Matrix Metalloproteinase Activity in Early-Stage Lung Cancer

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Summary
Background: The matrix metalloproteinases (MMPs)-2, -9 and -7 are thought to be associated with tumor invasion, metastasis, and angiogenesis. However, their possible roles in early-stage lung cancer are not clear. We measured the activity of MMP-2, -7 and -9 in early-stage lung cancer tissues. Material and Methods: Normal lung tissues and cancer tissues were collected from 60 consecutive stage-I non-small cell lung cancer (NSCLC) patients. The activities of MMP-2 and MMP-9 were determined by gelatin zymography, and the activity of MMP-7 was determined by casein zymography. Furthermore, the ratio of the active form of MMP-2 in tumor tissue (T) compared with normal tissue (N) was determined, and the survival in the groups with different MMP-2 T:N ratio was compared. Results: The activity of both MMP-2 and MMP-9 was detected in all cancer and normal tissues. Interestingly, MMP-9 activity was significantly reduced, whereas MMP-2 activity was significantly increased, in cancer tissues compared to normal tissues. The survival rate of the MMP-2 T:N ratio > 2.5 group was 57.45%, which was significantly reduced compared with that of the T:N ratio ≤ 2.5 group (86.78%). Conclusion: Our findings suggest that MMP-2, but not MMP-9 and MMP-7, may be implicated in early-stage tumor invasion, metastasis, and angiogenesis in NSCLC.
Introduction

Matrix metalloproteinases (MMPs), especially MMP-2, -9 and -7, may play crucial roles in invasion and metastasis of malignant tumors [1]. MMPs can degrade extracellular matrix (ECM) and intensify the attack of cancer cells on the normal tissue. In addition, they may play a key role in tumor angiogenesis [2]. Increased expression/activity of MMPs is also found in lung cancer patients [3]. Recently, MMP inhibitors have been developed to prevent and treat invasion, metastasis, and angiogenesis of malignant tumors including lung cancer. However, as far as we are aware, no clinical trial has so far achieved satisfying results. Although chemical components, clinical trial design, and patient selection might be important factors, a better understanding of the role of MMPs in the pathophysiology of malignant tumors may be needed [4]. In this study, we measured the activity of MMP-2, -9 and -7 in tumor tissues of early-stage non-small cell lung cancer (NSCLC).

Material and Methods

Tissue Collection

Tumor tissues and corresponding normal lung tissues from a total of 60 consecutive stage-I NSCLC patients (male: n = 49; female: n = 11) were collected during surgery between August 2009 and December 2010. Tissue samples were frozen with liquid nitrogen immediately after being removed and stored at –80 °C. According to the classification system of the International Union against Cancer (UICC), 36 cases were pT1N0M0Ia and 24 cases were pT2N0M0Ib. In addition, 25 cases were squamous carcinoma and 35 cases were adenocarcinoma. The age range of the patients was 44–86 years, and the average age was 63.3 ± 6.3 years. The basic clinical feature of the patients are summarized in table 1.

Gelatin Zymography

Gelatin zymography was used to measure the activity of MMP-2 and MMP-9 as described previously [5]. Briefly, tissue extracts were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel containing 0.1% gelatin and rinsed in dd-H₂O followed by incubation with a bulky volume of renaturation buffer (2.7% Triton-X 100 in dd-H₂O) at room temperature for 1 h with gentle shaking. Enzyme activity was developed in 50 mM Tris (pH 7.5), 0.2 M NaCl, 5 mM CaCl₂, and 0.2% Brij-35 at 37 °C for 18 h and stained with Coomassie Blue. MMP activity was analyzed with NIH image software (edition 1.62). Activities of pro-MMP-2 and MMP-2 were quantified at 66 and 62 kDa, respectively. Activity of MMP-9 was considered a lucid band at 92 kDa (fig. 1). MMP activities were calculated according to the method of Davies et al. [6]. Briefly, the ratio of activated MMP-2 to total MMP-2 activity (62 kDa / 66 kDa + 62 kDa) was calculated based on the gelatinolytic activities measured by computer-assisted image analysis. We then determined the T (tumor):N (normal tissue) ratio of the active form of MMP-2 by comparing the percentage of the active form in tumor tissue with that in normal tissue in each case.

Casein Zymography

Casein zymography was used for measuring MMP-7 activity. Tissue extracts were electrophoresed on an 8% polyacrylamide gel containing 2 mg/ml casein. After electrophoresis, gels were washed in 2.5% Triton-X 100 and incubated for 48 h at 37 °C in 50 mmol/l Tris-HCl (pH 7.4), 10 mmol/l CaCl₂, 1 mmol/l ZnCl₂, and 0.02% NaN₃, followed by staining with 0.1% Coomassie Blue. A pregnant woman’s amniotic fluid was used (with added sample buffer at a ratio of 1:1) as positive control. Activity of MMP-7 was quantified at 20 kDa.

