Stromelysin-3 Is Overexpressed by Stromal Elements in Primary Non-Small Cell Lung Cancers and Regulated by Retinoic Acid in Pulmonary Fibroblasts

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ABSTRACT

Stromelysin-3 (STR-3) is a recently characterized matrix metalloprotease (MMP) that was cloned on the basis of differential expression in benign and malignant breast tumors. This MMP has a unique processing mechanism and substrate specificity. Unlike previously characterized MMPs that are secreted as inactive zymogens, STR-3 is processed within the constitutive secretory pathway and secreted as an active enzyme. Although STR-3 has a characteristic MMP structure, the enzyme does not hydrolyze many of the extracellular matrix components that are substrates for other MMPs. However, STR-3 cleaves certain serine protease inhibitors (serpins), including the α1 proteinase inhibitor (α1 anti-trypsin). Because α1 proteinase inhibitor deficiency has a known pathogenetic role in pulmonary disease, the role of STR-3 in non-small cell lung carcinomas (NSCLC) is of great interest. STR-3 transcripts and proteins were significantly more abundant in primary NSCLC than in adjacent normal lung specimens in an extensive panel of stage I–III squamous cell and adenocarcinomas. The major form of STR-3 detectable in the primary NSCLC was the mature fully processed active enzyme. STR-3 transcripts and protein were primarily localized to NSCLC stromal elements, prompting analysis of STR-3 induction in normal pulmonary fibroblasts. Although STR-3 could be induced in normal pulmonary fibroblasts with growth factors (basic fibroblast growth factor and platelet-derived growth factor) and/or 12-O-tetradecanoylphorbol-13-acetate, STR-3 induction was inhibited by all-trans retinoic acid, a commonly used chemopreventive agent for aerodigestive tract malignancies. Taken together, these data suggest that STR-3 may be a novel marker and potential therapeutic target in NSCLC.

INTRODUCTION

Carcinoma of the lung is currently the leading cause of cancer-related deaths in men and women in the United States (1). Less than 20% of newly diagnosed lung cancer patients survive 5 years, because these patients frequently present with metastatic or micrometastatic disease that cannot be effectively treated (1). For these reasons, new approaches to micrometastatic or metastatic lung cancer are urgently needed.

One factor that distinguishes cancer cells is their ability to invade normal tissue and metastasize via blood vessels and lymphatics (2). A critical role may be played by MMPs that degrade the structural support network for normal and malignant cells and promote the neovascularization of tumor cell deposits (2-4).

STR-3 is a recently characterized MMP with a unique pattern of expression and substrate specificity (5). This MMP was originally identified by differential screening of a cDNA library from an invasive breast cancer surgical specimen with that from a benign breast fibroadenoma (5). Like other previously characterized MMPs, the predicted STR-3 amino acid sequence includes a "pro" peptide domain that facilitates secretion but is subsequently removed from the latent enzyme (3-5). The latent enzyme also contains a highly conserved "pro" domain that is cleaved when the enzyme is converted to its active form (5). STR-3 also includes additional unique sequence and a characteristic catalytic domain with three conserved histidine residues in a zinc-binding consensus sequence and a "hemopexin" domain with sequence similarity to the heme-binding proteins (3-5).

Of great interest, STR-3 transcripts are significantly and consistently more abundant in primary breast cancer specimens than in benign fibroadenomas (5). Furthermore, STR-3 is expressed at high levels in lymph nodes and distant organ sites containing metastatic breast cancer (5-7). In additional studies, STR-3 expression has been linked with local invasiveness of in situ breast cancers and head and neck basal cell skin cancers, implicating the enzyme in multiple epithelial malignancies (6-9). However, additional normal tissues that undergo extensive remodeling such as the placenta, uterus, and post lactation mammary glands also express the enzyme (5, 6, 10). Although STR-3 is overexpressed in primary tumors, the enzyme appears to be synthesized by cells in the interdigitating tumor stroma (5, 6).

The unique pattern of STR-3 expression has prompted great interest in the regulation of the enzyme and substrate specificity. Unlike previously characterized MMPs that are secreted as inactive zymogens, STR-3 is secreted as an active enzyme (11). The STR-3 protein contains a unique 10-amino acid insert between the pro- and catalytic domains that includes a recognition motif for the Golgi-associated pro-protein convertase, furin (12). Consequently, STR-3 is processed by furin to its enzymatically active form within the constitutive secretory pathway (12).

