Overexpression Level of Stromelysin 3 Is Related to the Lymph Node Involvement in Non-small Cell Lung Cancer1

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ABSTRACT

Proteases contribute to tumor invasion and metastasis via their potential to degrade basement membranes and extracellular matrix. Our aim was to compare the level of several proteases: urokinase-type plasminogen activator (u-PA), matrix metalloproteinase 2 (MMP-2; 72-kDa type IV collagenase, also known as gelatinase A), MMP-11 [also known as stromelysin 3 (STR3)], and cathepsins B and L in resected non-small cell lung cancer. Between June 1996 and March 1998, samples of lung tumor tissues were taken from 119 surgically treated patients. Thirty out of the 119 tumor samples were matched with corresponding adjacent normal tissue. u-PA was measured by a commercially available immunoluminometric assay. Metalloproteinases and cathepsins have been evaluated at the RNA level by Northern blot and quantified with a PhosphorImager. Expression of these proteases was compared to the following clinicopathological parameters: pathological diagnosis, tumor size, exposure to asbestos, radiotherapy, neo-adjuvant chemotherapy, tumor-node-metastasis stage, lymph node involvement, presence of metastasis, u-PA, MMP-2, MMP-11/STR3, and cathepsin B were significantly increased in tumor (the tumor:normal ratio was on average increased by 5.4-, 2.2-, 83.5-, and 2.2-fold, respectively). The tumor:normal ratio of MMP-11/STR3 was found to be significantly linked to the lymph node involvement (P < 0.05). Our results suggest that several proteases are involved in the invasive potential of non-small cell lung cancer and that the quantification of MMP-11/STR3 could represent an useful prognostic marker.

INTRODUCTION

NSCLC3 represents a heterogeneous group of cancers both biologically and histopathologically. In patients with NSCLC, the clinicopathological parameters, i.e., size of the primary tumor, involvement of regional lymph nodes, and presence of distant metastases, have been thus far the most important prognostic factors. Accordingly, they largely determine treatment that relies on surgery when the primary lesion is completely resectable in the absence of distant metastases. Nevertheless, the aggressiveness of lung carcinoma is not always related to the tumor-node-metastasis staging, and molecular markers of tumor aggressiveness are necessary to improve therapeutic planning. Angiogenesis and metastasis highly contribute to the development and progression of lung cancer. As a consequence, the determination and/or validation of markers of metastatic propensity turn out to be essential in the therapeutic management.

The metastatic propensity is linked to the cell capacity of degrading basement membranes and extracellular matrix. Many proteases, including u-PA, MMPs, and the cathepsins, have been thus far described as potentially involved in angiogenesis and metastasis (1, 2).

In comparison to other tumors, in particular breast cancer, the potential role of proteases in dissemination of lung tumors has not been as much studied. u-PA antigen level has been reported as not statistically associated with the prognosis of squamous and large cell lung carcinoma (3) or of adenocarcinoma (4). At the mRNA level, u-PA has been found expressed in stromal and/or cancer cells of lung carcinomas (5). Both epithelial and stromal u-PA expression were linked to the tumor size, and stromal u-PA expression was furthermore linked to the lymph node involvement (6). The MMP gelatinase A (MMP-2) has been described to be expressed in many lung tumors (7, 8). MMP-11 (STR3) has been studied in non-small cell lung carcinomas by in situ hybridization, immunohistochemical staining, and semiquantitative reverse PCR (6, 9, 10). STR3 was overexpressed in stromal cells in non-small cell lung carcinomas and was also expressed in epithelial cells in squamous and basaloid carcinomas (6, 9, 10). Stromal STR3 expression was linked to the tumor size and lymph node involvement in most lung carcinomas (6). Cathepsin L and, moreover, cathepsin B were shown at a higher level in NSCLC tissues than in surrounding nonmalignant tissues (11, 12). Cathepsin B immunostaining was associated with poor prognosis (13).