Follow-Up

Patients were followed at 3-month intervals after operation. Follow-up evaluation included blood examination and chest X-ray every 3 months and chest computed tomography every 6 months. The follow-up period ranged from 4 to 75 months (median 32 months). We compared the survival in the different MMP-2 T:N ratio groups.

Statistical Analysis

Results are expressed as the mean ± standard error (S.E.), and were compared by Student’s t test. Differences were considered significant at p ≤ 0.05. Survival was analyzed according to the Kaplan-Meier method, and differences in distribution were evaluated by the log-rank test.

Results

The activities of pro-MMP-2 and MMP-2 were quantified at 66 kDa and 62 kDa, respectively. As shown in figure 1, both pro-MMP-2 and MMP-2 activities were detected in tumor and normal lung tissues. However, activity of MMP-2 (62 kDa/62 kDa + 66 kDa) was significantly higher in tumor tissues (45.5 ± 8.4%) compared to normal lung tissue (25.9 ± 10.5%) (p < 0.001). The T:N ratio ranged from 1.2 to 3.8.
No difference was found between the MMP-2 activity in squamous carcinoma and adenocarcinoma. On the other hand, activity of MMP-9 was detected in both normal lung tissue and tumor tissues. Interestingly, activity of MMP-9 was significantly lower in tumor tissues (1,189.3 ± 537 pixel) than in normal lung tissue (1,557.7 ± 422.5 pixel) (p = 0.021) (fig. 1). There was no difference between the MMP-9 activity in squamous carcinoma and adenocarcinoma. As shown in figure 2, although we used different concentrations of tissue extracts and repeated the measurements several times, activity of MMP-7 was only detected in the positive control at 20 kDa, but neither in the tumor tissues nor the normal lung tissues.

**Follow-Up**

During the follow-up period, local and distant cancer recurrence was identified in 14 of the 60 (23.3%) stage-I patients. The sites of recurrence include bone in 2, contralateral lung in 5, brain in 3, mediastinal lymph nodes in 3, and in the same lung in 1 patient. Patients were divided into 2 groups according to differences in the MMP-2 T:N ratio. In the group with T:N ratio > 2.5, the survival rate was 57.45% which was significantly reduced compared with the group with T:N ratio ≤ 2.5 (86.78%) (p = 0.015, log-rank test) (fig. 3). The recurrence-free survival rate of the group with T:N ratio > 2.5 was 46.81% which was significantly reduced compared with the group with T:N ratio ≤ 2.5 (81.23%) (p = 0.001, log-rank test) (fig. 4).

**Discussion**

MMPs play a key role in cancer progression. MMPs such as interstitial collagenase (MMP-1) and type IV collagenases (MMP-2, MMP-9) are involved in the initial breakdown of collagen and basement membrane components during tumor growth and invasion. Overexpression of MMP-1, MMP-2 and MMP-9, MMP-11, MMP-13, MMP-14-17, MMP-24 and MMP-25, and TIMP-1 and TIMP-2 positively correlated with more advanced-stage disease and highly invasive and metastatic potential of carcinomas [7–9].

Previous studies found that protein expression of MMPs was upregulated in lung cancer, and their upregulation appeared to be associated with a poor prognosis. However, protein expression is not always representative of activity. Thus, in the present study, we measured the activities of MMPs. Several studies have shown that MMP-2 activity may be associated with a poor prognosis in lung cancer and other cancers [10]. We found that MMP-2 activity was significantly increased in tumor tissue of NSCLC. This finding is consistent with a previous study; using zymography and immunohistochemistry, Suzuki et al. [10] found that MMP-2 was detected in 5/5 NSCLC, whereas MMP-9 was detected in only 1/5 NSCLC, which suggested that MMP-2 may play a more important role in NSCLC invasion. We previously showed that MMP-2 has a high capacity to decompose tumor extracellular matrix tenascin-C [5]. In this study, we showed that the survival rate of patients with an MMP-2 T:N ratio > 2.5 was 57.45% which was significantly reduced compared to that of patients with a T:N ratio ≤ 2.5 (86.78%). Furthermore, recur-
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A number of studies have reported that protein and mRNA expression of MMP-7 was upregulated in certain lung cancer tissues [11]. Interestingly, we did not detect MMP-7 activity in cancer tissues of early-stage NSCLC. Thus, MMP-7 may not play an important role in invasion, metastasis, and angiogenesis of early-stage NSCLC. In addition, although MMP-9 activity was detected in cancer tissues, MMP-9 activity was surprisingly lower in cancer tissues compared to normal lung tissues. Thus, the role of MMP-9 in early-stage NSCLC needs to be further studied.

In summary, a significant increase in MMP-2, but not MMP-9 and MMP-7, was found in tumor tissue of early-stage NSCLC. Development of a selective MMP-2 inhibitor may be beneficial to prevent and treat invasion, metastasis, and angiogenesis of early-stage NSCLC.

Disclosure Statement

We declare that we have no conflict of interest.

References