Although STR-3 has a characteristic MMP structure, the enzyme fails to hydrolyze many of the extracellular matrix components (fibronectin, laminin, type IV collagen, gelatin, elastin, and type I collagen) that are substrates for other MMPs (11). However, the serine protease inhibitor (serpin), α1-proteinase inhibitor (α1-PI, α1-antitrypsin), was recently identified as a major STR-3 substrate (11). These data are of particular interest given the known pathogenetic role of α1-antitrypsin (α1-PI) deficiency in pulmonary disease (13) and the putative role for α1-PI in the direct suppression of tumor cell growth and invasion (14). For these reasons, we have evaluated the expression and regulation of STR-3 in a broadly representative panel of primary NSCLC and normal pulmonary fibroblasts.

MATERIALS AND METHODS

Patient Samples. Patients with NSCLC were rigorously staged with mediastinoscopy and radiographic studies including bone scan and head, chest, and abdominal computed tomography. Patients with stage I or II disease underwent immediate surgical resection, whereas patients with stage III disease either underwent immediate surgical resection or received neoadjuvant radiation therapy or chemotherapy prior to surgical resection. Primary NSCLC specimens and adjacent uninvolved normal lung samples were delineated by a

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2 The abbreviations used are: MMP, matrix metalloproteinase; STR-3, stromelysin-3; RA, all-trans-retinoic acid; α1-PI, α1 proteinase inhibitor; NSCLC, non-small cell lung cancer; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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dedicated pulmonary pathologist at the time of initial surgical resection. Thereafter, 100-mg aliquots were snap-frozen and stored in liquid nitrogen in the Respiratory Tumor Tissue Bank at Brigham and Women’s Hospital. All tissues were harvested and stored in accordance with established Institutional Review Board guidelines at Brigham and Women’s Hospital.

RNA Preparation and Analysis by Semiquantitative Reverse PCR. One hundred-mg specimens of selected primary tumors and adjacent “normal” lung from NSCLC patients were lysed in guanidine isothiocyanate and used to prepare total RNA. One μg of RNA from each sample was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) and oligo-dT primer as described previously (15).

The quality and quantity of each cDNA were initially evaluated by amplifying 20A of the resulting 20A total cDNA with human β-actin-specific oligonucleotide primers [5' sense, 5'-CAGCCATGTACGTTGCTATCCAG-3' (bp 2120–2140) and 3' antisense, 5'-GTTTCGTGGATGCCAAGGAC-3' (bp 2656–2634)] and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) for 19 cycles as described previously (15). Nineteen cycles of actin amplification were used because this cycle number was known to be in the linear range (15). Following amplification, samples were size fractionated and transferred to nitrocellulose blots (Schleicher & Schuell, Keene, NH) that were subsequently hybridized with a 32P-labeled internal actin probe [5'-GAGCAAGAGATGGCCAC-3' (bp 2401–2417; Ref. 15)], which identified the expected 400-bp fragment.

Immunoblotting of Paired Tumor and Normal Lung Specimens. Additional 100-mg aliquots of paired tumor and normal lung specimens were homogenized and sonicated in extraction buffer containing 50 mM Tris HCl (pH 7.0), 10% glycerol, and 1% SDS. After protein concentrations were determined (Bio-Rad Protein Assay), 50 μg of the paired tumor/normal sam-

Table 1. Characteristics of primary NSCLCs used to evaluate STR-3 abundance

<table>
<thead>
<tr>
<th>Pathological subtype</th>
<th>Stage</th>
<th>No. of patients</th>
<th>Neoadjuvant therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>I</td>
<td>14</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>Chemotherapy</td>
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<tr>
<td></td>
<td>III</td>
<td>18</td>
<td>XRT</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
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<td>7</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>Chemotherapy</td>
</tr>
<tr>
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<td>III</td>
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<td>21</td>
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Additional aliquots of the standardized cDNAs were then used to amplify STR-3 cDNAs with the following STR-3 oligonucleotide primers [5' sense, 5'-CCATGCAAGAGGAGCAGGAGCAG-3' (bp 361–384) and 3' antisense, 5'-GGATGACCAGAGCCACGACC-3' (bp 761–738); Ref. 5] under linear range conditions (29 cycles, 94°C denaturation, 68°C annealing, and 72°C extension, 1 min each). Samples were subsequently size fractionated on 1% agarose gels, transferred to nitrocellulose, and hybridized with a 32P-labeled STR-3 internal oligonucleotide probe [5' sense, 5'-GGATGACCAGAGCCACGACC-3' (bp 615–638); Ref. 5], which identified the expected 400-bp fragment.

