distribution of TIMP-1/MMP-9 ratio was generally characterized as a normal distribution (Fig. 3). The molecule weights of TIMP-1 and MMP-9 were 28.5 ku and 92 ku, respectively, and the TIMP-1/MMP-9 mass concentration ratio was converted into the molecule ratio at 5.56:1 according to the ratio of these two molecule weights.

**Discussion**

Distant metastasis of lung cancer is the major cause of disease aggravation and death. The process of metastasis consists of the detachment from primary lesion, infiltration through basement membrane (BM) and extracellular matrix (ECM), invasion into vascular or lymphatic vessels, maintenance in blood or lymph fluid, transfer from the circulation into the new site, neoangiogenesis and formation of metastatic lesions whereby the degradation of ECM and infiltration of BM by tumor cells is the initial step of malignant invasion and metastasis. Multiple protein enzymes deriving from tumor cells are capable of degrading ECM while MMPs are the most important. MMPs are mainly divided into five classes: collagenase, stromelysin, gelatinase, membrane type and others. Both MMP-9 and MMP-2 are gelatinases, whose substrates mainly consist of gelatin and non-fibrous type IV collagen, the major component of ECM, critical for the invasion and metastasis of lung cancer. Recent studies have shown that the strong potential of tumor metastasis was correlated to the high expression of MMP-9. TIMP is an in vivo natural inhibitor of MMP, which is synthesized and secreted by multiple cells, directly binds to activated MMP in a complex manner to inhibit its activity. The development of MMP inhibitors (MPIs) is ongoing, aiming to block the invasion and metastasis of tumor. However, the clinical outcomes were not acceptable, substantially restricting the clinical development and use of MPIs.

Gouyer et al. showed that both MMP-9 and TIMP-1 were highly expressed in lung cancer tissue, as a predictive factor of poor prognosis. Aljada et al. also showed that the expression of TIMP-1 was highly increased in postoperative lung cancer lesions and the patients with highly expressed TIMP-1 had their mortality risks higher than those with low expression by 90%. Previous domestic studies also confirmed that both MMP-9 and TIMP-1 were highly expressed in lung cancer tissues than normal lung tissues, and correlated to the invasion and metastasis of tumor; the MMP-9 positive rate in patients with survival period less than two years was significantly higher than those with survival period over two years, indicating the critical role of MMP-9 in the invasion and metastasis of lung cancer. And serum MMP-9 was shown to be higher in lung cancer patients than in normal individuals, considered as a potential new tumor marker of NSCLC. Both MMP-9 and TIMP-1 in the serum of lung cancer patients were higher than those in control group, and the dynamic balance between MMP-9 and TIMP-1 showed some abnormality, indicating the facilitation of disease progression and metastasis. 56% of lung cancer patients with low MMP-9 expression survived for more than one year while only 31% patients with high MMP-9 expression survived more than one year; the one-year survival rate of stage III lung cancer patients with low TIMP-1 expression reached 70% while that of patients with high TIMP-1 expression was only 20%. Therefore, serum MMP-9 and TIMP-1 were markers for lung cancer prognosis.

Our results showed that the positive rates of both MMP-9 and TIMP-1 were 100% in tumors in tumor-bearing mice and their serum MMP-9 and TIMP-1 were both significantly higher than those of normal control mice, confirming the high expression of MMP-9 and TIMP-1 in Lewis lung cancer. Moreover, MMP-9 in both tumor and serum and TIMP-1 in tumor were positively correlated to tumor weight and the number of lung metastasis, indicating the significant correlation of tumor, serum MMP-9 and tumor TIMP-1 to tumor cell proliferation and metastasis, used to evaluate the
invasion and metastasis extent of lung cancer. However, serum TIMP-1 was insignificantly correlated to tumor weight and the number of lung metastasis, possibly attributed to the regulation on the release of TIMP-1 from tumor into blood, which alleviated the correlation of serum TIMP-1 to tumor and metastasis in an unknown mechanism yet to be investigated. Additionally, our correlation analysis showed that expression of MMP-9 and TIMP-1 were positively correlated to serum concentrations of MMP-9 and TIMP-1, indicating the consistence between tumor and serum in terms of their expression. Serum MMP-9 and TIMP could be hereby used to evaluate their tumor counterparts.

