Thrombospondins I and II Messenger RNA Expression in Lung Carcinoma: Relationship with p53 Alterations, Angiogenic Growth Factors, and Vascular Density

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ABSTRACT

Thrombospondin (TSP) is a Mr 450,000 multifunctional matrix glycoprotein that interferes with tumor growth, angiogenesis, and metastasis. It has recently been shown that TSP expression is enhanced by the product of the p53 gene and that a down-regulation of TSP may be observed when alterations of the p53 protein occur. Moreover, a number of studies have demonstrated a regulatory activity of p53 on human vascular endothelial growth factor (VEGF), although additional investigations will be necessary to understand their relationship. In non-small cell lung carcinoma (NSCLC), neoangiogenesis, p53 alterations, and VEGF expression seem to have meaningful implications in the development and progression of this type of cancer. The aim of this study is to identify and quantitate TSP I and TSP II mRNA in NSCLCs with respect to p53 alterations, angiogenic growth factor expression, and microvascular density. A series of 24 cases of NSCLC were analyzed. Eleven of 24 of the cases were positive for TSP II mRNA, whereas 8 of 24 showed TSP I mRNA expression. A significant inverse association was found between TSP I mRNA and fibroblast growth factor (FGF) protein expression (P = 0.00001). Tumors with low FGF protein expression (≤40% of positive cells) presented a number of TSP I cDNA molecules, significantly higher than tumors expressing high levels of FGF protein. No association was found between TSP mRNA expression and other angiogenic growth factors (i.e., VEGF) or tumoral neovascularization. On the contrary, tumors with high levels of FGF showed a higher number of microvessels (P = 0.05). By PCR-single-strand conformational polymorphism analysis, we observed aberrations of the p53 gene in 19 of the 24 tumor samples. No association was found between p53 alterations and TSP mRNA expression. Instead, an interestingly significant association was found between the presence of p53 mutations and high VEGF protein expression (P = 0.01) and neovascularization (P = 0.03). Highly vascularized tumors showed higher VEGF protein expression (r = 0.45; P = 0.02). These data support the concept that in NSCLC, p53 exerts an important role in the control of neoangiogenesis. This influence is probably mediated by VEGF. The inverse association we found between TSP I and basic FGF suggests a different role of TSP I and TSP II in the angiogenic “switch,” supporting the hypothesis that especially TSP I may have a significant function in tumor angiogenesis.

INTRODUCTION

Growth and progression of tumors require many interactions between tumor cells and the host. The primary tumor needs a vascular bed providing the nutrients (1) and allows it to interact with the immune host system to avoid immune surveillance. TSP1 is a Mr 450,000 extracellular matrix glycoprotein member of the thrombospondin gene family, which has attracted interest as a potential regulator of tumor growth and metastasis (2). A number of studies have underlined two putative different roles of TSP I in tumor development and progression, either supportive or inhibitory (3–13). TSP I injection into mice 5 min before the injection of sarcoma cells potentiates the development of lung metastases (5), just as elevated levels of TSP I may promote malignant transformations and support the invasive nature of breast cancer (6, 9). On the other hand, a suppressive activity of TSP I in cancer cell proliferation and metastasis has been shown in various experimental models such as murine melanoma and lung and breast human cell lines (11, 12).

Recent results have hypothesized a regulation of TSP I by tumor suppressor genes such as p53 and NM23 (11, 14). Experiments in fibroblasts by patients with Li-Fraumeni syndrome have demonstrated that alterations of the p53 gene, in terms of loss of the wild-type allele, down-regulate TSP I, prompting the tumor cells toward an angiogenic phenotype. Moreover, interesting relations between TSP expression and biological and clinicopathological parameters have been found in human blad-

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The abbreviations used are: TSP, thrombospondin; FGF, fibroblast growth factor; bFGF, basic FGF; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; wt, wild type.
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der and breast cancers (15). Recently, Loganadanė et al. (16) have demonstrated that some proliferative cytokines such as interleukin 6 and bFGF inhibit TSP secretion in the subendothelium, and Patel et al. (17) have observed that in the hyperplastic rat thyroid, angiogenesis is accompanied by increases in bFGF and decreases in TSP I. In terms of interactions between tumor suppressor genes and/or oncogenes and angiogenic growth factors, i.e., VEGF, interesting results were obtained both in experimental and human models. Kieser et al. (18) showed that a mutant form of p53 was involved in the 12-O-tetradecanoylphorbol-13-acetate induction of the VEGF gene expression mediated by protein kinase C. Moreover, a suppressive effect of the VEGF gene expression on an adenovirus-transformed human fetal kidney cell line by wild-type p53 was demonstrated by Mukhopadhyay et al. (19).