Fig. 1. STR-3 transcript abundance in paired primary NSCLC and adjacent normal lung specimen cDNAs. A, actin standardization. The quality and quantity of each primary NSCLC and paired normal lung specimen cDNA was initially evaluated by actin semiquantitative reverse PCR (14). Paired tumor and normal lung actin reverse PCR samples from 58 patients with stages I–III adenocarcinoma and squamous cell carcinoma of the lung are shown. B, STR-3 transcript abundance. STR-3 transcript abundance in paired tumor and adjacent normal lung specimens from the same 58 patients was evaluated using semiquantitative reverse PCR. STR-3 hybridization signals in paired tumor and normal specimens were evaluated by scanning densitometry. Ninety-three percent of the evaluated primary NSCLC expressed significantly higher levels of STR-3 than adjacent normal lung (tumor versus normal lung absorbance values (mean ± SD): 1.38 ± 0.78 versus 0.265 ± 0.36; P < .001 (one-sided Student's t test)).
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RESULTS

STR-3 Abundance in Paired Primary NSCLC and Normal Lung Specimens. We initially assessed STR-3 transcript abundance in a broadly representative panel of paired primary NSCLC and adjacent "normal" lung specimens using semiquantitative reverse PCR. Paired tumor and normal lung sample RNAs were prepared from 58 patients with stages I-III adenocarcinoma (37 patients) and squamous cell carcinoma (21 patients; Table 1).

Initially, the panel of RNAs was reverse-transcribed and standardized to yield equivalent quantities of actin cDNA following amplification (Fig. 1A). Thereafter, additional aliquots of the standardized cDNAs were amplified for STR-3 under linear range conditions. Resulting PCR products were then size fractionated, blotted, and probed with a unique STR-3 internal oligonucleotide (Fig. 1B).

Amplified STR-3 cDNAs from paired tumor and normal lung specimens from patients with stages I-III adenocarcinoma or squamous cell carcinoma of the lung are shown in Fig. 1B. It is readily apparent that STR-3 reverse PCR products are consistently more abundant in primary NSCLCs than in adjacent normal lung specimens. Specifically, 93% of the evaluated primary NSCLCs expressed significantly higher STR-3 levels than adjacent normal lungs. Furthermore, three of the only four patients whose tumors had undetectable STR-3 (2 patients) or increased


![Fig. 2. STR-3 immunoblotting of paired primary NSCLC and adjacent lung lysates. Equivalent concentrations (50 µg) of paired tumor and normal lung lysates from 18 patients with stage I or III squamous cell carcinoma or adenocarcinoma were immunoblotted and analyzed for STR-3 protein. Seventeen of 18 primary tumors contained the Mr ~45,000 kDa processed active STR-3 enzyme (and additional lower molecular weight STR-3 proteins). The primary NSCLC expressed significantly higher levels of STR-3 than adjacent normal lung [tumor versus normal lung absorbance values (mean ± SD): 0.90 ± 0.60 versus 0.05 ± 0.05; P < .001 (one-sided student’s t test)].](image-url)
STR-3 in the adjacent normal lung (1 patient) had received neoadjuvant chemotherapy or radiation therapy for stage III disease prior to surgical resection (Fig. 1B). Taken together, these data indicate that STR-3 transcripts are more abundant in virtually all newly diagnosed stages I–III primary adenocarcinomas and squamous cell carcinomas of the lung.

To determine whether there were similar differences in STR-3 protein in paired NSCLC and normal lung specimens and to further characterize STR-3 protein products, we immunoblotted 18 representative paired samples from patients with stage I or III squamous cell or adenocarcinoma (Fig. 2) for STR-3. In recent studies, three forms of STR-3 have been identified in supernatants from cells transfected with the full-length STR-3 cDNA: (a) a presumptive full-length Mr ~65,000zymogen; (b) a Mr 45,000–50,000 form that lacks the NH2 terminal 97-amino acid propeptide but contains an intact carboxy terminus; and (c) a variably seen Mr ~28,000 breakdown product with weak caseinolytic activity (11, 20). The Mr ~45,000–50,000 kD STR-3 protein is the processed mature form of the enzyme that results from furin-mediated cleavage in the trans-Golgi network (12).