Cisplatin treated mice showed an inhibition rate of 41.18% and a lung metastasis inhibition rate of 39.02% whereby the expressions of serum MMP-9 and TIMP-1 were significantly downregulated and the positive rate of tumor MMP-9 was also decreased, a potential mechanism in which cisplatin inhibited lung cancer and its metastasis. And cisplatin treated mice also showed decreased MMP-9 and TIMP-1 in their serum compared to normal controls, whose mechanisms and targets were yet to investigated in terms of the inhibition on protein synthesis. Meanwhile, cisplatin had less effect on the positive rate of tumor TIMP, likely attributed to the limited effects of cisplatin on its expression, although cisplatin substantially inhibited the release of TIMP, less significantly decreased its expression in tumor. Thus, serum MMP-9 could be used as the outcome indicator of cisplatin regulating the tumor MMP-9 expression while in contrast serum TIMP could not be used for the evaluation of tumor TIMP-1.

It has been currently accepted that TIMPs directly bind to activated MMPs with non-covalent bond as a 1:1 complex, for example, TIMP-1 binds to MMP-9 and inhibits its activity. MMP and TIMP are hereby functionally balanced to maintain the dynamics of ECM. Multiple studies confirmed the high expression of TIMP-1 in lung cancer and its correlation to poor prognosis or factors and the dynamic balance between MMP and TIMP was considered critical for their intrinsic functional relationship. Many studies reported that TIMPs were less highly expressed than MMPs, which was not adequate to inhibit the activity of highly expressed MMPs, a possible explanation that the high expression of TIMP was otherwise correlated to poor prognosis. Some other studies found that TIMP-1 of higher concentration showed a growth factor-like effect in vitro, which otherwise promoted the growth of tumor cells. Our results showed that MMP-9 was positively correlated to MMP-9 and TIMP-1 in either tumor or serum; the serum TIMP-1/MMP-9 ratio was not correlated to tumor weight or lung metastasis or interventional measures, which showed a constant trend in all the mice examined (normal, tumor-bearing and intervened mice) as a normal distribution in the population, averaging at 1.72 or 5.56: 1 if converted into molecular ratio. Moreover, the intensity of tumor TIMP-1 expression also showed a trend over that of MMP-9, which was attributed to a potential physiological regulation mechanism of TIMP-1/MMP-9 whereby the highly expressed TIMP-1 derived from the response to the highly expressed MMP-9. Meanwhile, TIMP-1 also inhibited MMP-2, resulting in the TIMP-1/MMP-9 ratio beyond the theoretical value of 1:1. Tumors interacted with their hosts in some manner and their expression of tumor proteins were also regulated by the hosts, and mice were likely to allow TIMP-1 to be upregulated with the high expression of MMP-9 through such mechanism in the process of Lewis lung cancer progress, which regulated the dynamic balance between TIMP-1 and MMP-9 and maintained the ratio constant, deriving from the interaction between tumor cells and their hosts yet to be further investigated. A similar regulation mechanism was likely to be available for human lung cancer, possibly with a different constant remaining unknown. And such mechanism upregulated TIMP-1 while maintained the TIMP-1/MMP-9 ratio constant, resulting in substantial increase in TIMP-1 absolute level when its function switched into facilitating the growth of tumor cells. Therefore, although extrinsic MPIs were supplemented to
interfere with the TIMP-1/MMP-9 ratio, their effects on tumor cell invasion and metastasis were still restricted. The unacceptable clinical outcomes were reasonable. And the regulation mechanism is to be investigated in further studies due to its complexity.

References