p53, neoangiogenesis, and some angiogenic growth factors (i.e., VEGF) have been shown to be significantly related to each other and to influence the development and progression of NSCLC (20–22). The purpose of our study is to further investigate the relationship between p53, neoangiogenesis, and angiogenic regulators such as TSP and angiogenic growth factors to better understand the biological profile of this type of cancer.

MATERIALS AND METHODS

Patients and Tumor Tissues

Twenty-four lung cancer samples were obtained from patients who underwent wedge resection, lobectomy, or pneumonectomy at the Department of Surgery of Pisa University. There were 20 males and 4 females with a combined median age of 64 years (range, 40–80 years). Tumor specimens were obtained at resection, in part immediately snap frozen for RT-PCR and SSCP analysis and in part fixed in formalin and embedded in paraffin for histological and immunohistochemical processing. The pathological features of the samples were classified according to the WHO histological criteria (23) and to the guidelines of the American Joint Committee for Cancer Staging (24).

Immunohistochemistry

Growth Factor Expression. Immunostaining for FGF and VEGF was performed using the ABC method in formalin-fixed, paraffin-embedded tissue samples. Sections were dehydrated in xylene, taken through ethanol, and then incubated with 0.3% hydrogen peroxidase in methanol for 10 min to block the endogenous peroxidase activity. After washing with PBS and incubation for 30 min with 10% normal goat serum, the sections were incubated overnight with anti-FGF and anti-VEGF polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution: 1:100 and 1:50, respectively). Anti-bFGF is a goat polyclonal IgG raised against an epitope corresponding to amino acids 40–63 mapping within the terminal domain of the bFGF precursor of human origin. Anti-VEGF is raised against a synthetic peptide corresponding to amino acid residues 1–191 of human VEGF; it recognizes the 165, 189, and 121 amino splicing variants of VEGF. After the primary antibodies, biotinylated anti-goat and anti-rabbit IgG (Vector Laboratories, Burlingame, CA) were applied and followed by detection using the ABC method (Vector Laboratories). Light counterstaining was performed with hematoxylin. Normal goat and rabbit immunoglobulin G substituted the primary antibody as negative controls. The FGF and VEGF expressions were evaluated as a percentage of positive cells in a total of at least 1000 tumor cells. Tumor sections with no FGF and VEGF immunoreactive cells were considered as negative. The median values of the two series (40% of positive cells in both cases) were used as cutoff values to distinguish low from high FGF and/or VEGF-expressing tumors.

Microvessel Detection and Counting. The method of microvessel detection and counting has been described previously (21). Briefly, intratumor microvessels were highlighted with anti-CD34 Mab (QB-END 10; Novocastra Laboratories, Newcastle, UK) diluted 1:100, after heating the sections in a microwave oven twice for 5 min at 700 W in citrate buffer (pH 7.6). Biotinylated anti-mouse IgG (Vector) was applied for immunoreaction, followed by detection using the ABC method. A single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessel, tumor cells, and other connective tissue elements (Fig. 1, left panel). Each sample was examined under low power (×10 objective lens and ×10 ocular lens) to identify the three regions of the section with the highest number of microvessels. A ×250 field (×25 objective lens and ×10 ocular lens; 0.74 mm²/field) was counted in each of these three areas, and the average counts of the three fields were recorded. Large vessels with thick muscular walls were excluded in the counts. The lumen was not required to identify a vessel.

Competitive RT-PCR for TSP

RNA Extraction. Frozen lung tissue samples were homogenized in liquid nitrogen, and total RNA was extracted using a RNA extraction reagent, ULTRASPEC RNA (Biotech Laboratories, Inc., Houston, TX), according to the standard acid guanidinium-phenol-chloroform method.