As indicated in Fig. 2, the Mr ~45,000–50,000 kD active STR-3 enzyme and additional lower molecular weight STR-3 proteins are readily apparent in 17 of 18 early and late stage NSCLCs; these STR-3 proteins are expressed at significantly higher levels in the primary NSCLCs than adjacent normal lungs [tumor versus normal lung absorbance values (mean ± SD) 0.90 ± 0.60 versus 0.05 ± 0.05; P < 0.001]. These data identify the Mr 45,000–50,000 kD STR-3 processed enzyme as the major form detectable in primary NSCLC and confirm the overexpression of the enzyme in this malignancy.

**STR-3 Localization in Primary NSCLC Specimens.** To specifically identify STR-3-producing cells in the primary NSCLCs, we performed STR-3 in situ hybridization on serial sections of representative tumors. Representative NSCLCs, such as the squamous cell carcinoma shown in Fig. 3, were examined by light microscopy to delineate tumor and stromal elements and by dark-field microscopy to visualize autoradiographic signals. As indicated in Fig. 3, a and b, STR-3 transcripts were localized to stromal cells in direct proximity to tumor cells. To confirm the specificity of the STR-3 hybridization signals, additional primary tumor sections were also hybridized with the corresponding sense STR-3 probe (Fig. 3, c and d), which produced no specific signals.

To further characterize STR-3-producing cells in the paired NSCLC and adjacent normal lung specimens, we immunostained serial sections of representative tumors and adjacent normal lung with anti-STR-3 and anti-keratin mAbs or control mouse immunoglobulin (Fig. 4). In the two representative primary squamous cell carcinomas shown in Fig. 4 (rows 1 and 2), tumor cells are delineated by their keratin positivity (solid arrows), and stromal elements are characterized by their lack of keratin staining (open arrows). It is readily apparent that STR-3 immunostaining is most prominent in the stromal elements surrounding the keratin-positive tumor cells. In contrast, normal lung specimens (such as shown in Fig. 4, row 3) lacked detectable STR-3 stromal staining. These data are consistent with the RNA studies demonstrating quantitative differences in STR-3 abundance in NSCLCs and adjacent normal lungs (Fig. 1B) and localization of STR-3 transcripts to stromal elements in primary NSCLC (Fig.
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Fig. 4. STR-3 immunolocalization in primary NSCLC and normal lung specimen. Serial sections of two representative squamous cell carcinomas (rows 1 and 2) and adjacent normal lung (row 3) were immunostained with anti-keratin, anti-STR-3, or control mouse immunoglobulin. In two representative primary squamous cell carcinomas (tumors 1 and 2), tumor cells (solid arrows) are delineated by their keratin positivity, and stromal elements (open arrows) are characterized by their lack of keratin staining. As indicated in additional serial sections, STR-3 immunostaining is most prominent in stromal elements (open arrows) surrounding the keratin-positive tumor cells (solid arrows). In contrast, the representative normal lung specimen (row 3) has detectable keratin staining but lacks detectable STR-3 stromal staining.

3). These studies (Figs. 3 and 4) also confirm earlier reports of STR-3 as a stromal cell product in other epithelial malignancies (5–7, 9).

Factor-mediated Induction of STR-3 in Normal Pulmonary Fibroblasts. The dramatic overexpression of STR-3 by stromal cells in primary NSCLC and other epithelial malignancies suggests that STR-3 may be secreted by stromal cells in response to tumor-derived factors. For this reason, it is noteworthy that cytokines, including bFGF and PDGF and compounds such as TPA, reportedly induce STR-3 expression in fetal lung fibroblasts (CCL-153; Ref. 5). To further characterize STR-3 induction in fetal lung fibroblasts and adult pulmonary stromal elements, we treated CCL-153 cells and pulmonary fibroblasts derived from a normal adult donor (CCL-210; American Type Culture Collection) with bFGF, PDGF, or TPA and subsequently evaluated STR-3 transcript abundance. As indicated in Fig. 5, bFGF, PDGF, and TPA induce STR-3 transcripts in CCL-153 fetal lung fibroblasts, whereas TPA...
most effectively induces STR-3 transcripts in CCL-210 adult pulmonary fibroblasts.

**STR-3 Induction in Pulmonary Fibroblasts: Modulation by RA.** Because STR-3 is consistently overexpressed in primary NSCLC (Figs. 1B and 2) and linked to local invasion in related epithelial malignancies (7, 9), this MMP may be an important therapeutic target for chemoprevention strategies. For example, it is possible that chemopreventive agents such as RAs (21–23) reduce the incidence of subsequent head and neck lung cancers, in part, via their effects on STR-3. Pulmonary fibroblasts express functional RA receptors, providing a means by which RA may modulate pulmonary stromal cell products such as STR-3 (24).