In an attempt to deal with proteases as molecular markers

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; α1-PI, α1-proteinase inhibitor; MMP, matrix metalloproteinase; STR3, stromelysin 3; u-PA, urokinase-type plasminogen activator.
in lung carcinomas, we have quantified the expression of the above mentioned proteases in a series of non-small cell lung tissues and then studied the relationships with the prognostic clinicopathological factors.

PATIENTS AND METHODS

Patients. From June 1996 to March 1998, 119 patients (93% men; mean age, 59 years; range, 29–76 years) who underwent surgery for NSCLC in the Department of Surgery (Centre Hospitalier Régional Universitaire de Lille) were included in this study (Table 1). Only patients completely resected in a potentially curative way for adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and neuroendocrine non-small cell lung carcinoma were enrolled in the study. Patients with localized metastatic spread in only one site treated by surgery were also included in the study. Patients who had a metastatic spread in more than one site were excluded from the study. Patients who died of surgical complications during the postoperative course were excluded from the study. We also excluded patients with an extrapulmonary cancer history and patients with a synchronous lung lesion histologically different from NSCLC.

Tissue Samples. Tumor specimens of NSLC were taken from 119 patients. Thirty tumor samples were matched with corresponding adjacent normal tissue, which was taken at a minimal distance of 5 cm from the tumor margin. At the time of collection, tissue samples were divided into three parts: one part was submitted to pathological study, and the other two were snap frozen in liquid nitrogen and stored at −80°C until respective treatment for protein or RNA extraction. In some cases, the tissue sample could only be divided into two parts.

Immunoaassay of u-PA. Tissue extracts were prepared by homogenization in 10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5 mM Na₂MoO₄, 1 mM monothioglycerol buffer. Supernatants were collected after centrifugation at 15,000 × g for 3 h. u-PA was evaluated by an immunoluminometric assay using LIA kits provided by Byk-France (LIA-mat u-PA, AB Sangtec Medical, Bromma, Sweden). This assay is a monoclonal two-site incubation immunoluminometric assay (sandwich principle), which evaluates free u-PA, and also receptor and PAI-1-bound u-PA. The immunological reaction is detected by a light reaction through the oxidation of the isoluminol bound to antibodies.

Protein content of the cytosolic extracts was determined by the BCA protein kit test from Pierce using BSA as a control. The results were expressed in ng of protease per mg of protein.

mRNA Levels of MMP-2, MMP-11 (STR3), Cathepsin B, and Cathepsin L. Total cellular RNA was isolated after homogenization of lung tissue in guanidinium isothiocyanate and centrifugation through cesium chloride gradients.

Fifteen μg of total cellular RNA were electrophoresed on 0.9% agarose-2.3 M formaldehyde gels and subsequently transferred onto nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Rainham, United Kingdom). The membranes were hybridized overnight with the 32P-labeled probes (Random primed DNA labeling kit; Roche Molecular Biochemicals, Meylan, France). The MMP-2 probe was obtained from the ATCC. The STR3, cathepsin B, and cathepsin L probes spanned nucleotides 346-2105 (14), 147-1011 (15), and 604–999 (16), respectively. After hybridization, the blots were exposed to a PhosphorImager screen (Molecular Dynamics). For normalization, the membrane was hybridized with the human actin cDNA probe. The results were expressed as the ratio between the labeling obtained after hybridization with the proteinase cDNA probe and the labeling obtained after hybridization with the actin cDNA probe.

Statistical Analysis. Two groups of patients were considered: a first group included the 30 patients for whom matched tumor and normal tissues were available, and a second group included all 119 patients. In the first group, we have calculated for each patient the tumor:normal ratio of expression for the different proteases.

The expression of proteases was compared with the following clinicopathological parameters: histological type, tumor size, exposure to asbestos, preoperative radiotherapy, neoadjuvant chemotherapy, histopronostic grading, lymph node involvement, and presence of metastasis.