RT-PCR Analysis. A constant amount of total RNA (5 μg) was reverse-transcribed at 42°C for 60 min in a total 20-μl reaction volume using the 1st-Strand cDNA Synthesis kit (Clontech Laboratories, Inc., Palo Alto, CA); cDNA was incubated at 95°C for 5 min to inactivate the reverse transcriptase and served as a template DNA for 30 rounds of amplification using the Gene Amp PCR System 2400 (Perkin-Elmer Applied Biosystem, CA). PCR was performed in a standard 50-μl reaction mixture consisting of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂ (pH 8.3), 0.2 mM deoxynucleotide triphosphates, 20 pM of each sense and antisense primer, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Foster City, CA). PCR primers for TSP I cDNA were as follows: forward primer, 5’-CGTCTCTGTCCTCAGATCATG-3’ (position 99–118); and reverse primer, 5’-GCGCAGGACACCCTTTTGGCAG-3’ (position 1115–1135). TSP II primers were 5’-CAAGGCTCTGCCTGTCTGCA-3’ (position 2054–2073) and 5’-GGGCACCTCCTCCATTGCTG-3’ (position 2614–2633) for 3’ and 3’ primers, respectively (15). Amplification was performed for 60 s at 94°C, 1 min at 55°C, and 1 min at 72°C. Finally, an additional extension step was performed for 2 min. As negative control, the DNA template was omitted in the reaction. Amplified PCR products were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The presence of a single 412-bp band amplified with primers specific for glycer-
aldehyde-3-phosphate dehydrogenase with the same cDNAs was used as internal control under identical conditions. For glycer-
aldehyde-3-phosphate dehydrogenase, the forward primer was 5'-CGATGCTGCGCTAGTAC-3', and the reverse primer was 5'-CTTCAGCTAGGGATGACC-3' (25).

Quantitative PCR Reaction. To obtain the quantitation of TSP mRNA levels, we used a technique based on a compet-
itive PCR approach using a nonhomologous internal standard called COMPETITOR (PCR MIMIC Construction kit; Clon-
tech). The method involves amplification of a heterologous DNA fragment (BamHI/EcoRI 574-bp fragment of v-erbB) with a pair of composite primers that contain the target primer sequences contiguous to a sequence that anneals the heterolo-
gous DNA fragment. During amplification, the target primer sequences were incorporated into the products. For this reason, we refer to this heterologous competitor fragment as COMPET-
ITOR because it “competes” with the target gene for primer annealing and amplification. Two successive PCR amplifications were performed to construct the COMPETITOR. In the first PCR reaction, two composite primers were used, each having the TSP I/TSP II target gene primer sequence attached to a short, 20-nucleotide stretch of sequence designed to hybridize to opposite strands of the heterologous DNA fragment. The two composite primers and 2 ng of the heterologous DNA were added to a PCR reaction. During amplification of the DNA fragment, the target primer sequences were incorporated into the PCR product. A dilution of this 597 bp for TSP I and 506 bp for the TSP II PCR product was used to perform a second PCR using the target TSP primers, ensuring that the complete target primer sequences has been incorporated into the COMPETI-
TOR. At this point, we removed the primers and the reaction components by passing the secondary PCR product through a

PCR-SSCP Analysis

DNA Extraction. Tissue samples were mechanically disrupted in liquid nitrogen and lysed by proteinase K. DNA extraction was then performed using the spin column procedure (QIAamp Tissue kit; Qiagen). The purification procedure com-
prised three steps: loading of the entire lysate onto the spin column with adsorption of DNA to the silica membrane; re-
moval of residual contaminants by washing in the centrifugation step; and elution in sterile water.

PCR-SSCP Screening for p53 Gene Mutations. The eluted DNA was used as template in a standard 20-μl PCR reaction mixture consisting of 10 μl Tris-HCl, 50 μl KCl, 1.5

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nodal metastatic involvement at diagnosis. In our analysis, no significant association was found between TSP and pathological parameters, as reported in Table 2 (Kruskal-Wallis and Mann-Whitney U test).