For these reasons, we evaluated the effects of RA pre-treatment on STR-3 induction in pulmonary fibroblasts. In brief, CCL-153 and CCL-210 cells were pretreated with 10^{-5} M RA or vehicle alone and subsequently cultured in RA* or RA- serum-free medium containing bFGF, PDGF, TPA, or vehicle alone. Cells were harvested thereafter for mRNA isolation and Northern analysis (Fig. 5). Northern blots were initially probed with glyceraldehyde-3-phosphate dehydrogenase to assure equal loading of sample mRNAs and subsequently analyzed for STR-3 transcript abundance (Fig. 5).

As indicated, RA pretreatment completely inhibited bFGF-, PDGF-, and TPA-mediated induction of STR-3 in fetal lung fibroblasts (CCL-153) and TPA-mediated induction of STR-3 in adult pulmonary fibroblasts (CCL-210; Fig. 5). Taken together, these data support the possibility that RA may reduce STR-3 expression in pulmonary stromal cells.

**DISCUSSION**

STR-3 transcripts and protein are significantly more abundant in primary NSCLC of all stages and pathological subtypes than in paired adjacent normal lung tissue. Moreover, the furin-processed active STR-3 enzyme is detectable in the majority of NSCLC. Because furin itself is reported to be more abundant in primary NSCLC than in normal lung tissue (25), STR-3 precursors may also be processed more efficiently in NSCLC.

Although STR-3 is consistently overexpressed in primary NSCLC, the protein is primarily produced by interdicting stromal cells rather than the tumor itself. For these reasons, it is noteworthy that specific growth factors induce STR-3 in normal pulmonary fibroblasts and that STR-3 induction is inhibited by RA. Although these studies do not identify the specific factors that induce STR-3 in primary NSCLC, the data suggest that tumor-derived factors may induce STR-3 expression in surrounding stromal cells and that STR-3 regulation may have direct clinical significance. It is, therefore, of interest that MMP inhibitors with promising antitumor activity also inhibit STR-3 (11).

The fact that α1-PI is a major STR-3 substrate underscores the likely pathological significance of STR-3 overexpression in NSCLC. α1-PI is the predominant inhibitor of human neutrophil elastase in plasma and the lower respiratory tract (13, 26). Deficiencies of this STR-3 substrate result in unopposed elastase-mediated alveolar sepal injury and subsequent emphysema (13). Since α1-PI can bind to the extracellular matrix and inhibit its elastase-mediated proteolysis, STR-3 may indirectly promote elastase-mediated destruction of pulmonary extracellular matrix in NSCLC (26). These data are of particular interest because elastase activity has been linked with metastatic potential in other epithelial malignancies (27, 28). STR-3 may also reduce direct inhibitory effects of α1-PI on anchorage-independent tumor cell growth (14). STR-3 also degrades α2-antiplasmin (11), prompting speculation that STR-3 may indirectly increase local plasmin levels and promote plasmin-mediated conversion of additional proMMPs to their active forms in NSCLC (29). These data suggest that STR-3 may have a broader role in regulating the function of additional serpins at the tumor-stromal cell interface (11) and that the enzyme may hydrolyze additional as yet uncharacterized substrates.

STR-3 is overexpressed in NSCLC of all pathological stages and subtypes, indicating that STR-3 induction may be an early consequence of malignant transformation. In this regard, it is noteworthy that STR-3 has been linked with local invasion in breast and head and neck cancers and that STR-3 has been identified in malignant but not premalignant colonic lesions (7, 9, 30). The near uniformity of STR-3 overexpression in NSCLC suggests that it may be possible to define pathological levels of STR-3 in putum and to use this information to develop STR-3-based screening assays for NSCLC. Furthermore, RA-mediated inhibition of STR-3 induction in pulmonary stromal elements may provide a partial explanation for the efficacy of RAs as chemopreventive agents for aerodigestive tract malignancies. Therefore, RA may be modulating primary stromal cell products (24) as well as suppressing squamous metaplasia (22). Although RAs are known to modulate the expression of other MMPs (31–33), STR-3 is the only MMP that is near-uniformly overexpressed in primary NSCLCs. For these reasons, STR-3 may be a novel surrogate marker and potential therapeutic target in these diseases.

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