Matrix Metalloproteinase-12 Expression Correlates with Local Recurrence and Metastatic Disease in Non–Small Cell Lung Cancer Patients

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ABSTRACT

Purpose: Non–small cell lung cancer (NSCLC) is a very common and aggressive malignancy. Survival after resection of tumor is especially determined by the occurrence of distant metastasis. Matrix metalloproteinases (MMP) support this metastatic process by degradation of the extracellular matrix.

Experimental Design: We used DNA microarray technology to examine the expression of 22 MMPs in 89 surgically treated NSCLC patients. Validation of microarray results was done using reverse transcription-PCR and immunohistology.

Results: MMP-1, MMP-9, and MMP-12 expression was significantly increased in tumors versus corresponding lung tissues. MMP-12 expression significantly correlated with local recurrence and metastatic disease. Multivariate Cox regression analysis revealed MMP-12 expression as an independent prognostic factor for tumor relapse–free interval. Gene expression analysis of 158 healthy tissues from 32 different organs revealed no MMP-12 expression in these organs and immunohistology identified MMP-12 protein in NSCLC only in tumor cells.

Conclusions: MMP-12 might be not only a prognostic marker, but also a valuable therapeutic target.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide in men and women. As 86% of the people who are diagnosed with lung cancer die of the disease within 5 years, the 169,000 new cases identified in the United States in 2002 will lead to more deaths than breast, colon, prostate, and cervical cancers combined (1). The pathogenesis of lung cancer remains highly elusive due to its aggressive nature and considerably heterogeneity as compared with other cancers. Many lung cancer patients have distant metastases or occult hematogenous and lymphatic spread of tumor cells at diagnosis, thus accounting for poor prognosis of this disease (2).

Metastasis is the final stage in tumor progression from a normal cell to a fully malignant cell. One of the initial steps in the metastatic process involves degradation of different components of the extracellular matrix and requires the action of proteolytic enzymes such as serine-, cysteinyl- and aspartyl-proteinases as well as matrix metalloproteinases (MMP). MMPs are a family of more than 20 secreted or transmembrane proteins that are capable of digesting extracellular matrix and basement membrane components under physiologic conditions (3). According to their substrate specificity and structure, MMPs are classified into five subgroups: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), as well as metalloelastase (MMP-12), the membrane-type MMPs (MMP14, MMP15), and other MMPs (e.g., MMP-19, and MMP20; ref. 4). Reports have shown correlations between the degradation of the basement membrane by MMPs and the metastatic potential of tumor cells (5, 6). The clinical relevance of MMPs in non–small cell lung cancer (NSCLC) is still under discussion. Several MMPs, especially MMP-2 and MMP-9, seem to correlate with early cancer-related deaths in NSCLC (7, 8). First, clinical trials have successfully investigated the efficacy of MMP inhibitors in advanced cancer (9).

The present study was done to assess differentially expressed MMPs in NSCLC by DNA microarray technology and their impact on disease-free interval after surgical resection. The aim was the evaluation of patients with high risk of early metastasis, who may provide candidates for neoadjuvant chemotherapy or MMP-inhibitory therapy.

MATERIALS AND METHODS

Patients and Samples. Tumor and control lung tissue samples were obtained from 89 consecutive patients with NSCLC, who underwent pulmonary resection surgery between 1999 and 2001 (Table 1). The use of human tissues was approved by the local ethics committee and the patients gave informed consent. Only patients with clear histologic classification as NSCLC (adenocarcinoma or squamous cell carcinoma) and without neoadjuvant chemotherapy or radiotherapy were admitted to the study. Immediately following resection, the tumor tissue and other MMPS (e.g., MMP-19, and MMP20; ref. 4). Reports have shown correlations between the degradation of the basement membrane by MMPs and the metastatic potential of tumor cells (5, 6). The clinical relevance of MMPs in non–small cell lung cancer (NSCLC) is still under discussion. Several MMPs, especially MMP-2 and MMP-9, seem to correlate with early cancer-related deaths in NSCLC (7, 8). First, clinical trials have successfully investigated the efficacy of MMP inhibitors in advanced cancer (9).