**Relationship between TSP mRNA Expression and p53 Alterations.** The highly preserved regions of the p53 gene, exons 4–8, were amplified by PCR. Altered mobilities of amplified DNA fragments, indicating a structural aberration of the p53 gene, were observed in 19 of the 24 (79.1%) cases analyzed. Examples of mutations detected in exons 4–8 using SSCP screening are shown in Fig. 2. The alterations were distributed among the following exons: 1 in exon 4, 5 in exon 5, 11 in exon 6, 9 in exon 7, and 2 in exon 8. When the number of cDNA molecules of both TSP I and TSP II, evaluated by competitive PCR, was compared with p53 alterations of the tumors, no association was found between TSP mRNA and the presence of p53 mutations (Fig. 3), even if the TSP I mRNA expression was higher in wt p53. No association was found between p53 alterations and TSP, even when the two histological subgroups were separately analyzed (data not shown).

**Relationship among TSP, Angiogenic Growth Factors, and Neoangiogenesis.** VEGF and FGF immunostaining was observed and evaluated in the cytoplasm of neoplastic cells; moreover, some stromal cellular components, such as inflammatory and fibrohistiocytic cells, occasionally expressed VEGF and FGF proteins, even if this kind of expression was not compared with TSP mRNA in this study. Table 3 reports the association between TSP I and TSP II and some biological parameters such as microvessel density, FGF, and VEGF. No difference was observed in the TSP mRNA expression between tumors with different vascular densities or different VEGF expression. On the contrary, a significant inverse association was found between FGF protein expression and TSP I. Tumors with low FGF expression (<40% of positive cells) showed a significantly higher expression of TSP I cDNA molecules, whereas no TSP I expression was detected in highly expressing bFGF tumors ($P = 0.00001$; Table 3; Fig. 4; Mann-Whitney U test). No equal association was found between FGF and TSP I, when squamous carcinomas and adenocarcinomas were examined separately (data not shown).

**p53 Mutations and VEGF Protein Expression.** When we analyzed p53 mutations in comparison with the VEGF protein expression, we observed that tumors with aberration of the p53 gene showed a significantly higher VEGF protein expression than tumors without p53 mutations (Mann Whitney U test, $P = 0.01$; Fig. 5). Moreover, the VEGF protein expression was significantly correlated with the number of microvessels evaluated in the most intense areas of neovascularization of the tumors (hot spot; Spearman’s test: $r = 0.45$; $P = 0.03$; Fig. 6).

**p53 Mutations and Neangiogenesis.** As shown in Fig. 7, tumors showing p53 mutations were significantly more vascularized than tumors without p53 gene alterations. As a matter of fact, the mean microvessel count in p53 mutated cancer was $38.3 \pm 26.7$ compared with $11.4 \pm 4.8$ of the wt p53 tumors (Mann-Whitney U test; $P = 0.03$).

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**Table 1** TSP I and TSP II mRNA expression in NSCLC

<table>
<thead>
<tr>
<th>TSP expression</th>
<th>No. of cases/total</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSP I</td>
<td>8/24</td>
<td>153.5 ± 75</td>
</tr>
<tr>
<td>TSP II</td>
<td>11/24</td>
<td>313 ± 78.6</td>
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</table>

**Table 2** TSP I and TSP II mRNA expression according to clinicopathological characteristics

<table>
<thead>
<tr>
<th>Patient and tumor characteristics</th>
<th>No. of cases (%)</th>
<th>TSP I expression (mean)</th>
<th>TSP II expression (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>50.8</td>
<td>53.3</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>104.2</td>
<td>50.6</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\leq 64$</td>
<td>12</td>
<td>68.9</td>
<td>67.4</td>
</tr>
<tr>
<td>$&gt;64$</td>
<td>9</td>
<td>44.5</td>
<td>33.5</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous</td>
<td>10</td>
<td>72.4</td>
<td>49</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>9</td>
<td>47.8</td>
<td>51.2</td>
</tr>
<tr>
<td>Anaplastic large cell</td>
<td>2</td>
<td>36.5</td>
<td>80</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$T_1$</td>
<td>5</td>
<td>62.1</td>
<td>77.2</td>
</tr>
<tr>
<td>$T_2$</td>
<td>13</td>
<td>41.2</td>
<td>42.1</td>
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<tr>
<td>$T_3$</td>
<td>3</td>
<td>127.3</td>
<td>59</td>
</tr>
<tr>
<td>Node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N_0$</td>
<td>13</td>
<td>46.8</td>
<td>50.5</td>
</tr>
<tr>
<td>$N_1$</td>
<td>1</td>
<td>110.6</td>
<td>57</td>
</tr>
<tr>
<td>$N_2$</td>
<td>7</td>
<td>72.5</td>
<td>56.8</td>
</tr>
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</table>
DISCUSSION

