Elevated Levels of Circulating Plasma Matrix Metalloproteinase 9 in Non-Small Cell Lung Cancer Patients

Toshihiko Iizasa, Takehiko Fujisawa, Makoto Suzuki, Shin-ichirou Motohashi, Kazuhiro Yashufuku, Tomohisa Yasukawa, Masayuki Baba, and Mitsutoshi Shiba

Department of Surgery, Institute of Pulmonary Cancer Research, Chiba University School of Medicine, Chiba 260-8670, Japan

ABSTRACT

Elevated expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 have been implicated as playing important roles in tumor invasion and metastasis in various tissues. We investigated the relationship between circulating plasma MMP-9, its expression in tumor samples, and other clinical features in patients with non-small cell lung cancer (NSCLC). A series of 73 patients (45 men and 28 women) who underwent surgery for NSCLC was used in this study. Preoperative plasma concentrations of MMP-9 were examined using a one-step sandwich enzyme immunoassay. Expression levels of MMP-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were measured in 24 tumor samples by immunohistochemistry. The plasma concentration of MMP-9 in NSCLC patients (71.0 ± 60.2 ng/ml) was significantly elevated compared to that of healthy volunteers (P < 0.0001). MMP-9 concentrations were elevated in 33 of 73 cases (45.2%), compared with a cutoff value of the mean ± 2 SD in healthy volunteers. There were statistically significant differences in MMP-9 concentration in adenocarcinoma versus squamous cell carcinoma (P = 0.014) and adenocarcinoma versus large cell carcinoma (P = 0.014). Five of 24 patients (20.8%) had positive immunohistochemical MMP staining of the tumor cell cytoplasm, and two cases had positive staining in the surrounding stromal cells. Plasma MMP-9 concentrations were elevated in 45.2% of NSCLC patients; however, this elevation did not seem to correlate with MMP-9 production by cancer and stromal cells. We concluded that the MMP-9 ELISA could be a beneficial adjunct for assessing the tumor burden of NSCLC, especially for types of squamous cell carcinoma and large cell carcinoma.

INTRODUCTION

The MMP family of enzymes is characterized by the presence of a zinc ion at the catalytic domain and is responsible for the proteolytic degradation of the extracellular matrix (1, 2). MMPs are expressed in many physiological conditions including embryogenesis and tissue remodeling after injury as well as in various pathological processes involving tissue destruction (3) such as arthritis, cancer, and osteoporosis.

Several steps are required to develop malignant tumor cell metastasis. The first critical phase is the destruction and penetration of the basement membrane, which is part of the extracellular matrix, by the tumor cells. Reports have demonstrated correlations between the degradation of the basement membrane and the metastatic potential of MMPs (4, 5).

MMP family members MMP-2 and MMP-9 degrade type IV collagen, one of the main constituents of the basement membrane. Physiologically, MMP-2 is produced by fibroblasts (6, 7), whereas MMP-9 is produced mainly by neutrophils (8) and macrophages (6–9).

Expression of MMP-2 and MMP-9 is elevated in some malignant tumor tissues including breast cancer (10–13), colon cancer (8, 9, 14), brain tumors (15, 16), and other malignancies (17). Expression has also been detected in samples of NSCLC. MMP-2 and MMP-9 appear to be expressed in NSCLC tumor cells and the surrounding stromal cells, although the expression of MMP-9 in tumor cells is weak compared with that of MMP-2 by Northern blotting, in situ hybridization, and gelatin zymography (18, 19). In the present study, we focused on the levels of released MMP-9 in the plasma of patients with NSCLC and healthy volunteers using a one-step sandwich enzyme immunoassay with a recently developed anti-MMP-9 monoclonal antibody (20). The antibody was also used to measure the expression of MMP-9 in NSCLC tumor samples by immunohistochemistry to determine the site of plasma MMP-9 production. We examined the clinical implications of circulating MMP-9 by investigating the relationship between MMP-9 plasma levels, MMP-9 expression in tumor samples, and other clinical features of NSCLC patients.

MATERIALS AND METHODS

Patient Plasma and Tumor Tissue Samples. The study group consisted of 73 patients with NSCLC (45 males and 28 females) ranging in age from 48–90 years, with a mean age of 66.7 years. NSCLC was diagnosed histologically in surgically
resected tissues. Blood samples were drawn before surgery and at 2-week intervals after surgery. Histological types included 38 adenocarcinomas, 25 squamous cell carcinomas, 8 large cell carcinomas, and 2 adenosquamous cell carcinomas. Pathological staging revealed 18 patients with stage IA disease, 12 patients with stage IB disease, 3 patients with stage IIA disease, 9 patients with stage IIB disease, 10 patients with stage IIIA disease, 18 patients with stage IIIB disease, and 3 patients with stage IV disease (21). Histological type, pathological stage, and tumor-node-metastasis (TNM) classification were classified according to the criteria of the American Joint Committee on Cancer (21, 22) and the Japan Lung Cancer Society (23). No patient received treatment for NSCLC before surgery. A C-reactive protein test was used as a marker of inflammation and tissue destruction. Healthy volunteers (n = 138) were tested for plasma concentration of MMP-9 as a control group.