The present study was done to assess differentially expressed MMPs in NSCLC by DNA microarray technology and their impact on disease-free interval after surgical resection. The aim was the evaluation of patients with high risk of early metastasis, who may provide candidates for neoadjuvant chemotherapy or MMP-inhibitory therapy.
was prepared from total RNA by in vitro transcription after synthesis of double-stranded cDNA using standard protocols. After cRNA-fragmentation and hybridization with microarrays (EOS-K), signals were detected with streptavidin-phycocerythrin. Signal enhancement was done using biotinylated goat anti-streptavidin antibodies. Arrays were washed and stained with the GeneChip Fluidics Station 400 and scanned with a GeneArray Scanner. Primary image analysis was done by using Microarray Suite 5.0. Altogether, 49 squamous cell carcinomas, 40 adenocarcinomas and 15 corresponding control lung samples were analyzed. All expression values below 60 were set to 60. To identify specific genes that were differentially expressed in tumors as compared with lung tissue, we used a criterion that marks differential gene expression at an approximate significance level (determined by Bonferroni method) of $8.0 \times 10^{-7}$ using Student’s $t$ test and a fold-change cutoff of 2.0 and 0.5 for up-regulated and down-regulated genes, respectively. Calculation of fold-change was done by dividing the mean expression level of a gene in the tumor samples by the mean expression level of the same gene in the lung samples.

**Body Map.** The evaluation of MMP-12 expression in other healthy organs was done with an EOS-Biotechnology own body map list, containing expression data collected by the same microarray technology. This gene expression database encloses expression values of 158 healthy tissues (brain, larynx, lip, pharynx, salivary gland, heart, aorta, breast, thymus, esophagus, omentum, stomach, intestine, small bowel, colon, rectum, liver, pancreas, lien, kidney, adrenal gland, bladder, ureter, urethra, cervix, ovary, skin, muscle, diaphragm, and lymph node).

**Reverse Transcription-PCR.** Validation of microarray results was done on 23 tumor and 6 lung samples initially used for gene chip analysis. For evaluation of predictive value, previously uncharacterized tumor probes were included (see RESULTS). Tissue was lysed in TRIzol (Life Technologies), RNA prepared and converted to first-strand cDNA by use of oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions. Success of cDNA synthesis was monitored by PCR with human $\beta$-actin primer 5'-CATCGTGATGGACTCCGGTG-3' and 5'-GCTGGAAGGTGGACCGAG-3', amplifying after 21 cycles a cDNA product of 610 bp in size. For analysis of MMP-12 expression, primers specific for human MMP-12 transcript were designed (sense, 5'-GGCTGAAGGTTTCTGA-3'; antisense, 5'-TTTGGTGTACGTTGGAG-3') enabling specific amplification of a 461-bp fragment. PCR amplification was run for 24 to 32 cycles (see RESULTS).

**Immunohistology.** To evaluate and localize MMP-12 expression in tumor tissue, immunohistology was done on four NSCLC specimens with high MMP-12 expression (adenocarcinoma, $n = 2$; squamous cell carcinoma, $n = 2$) and their corresponding lung tissue. Four-micron-thick sections were prepared from formalin-fixed and paraffin-embedded tissues mounted on Star Frost adhesive-slides, dried for 2 hours at 60°C and dewaxed in xylene and graded ethanol. Nonspecific protein staining was blocked by preincubation for 30 minutes with 5% normal goat serum in 1% normal horse serum. The sections were then incubated with mouse monoclonal antibody against human MMP-12 overnight at 4°C.