Statistical analyses were carried out using StatView statistical software on a personal computer. The differences between tumoral tissue and normal adjacent tissue were examined using the Wilcoxon signed rank test. The difference between two independent groups was determined by the Mann-Whitney U test, and the significance of differences among more than two groups was determined by Kruskal-Wallis one-way analysis. Values of P less than 0.05 in two-tailed analyses were considered significant.

RESULTS

Concentration of u-PA Antigen. The distribution of u-PA in lung carcinoma and their control lung tissue is illustrated in Fig. 1. In the 30 tumor samples, the concentration of u-PA was found to be statistically higher than in the correspond-
ing control lung tissue ($P < 0.0001$, Table 2). The mean fold increase was 5.4 (Table 2).

Expression of MMP-2, MMP-11/STR3, Cathepsin B, and Cathepsin L Genes. As shown in Fig. 2, MMP-2, MMP-11/STR3, and cathepsin L transcripts were respectively identified as single autoradiographic bands of 3.1, 2.4, and 1.2 kb. Cathepsin B transcripts appeared as two autoradiographic bands of 4 and 2.3 kb. The level of protease transcripts in lung carcinoma and their control lung tissue was quantified by direct measurement of radioactivity on the membranes and then normalized to actin expression. The degree of variation in MMP-2, MMP-11/STR3, cathepsin B, and cathepsin L gene expression in neoplastic and control tissues is illustrated in Fig. 1. In the 30 tumor samples, the expression of MMP-2, MMP-11/STR3, and cathepsin B genes was found to be statistically higher than in the corresponding control lung tissue ($P < 0.005$, Table 2). The

Table 2  Comparison of u-PA antigen, MMP-11/STR3, MMP-2, cathepsin B and L mRNA levels in the 30 matched pairs of tumor and normal lung tissues

<table>
<thead>
<tr>
<th></th>
<th>Tumor tissue</th>
<th>Normal tissue</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-PA antigen</td>
<td>0.247 (0.395)</td>
<td>0.046 (0.030)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>STR-3 mRNA</td>
<td>0.039 (0.064)</td>
<td>0.0005 (0.0005)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MMP-2 mRNA</td>
<td>0.093 (0.107)</td>
<td>0.042 (0.060)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Cathepsin B mRNA</td>
<td>0.241 (0.271)</td>
<td>0.111 (0.100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cathepsin L mRNA</td>
<td>0.064 (0.059)</td>
<td>0.041 (0.038)</td>
<td>NS' (0.1446)</td>
</tr>
</tbody>
</table>

*a* Mean (SD).

*b* n = no. of patients.

't* NS, not significant.
mean fold increase was of 2.2 for MMP-2, 83.8 for MMP-11/STR3, and 2.2 for cathepsin B (Table 2). In contrast, no statistically significant difference was found for cathepsin L between the expression in tumor and nontumor tissue (Table 2).

Level of u-PA, MMP-2, MMP-11, Cathepsin B, and Cathepsin L in Relation to Clinicopathological Parameters. The relationships between the tumor:normal ratio of protease expression and the conventional clinicopathological parameters with prognostic significance in lung cancer were analyzed (Table 3).

u-PA, MMP-2, cathepsin B, and cathepsin L were not related to any of these clinicopathological parameters. In contrast, the tumor:normal ratio of MMP-11/STR3 was found significantly linked to the lymph node involvement ($P = 0.0433$ for the lymph node status and $P = 0.0373$ for the capsular rupture; Table 3 and Fig. 3).

We then analyzed the correlation between the levels of protease expression in the 119 tumor samples and the clinicopathological parameters (Table 4). Although not statistically significant, a trend ($P = 0.0866$) was particularly observed between the MMP-11/STR3 expression and the lymph node status.