**One-Step Sandwich Enzyme Immunoassay for MMP-9.** Plasma MMP-9 concentrations were measured with a one-step sandwich enzyme immuonoassay kit (Fuji Chemical Industries, Toyama, Japan) using the antihuman MMP-9 monoclonal antibody 56-2A4 (20) that binds both the inactive proform and the active form of MMP-9. Each plasma sample (10 μl) was mixed with 100 μl of 50 μg/liter horseradish peroxidase-conjugated anti-proMMP-9 IgG. Aliquots (100 μl) were transferred to microplate wells coated with anti-proMMP-9 IgG. Plates were incubated for 60 min at room temperature without shaking and washed three times with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Microplate wells were then incubated with 100 μl of 0.15 M citric acid-sodium phosphate buffer (pH 4.9) containing 2.0 g/liter o-phenylenediamine and 0.02% (v/v) hydrogen peroxide for 20 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M sulfuric acid (final concentration, 0.5 M), and absorbance was measured at 492 nm. Plasma concentrations of proMMP-9 were calculated using a standard curve (20).

**MMP and TIMP Immunohistochemistry.** Expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 was confirmed immunohistochemically in 24 patient tumor samples to clarify the localization of expression as described previously (24). Each sample was fixed with peroxides-lysine-paraformaldehyde fixative for 18–24 h at 4°C after treatment with monensin (Wako Inc., Tokyo, Japan) in RPMI 1640 at 37°C for 3 h and then embedded in paraffin wax. Immunohistochemical staining was performed using the Catalyzed Signal Amplification System (DAKO, Carpinteria, CA), a modified streptavidin biotin method, with mouse antihuman monoclonal antibodies to MMP-2 (42-D511), MMP-9 (56-2A4), TIMP-1 (147-6D11), and TIMP-2 (67-4H11; Ref. 24; Fuji Chemical Industries) according to the manufacturer’s instructions.

**Statistical Analysis and Determination of a Normal Range for MMP-9 Concentration.** Comparison of MMP-9 concentrations between healthy controls and NSCLC patients was evaluated by the Mann-Whitney U test. A normal range of MMP-9 was calculated as the mean in controls ± 2 SD.

The statistical significance of differences between each clinical feature and plasma concentration of MMP-9 was estimated by the χ² test. Correlations between elevated cases of plasma MMP-9 and positive cases of immunohistochemical staining for MMP-2, MMP-9, TIMP-1, and TIMP-2 were also estimated by the χ² test. The correlation of plasma MMP-9 concentration with tumor size and C-reactive protein test was evaluated with a simple regression analysis. Differences were regarded as statistically significant at P < 0.05.

**RESULTS**

**Concentrations of MMP-9 and Clinical Features in 73 Primary NSCLCs.** As shown in Fig. 1, the plasma MMP-9 concentrations in the 73 NSCLC patients (71.0 ± 60.2 ng/ml, mean ± SD) were significantly higher than those in the 138 healthy controls (36.3 ± 13.2 ng/ml; P < 0.0001). We determined the normal range of plasma MMP-9 concentration as 11.4–59.4 ng/ml (mean in 132 controls ± 2 SD). A total of 33 of 73 (45.2%) patients demonstrated MMP-9 concentrations above the upper limit of the normal range. The MMP-9 concentrations of all cases (including the already elevated cases) increased for several weeks after surgery and returned to levels within the normal range 4–8 weeks after surgery (data not shown).

There were statistically significant differences in plasma MMP-9 levels in adenocarcinoma versus squamous cell carcinoma (P = 0.014) and adenocarcinoma versus large cell carcinoma (P = 0.014; Table 1). However, no significant correlations between other clinical features and plasma concentrations.
of MMP-9 were observed. We were also unable to demonstrate a correlation between MMP-9 concentration and tumor size (correlation coefficient $r = 0.161; P = 0.1733$) and C-reactive protein test (correlation coefficient $r = 0.245; P = 0.1865$; Fig. 2, A and B).