**Table 1  Clinical and pathologic characteristics of patients and their tumors**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients</th>
<th>Mean age (y)</th>
<th>Male/female ratio</th>
<th>Smoking/nonsmoking ratio</th>
<th>Surgical procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>89</td>
<td>65.5</td>
<td>71/18</td>
<td>60/29</td>
<td></td>
</tr>
<tr>
<td>Segment resection</td>
<td>5 (5.5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobectomy</td>
<td>64 (72%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bilobectomy</td>
<td>2 (2.2%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonectomy</td>
<td>12 (13.5%)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>49 (55%)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Adenocarcinoma</td>
<td>40 (45%)</td>
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<tr>
<td>Tumor-node-metastasis staging</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>31 (34.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>23 (25.8%)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>29 (32.6%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>6 (6.8%)</td>
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<tr>
<td>Grading</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Well/moderately well differentiated</td>
<td>25 (28%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated/undifferentiated</td>
<td>64 (72%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual tumor situation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R0</td>
<td>75 (84.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>6 (6.8%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>8 (9%)</td>
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5 minutes with 1:10 diluted Normal Horse Serum (Vector Laboratories). The slides were incubated with 1:10 diluted monoclonal anti-human MMP-12 hemopexin-like domain antibody Clone 4D2 (RD Systems, Wiesbaden, Germany) for 30 minutes at 37°C. After addition of biotinylated secondary antibody (DCS, Hamburg, Germany) the slides were incubated with Avidin-Biotin Complex reagent (DCS) for another 10 minutes. Antibody localization was visualized with 3- amino-9-ethyl-carbazol and H2O2, followed by a final counter-staining with hematoxylin. All slides were rinsed with phosphate-buffered saline after each step of the procedure, except after application of the normal serum. Controls were prepared without primary antibody.

Statistical Analysis. Differences in the frequency of MMP-positive sections in subgroups of patients were analyzed by Pearson's χ² test. For analysis of follow-up data, life table curves were calculated with Kaplan-Meier methods, and survival distributions were compared by use of log-rank statistics. The Cox proportional hazards model was applied for multivariate analysis using the Statistical Package for the Social Sciences software program (SPSS, Inc., Chicago, IL). Only patients with confirmed post-operative R0 status were admitted to the analysis of the disease-free survival. The threshold for statistical significance was chosen as P = 0.05.

RESULTS

Relative Expression of Matrix Metalloproteinases in Non–Small Cell Lung Cancer and Control Lung. Comparison of expression profiles from control lung and NSCLC revealed changes in gene expression in a total of 344 (0.6%) genes according to the chosen levels of significance (P < 8.0 × 10⁻⁵) and fold-change (cutoff of 2.0). Most of the 22 investigated MMPs were not differentially expressed in control lung and tumor tissue (Table 2A). Only MMP-1, MMP-9, and MMP-12 were differentially expressed. MMP-10 and MMP-11 expression achieved only one of the quality objectives for differentially expressed genes, level of significance or fold-change. Lung tissues showed no or only minimal expression of MMP-1, MMP-10, and MMP-12, whereas MMP-9 and MMP-11 were also coexpressed at a high level in lung tissue. The highest fold-change (4.1) and the highest level of significance (1.4 × 10⁻²⁰) was reached by MMP-12. Because the expression values of these MMPs were highly variable in the tumor samples, we individually determined cutoffs for the classification into groups of low and high expression. The cutoffs were defined by an expression level of about 150% of the controls (cutoffs for MMP-1, -10, and -12 was 100 relative expression units; for MMP-11, 300 relative expression units; and for MMP-9, 400 relative expression units, respectively).

Matrix Metalloproteinase-12 Predicts Tumor Relapse. Of the 75 R0-resected patients, two died within 30 days (hospital mortality), two succumbed to cancer-unrelated diseases and one woman died of a second primary tumor. This resulted in 70 R0-resected patients for the analysis of tumor relapse. Tumor relapse was defined as local recurrence, distant metastasis, or both. Within the follow-up period (median 26.3 months), 30 (42.8%) of the eligible 70 patients had tumor relapse. Local recurrence occurred in 5 (7.1%) and distant metastases in 20 (28.6%) patients, 6 (8.6%) patients suffered from both. There were no significant correlations between MMP-1, -9, -10, and -11 expression and the incidence of tumor relapse (Table 2B). However, among the 56 patients with high MMP-12 expression in the tumor tissues, 29 (51.7%) developed local recurrence and/or distant metastases, whereas only 2 (14.3%) of 14 with low MMP-12 expression in the tumor tissues had tumor relapse. Interestingly, MMP-12 expression level significantly correlated with tumor relapse (P = 0.04).