DISCUSSION

Much research, including studies on human tumor tissue samples and in vitro or in vivo experiments on cell cultures or animal models, has demonstrated the key role of proteases in tumor spread and metastasis. In particular, investigation by
immunohistochemistry and in situ hybridization on human tumors has shown overexpression of proteases by the stromal cells at the periphery of tumors, suggesting the involvement of proteases in the extension of the malignant process. The aim of this study was to quantify the expression of several proteases and to study their relationship with the recognized clinicopathological prognostic variables, in an attempt to determine whether overexpression of some proteases could be particularly relevant to the malignancy of lung cancer.

Among the proteases studied here, our results show that u-PA, MMP-2, MMP-11/STR3, and cathepsin B levels are significantly increased in lung carcinoma in comparison with adjacent normal tissue (the tumor:normal ratio was on average increased by 5.4-, 2.2-, 83.5-, and 2.2-fold, respectively). For cathepsin L, no statistically significant difference was observed. In situ hybridization and immunohistochemistry studies in lung cancer showed that most proteases, including u-PA and MMP-11/STR3, were found to be predominantly expressed in stromal cells, suggesting an active role in the local peritumoral region (6, 9, 10). Besides, the enzymatic activity of protease in the extracellular space is also regulated by specific protease inhibitors. The proteolysis of extracellular matrix components in the processes of tumor invasion and metastasis is finally controlled by the balance between proteases and protease inhibitors (17).

For MMP-2 and cathepsin B, which were only slightly increased in lung tumor tissue (by 2.2-fold), no correlation appeared with any of the clinicopathological prognostic variables. Although u-PA level was found to be more increased (by 5.4-fold), no relationship could be found between u-PA level and any of the clinicopathological prognostic variable in both statistical studies. Pedersen et al. (3, 4) also reported the absence of a statistically significant association of u-PA level with age, sex, tumor size, stage, extent of surgery, and number of tumor-positive mediastinal lymph nodes in squamous carcinomas, large cell carcinomas, and adenocarcinomas of the lung. These observations contrast with findings in other types of cancer, in particular breast cancer, in which u-PA level is an independent prognostic factor of relapse-free survival (18–21). However, stromal u-PA expression assessed in non-small cell lung carcinomas by in situ hybridization was correlated to the tumor size and lymph node involvement (6).

The mean increase in MMP-11/STR3 level in lung carcinoma was much higher (by 83.5-fold). A previous semiquantitative analysis of STR3 expression in 58 paired tumor and normal tissue from NSCLCs has been investigated by reverse
### Table 4: Relationships between the levels of protease expression in the 119 tumor samples and clinicopathological parameters

<table>
<thead>
<tr>
<th>Prostate</th>
<th>Pathological parameter</th>
<th>Mean</th>
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<td>Histological type</td>
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<td>Large cell carcinoma</td>
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<tr>
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<td>Neuroendocrine transthectomy</td>
<td>0.097</td>
<td>0.056</td>
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<td>Large cell carcinoma</td>
<td>0.374</td>
<td>0.346</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Tumor stage</td>
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<td></td>
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<tr>
<td></td>
<td>T1</td>
<td>0.275</td>
<td>0.079</td>
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<tr>
<td></td>
<td>T2</td>
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</tr>
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<td></td>
<td>T3</td>
<td>0.246</td>
<td>0.092</td>
<td>0.028</td>
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<tr>
<td></td>
<td>T4</td>
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<td>0.011</td>
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<td>Lymph node status</td>
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<td></td>
<td>N1</td>
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<td>0.023</td>
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<td>Prostate-related radiation</td>
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<tr>
<td></td>
<td>Yes</td>
<td>0.184</td>
<td>0.098</td>
<td>0.023</td>
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<tr>
<td></td>
<td>No</td>
<td>0.242</td>
<td>0.092</td>
<td>0.023</td>
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<tr>
<td></td>
<td>Prostate-related chemotherapy</td>
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<td>0.023</td>
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<tr>
<td></td>
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- Table 4 relationships between the levels of protease expression in the 119 tumor samples and clinicopathological parameters.

**REFERENCES**

Proteases in Lung Cancer


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