**Immunohistochemical Staining of Tumor Samples for MMP-2, MMP-9, TIMP-1, and TIMP-2.** A total of 5 of 24 patients (20.8%) had positive immunohistochemical staining for MMP-9 in the cytoplasm of tumor cells; however, none of these patients exhibited elevated plasma MMP-9 levels. The stromal cells surrounding the tumor stained positively in two cases (Table 2), and macrophages stained positively in nine cases. Positive staining for MMP-2, TIMP-1, and TIMP-2 was demonstrated in 11 cases (45.8%), 7 cases (29.2%), and 16 cases (66.7%) respectively ($n = 24$; Table 2). Positive immunostaining for MMP-2, MMP-9, TIMP-1, and TIMP-2 showed no significant association with plasma concentrations of MMP-9.

**DISCUSSION**

Type IV collagenases, including MMP-2 and MMP-9, destroy basement membranes, which may be the first barrier to tumor metastasis. Thus, it is believed that elevated expression levels of MMP-2 and MMP-9 in various cancer tissues may play important roles in tumor cell invasion and metastasis (25–29). However, many questions still remain regarding the regulatory mechanisms and clinical significance of these enzymes (30). In this study, we have demonstrated elevated plasma MMP-9 concentrations in 45.2% of patients with NSCLC. The frequency of elevated plasma MMP-9 levels was significantly higher in cases of squamous cell carcinoma and large cell carcinoma compared with adenocarcinoma. Due to tissue remodeling after surgical resection of tumor tissue, MMP-9 levels increased in all patients, including those cases with preoperative elevated MMP-9 levels. These elevated plasma concentrations of MMP-9 decreased to levels within the normal range 4–8 weeks after tumor resection.

Using the same MMP-9 antibody, other researchers have also reported increased MMP-9 concentrations in 53% of gastric cancer patients (21 % ± 3 mg/ml; Ref. 31) and 56% of hepatocellular carcinoma patients (13–660 ng/ml; Ref. 32). Using an anti-MMP-9 monoclonal antibody that they developed, Zucker et al. (33, 34) have reported elevated plasma concentrations of MMP-9 in 23% of gastrointestinal cancer patients and 32% of breast cancer patients; however, they could detect only 1 case of elevated MMP-9 in 24 patients with NSCLC. The discrepancy is probably due to differences in the affinity and avidity of the

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**Table 1** Correlations between patient backgrounds and plasma concentrations of MMP-9

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* $^a$ Chi-squared test.
* $^b$ Adenocarcinoma versus squamous cell carcinoma.
* $^c$ Adenocarcinoma versus large cell carcinoma.
MMP-9 monoclonal antibodies. Based on the results of the present study, the concentration of plasma MMP-9, as determined by an ELISA using the antihuman MMP-9 monoclonal antibody, could be a novel tumor marker for NSCLC, especially for squamous cell carcinoma and large cell carcinoma. Plasma MMP-9 concentrations were not statistically associated with any clinical feature except histological type. This is the first report to demonstrate an elevated plasma concentration of MMP-9 in NSCLC patients and a correlation between clinical features and the expression of MMPs and TIMPs.

We used immunohistochemistry to investigate the expression of MMP-9 in NSCLC tumor samples to determine the site of plasma MMP-9 production. Surprisingly, the frequency of tumor samples expressing MMP-9 was much less than the frequency of cases with elevated plasma MMP-9. The concentration of plasma MMP-9 was not associated with the expression of MMP-9 in tumor samples or with tumor size. The expression of MMP-2, TIMP-1, and TIMP-2 also revealed no correlation of MMP-9 in tumor samples or with tumor size. The expression of MMP-9, are involved in various pulmonary diseases, such as emphysema, bronchiectasis, and interstitial fibrosis, involved in inflammation and tissue destruction. However, the plasma MMP-9 levels were not associated with C-reactive protein test results in our study. Also, Brown et al. (19) found no obvious association between the presence of infiltrating inflammatory cells in tumor samples and the expression of MMP-9. These data suggest that the elevated plasma concentrations of MMP-9 are not due to inflammation correlating with the tumor.

In conclusion, we observed elevated plasma MMP-9 concentrations in 45.2% of NSCLC patients compared with healthy controls. The mechanisms responsible for the elevation of plasma MMP-9 levels remain obscure. Our results demonstrate that plasma MMP-9 does not seem to be directly produced by cancer or stromal cells, but that there may be other sites responsible for increased plasma levels of MMP-9 that correlate with the existence of tumor tissues. The MMP-9 assay system could be a beneficial adjunct to assess the tumor burden of NSCLC, particularly for types of squamous cell carcinoma and large cell carcinoma.

### ACKNOWLEDGMENTS

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### REFERENCES


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