Matrix Metalloproteinase-12 Expression Significantly Correlated with Relapse-Free Survival. Kaplan-Meier analysis of tumor relapse-free survival, comparing patients with MMP-12 low expressed (n = 14) to MMP-12 high expressed tumors (n = 56), showed a significant correlation (P = 0.02) between high MMP-12 expression and an unfavorable outcome (Fig. 1). The 3-year tumor relapse-free rate in patients with high MMP-12 Expression was 32% and 85% in patients with low MMP-12 tumor expression. Expression of MMP-12, sex, age, histology, tumor-node-metastasis staging, and grading were tested for independence of a possible prognostic value (Table 3). The Cox proportional hazards model showed that MMP-12 expression was a significant (P = 0.04) independent prognostic predictor for disease-free survival in patients with resected NSCLC. The relative risk for tumor relapse in all R0-resected patients was 4.8-fold higher in patients with high expression of MMP-12 in the tumor tissue. The other possible risk factors did not significantly correlate to tumor recurrence.

No Coexpression of Matrix Metalloproteinase-12 in Healthy Tissue. To examine the prevalence of MMP-12 in other healthy organs, we compared our MMP-12 expression values to data of an EOS-Biotechnology own body map list. Except for two samples (colon and lymph node) all other tissues showed no MMP-12 expression (Fig. 2).

Verification of Array Data by Reverse Transcription-PCR and Immunohistology. To verify the results of our microarray studies, reverse transcription-PCR and immunohistology were done.

Differences in expression patterns for MMP-12 could be confirmed in 23 NSCLC and 6 control lungs by reverse transcription-PCR (Fig. 3A). All patients with tumor relapse had moderate to high levels of MMP-12 expression. As graphically depicted in Fig. 3B, there was a high correlation (KK = 0.83) between reverse transcription-PCR and oligonucleotide array results for all 23 tumor probes. All samples without tumor relapse were focused in the left lower corner of the graph.

Immunostaining for MMP-12 showed a weak to moderate, sometimes patchy positive reaction of the tumor tissue. The staining was restricted to the cytoplasm of the tumor cells only (Fig. 4). Concerning the intensity of the immunoreaction, we did not notice remarkable differences between adenocarcinomas and squamous cell carcinomas. There was an invariable weak to moderate positive immunostaining of alveolar macrophages and bronchiolar epithelia of the surrounding lung tissue, serving as an internal positive control.
DISCUSSION

Tumor invasion and metastasis require controlled degradation of extracellular matrix. MMPs play a crucial role in this breakdown of collagen and basement membrane components. In the present study, we investigated the gene expression of MMPs in NSCLC and the lung. Whereas previous studies (7, 8, 18) compared expression of a few members of the MMP family by immunohistology, genome-wide DNA microarray analysis enabled us to investigate 22 MMPs simultaneously. In our study, MMP-1, MMP-9, and MMP-12 were expressed more strongly in the NSCLCs of both squamous epithelial and adenoid morphology than in the corresponding nonmalignant lung tissues, where expression of MMP-1 and MMP-12 was generally low or absent. The strong MMP-1 and MMP-9 expression in NSCLC (8, 18, 19), as well as in SCLC (20), has been previously described.

The present study shows for the first time that MMP-12 is highly expressed in NSCLC and strongly correlates with local recurrence as well as metastatic disease in patients with resected NSCLC.

MMP-12 was primarily identified as an elastolytic metalloproteinase secreted by inflammatory macrophages (21). This enzyme has broad substrate specificity, including extracellular matrix proteins such as type I gelactin, fibronectin, laminin,
vitronectin, proteoglycans, and fibrin and it can also produce angiostatin from plasminogen. Expression of MMP-12 in vivo has previously been described in hepatocellular carcinoma (22), colorectal carcinoma (23), vulva carcinoma (24), renal cell carcinomas (25), skin cancer (26), pancreatic cancer (27), and in esophageal carcinoma (28). However, the role of MMP-12 in tumor pathogenesis seems to depend on the type of tissue involved. In contrast to our study with NSCLC, MMP-12 overexpression in hepatocellular as well as in colorectal carcinoma closely correlated with better prognosis, which was explained by the antiangiogenic function of MMP-12 on the basis of the generation of angiostatin from plasminogen. Angiostatin inhibits endothelial cell proliferation, thereby possibly leading to a reduction of metastatic potential (22, 29).

On the other hand, the macrophages of MMP-12−/− mice had a markedly diminished capacity to degrade extracellular matrix components and were also essentially unable to penetrate reconstituted basement membranes in vitro and in vivo (30). However, MMP-12 is not only required for macrophage-mediated extracellular matrix proteolysis. Immunohistochemical investigations of MMP-12 in the present NSCLC as well as in a esophageal squamous cell carcinoma study showed homogenous staining in the tumor cells, whereas no significant staining was seen in the lung or esophagus. Therefore, it is postulated that the tumor cell–related MMP-12 expression might be correlated with tumor growth, invasion, and metastasis (24). In fact, Ding et al. (28) found a direct significant correlation between MMP-12 mRNA expression and the depth of esophageal wall invasion as well as the frequency of lymph node metastasis in esophageal squamous cell carcinomas. Corresponding with our data, a positive correlation of MMP-12 expression and tumor aggressiveness has been shown for vulva carcinoma and skin cancer. The level of MMP-12 expression correlated with epithelial dedifferentiation and histologic aggressiveness (24, 26).

### Table 3: Multivariate Cox regression analysis of potential risk factors for tumor relapse in patients with R0 resected NSCLC

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>RR</th>
<th>95% Confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex Male</td>
<td>0.77</td>
<td>0.28-2.10</td>
<td>0.61</td>
</tr>
<tr>
<td>Sex Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y) ≤65</td>
<td>0.75</td>
<td>0.36-1.56</td>
<td>0.44</td>
</tr>
<tr>
<td>Age (y) &gt;65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology Squamous cell carcinoma</td>
<td>1.77</td>
<td>0.77-4.05</td>
<td>0.17</td>
</tr>
<tr>
<td>Histology Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-node-metastasis stage I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor-node-metastasis stage II</td>
<td></td>
<td>0.28-2.25</td>
<td>0.67</td>
</tr>
<tr>
<td>Tumor-node-metastasis stage III</td>
<td></td>
<td>0.76-4.36</td>
<td>0.17</td>
</tr>
<tr>
<td>Grading well/moderately well differentiated</td>
<td>0.85</td>
<td>0.35-2.06</td>
<td>0.73</td>
</tr>
<tr>
<td>Grading poorly differentiated/undifferentiated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-12 Low expression</td>
<td>4.86</td>
<td>1.06-22.26</td>
<td>0.04</td>
</tr>
<tr>
<td>MMP-12 High expression</td>
<td></td>
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![Fig. 2](image_url)  
**Fig. 2** MMP-12 expression of NSCLC and corresponding lung in correlation to MMP-12 expression data from 158 tissues of healthy organs (body map).
inversely correlated with prognosis. Survival of patients with pancreatic cancer overexpressing MMP-12 mRNA was significantly shorter as compared with patients with a tumor that did not overexpress MMP-12 (27).

Our body map data demonstrating low or missing expression of MMP-12 in healthy tissues support the concept of adjuvant therapy with MMP inhibitors in NSCLC. However, the first phase III studies with broad-spectrum MMP inhibitors revealed no significant differences in survival in a NSCLC prinomastat study as well as in a SCLC marimastat trial (31). Selective MMP inhibitors might be of more benefit, because BAY 12-9566 for instance, a potent inhibitor of MMP-2, -3, and -9 prolonged progression-free interval of NSCLC patients in an adjuvant therapy (9). ONO-4817, a third-generation MMP inhibitor against MMP-2, -8, -9, -12, and -13, suppressed progression of lung micrometastasis of MMP-expressing tumor cells in nude mice in combination with docetaxel (32). Phase III clinical trials with Neovastat, a multifunctional angiogenic agent against vascular endothelial growth factor, MMP-2, -9, and -12 are currently under investigation in patients with unresectable NSCLC (33).

In summary, we show for the first time that MMP-12 is highly expressed in NSCLC tumors but not in control lung tissue and that MMP-12 expression in NSCLC tumor correlates positively with the metastatic potential as well as shortened relapse-free interval in patients with NSCLC. Therefore, MMP-12 expression might be suitable for identification of patients at high risk for early tumor relapse that might benefit from adjuvant therapy. Furthermore, the present study might support the concept of selective MMP inhibitor therapy in NSCLC patients with high expression of MMP-12 in tumor tissue.

**REFERENCES**

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