The aim of this study was to investigate the relationship between the expression of thrombospondin mRNA, p53 alterations, neoangiogenesis, and some angiogenic growth factors in NSCLC. The study was supported by our previous analyses, which underlined an important role for neoangiogenesis, as well as p53 alterations in the development and progression of NSCLC (20-22). We found that TSP I and TSP II were both expressed in tumor samples, although more tumors expressed TSP II, and the mean value of cDNA molecules was slightly higher for TSP II than for TSP I. Similar data have also been reported by Bertin et al. (15), who found a concomitant expression of both TSP I and TSP II mRNAs in breast cancer tissues. In this study, an interesting inverse association was observed between TSP I mRNA expression and bFGF expression, which suggests that bFGF and TSP I could strictly interact in the control of tumor angiogenesis. In this respect, bFGF has been reported recently to inhibit TSP secretion, because its incorporation into the subendothelial matrix is inversely proportional to cell density (16). Furthermore, interesting experiments in vitro support this data, showing that TSP is able to bind bFGF through a domain within its Mr 140,000 fragment, a mechanism that might affect bFGF interaction with endothelial cells and association with the extracellular matrix (25). TSP I can antagonize the stimulation of endothelial cell growth and motility induced by bFGF (16, 17). In fact, TSP I binds avidly to heparin, sulfatide, and heparan sulfate proteoglycans through an NH$_2$-terminal heparin binding domain, through secondary heparin binding sites in type 1 repeats, and possibly through additional undefined sites (2). The inverse association found in our series between the TSP I and bFGF protein expression is probably the result of this complicated

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**Fig. 2** PCR-SSCP detection of p53 mutation in human lung tumors. Exon 4, aberrant band patterns in samples 1 and 3. Exon 5, aberrant band patterns in samples 2 and 4. Exon 6, aberrant band patterns in sample 3. Exon 7, aberrant band patterns in samples 3 and 4. Exon 8, aberrant band patterns in sample 1.

**Fig. 3** Relationship between p53 mutations and TSP I/TSP II mRNA expression in NSCLC. TSP I and TSP II mRNAs were expressed as number of VEGF cDNA molecules, calculated by the ratio of amplified target to competitor PCR products. Bars, SD.

**Table 3** TSP I and TSP II mRNA expression according to biological characteristics

<table>
<thead>
<tr>
<th>Patient and tumor characteristics</th>
<th>No. of cases</th>
<th>TSP I expression (mean)</th>
<th>TSP II expression (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVD $^\alpha$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤14</td>
<td>10</td>
<td>59.6</td>
<td>53.3</td>
</tr>
<tr>
<td>&gt;14</td>
<td>11</td>
<td>57.4</td>
<td>52.6</td>
</tr>
<tr>
<td>VEGF expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>11</td>
<td>46.5</td>
<td>62.5</td>
</tr>
<tr>
<td>&gt;40</td>
<td>10</td>
<td>69.3</td>
<td>42.3</td>
</tr>
<tr>
<td>FGF expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>7</td>
<td>85.4</td>
<td>82.4</td>
</tr>
<tr>
<td>&gt;40</td>
<td>7</td>
<td>7</td>
<td>27.7</td>
</tr>
</tbody>
</table>

$^\alpha$ MVD, microvessel density.
interaction. Because tumors showing high levels of bFGF protein expression presented a trend toward an increased angiogenesis, tumoral neoangiogenesis might also be influenced by the relationship between TSP I and bFGF. The lack of correlation between TSP and p53 alterations in our study disagrees with data from other groups, who noticed either in the in vitro (14) or in the in vivo (26) models a significant association between p53 alterations and TSP down-regulation. However, in our study, we observed a significant association between p53 mutations and one of the most important neoangiogenesis regulators, i.e., VEGF. Moreover, p53 mutated cancer also showed a significantly higher mean vascular count than wt tumors. In our previous analyses (22), we have already underlined that in NSCLC, the p53 protein overexpression is significantly associated with the VEGF and with vascular count, and that the latter correlates directly with the VEGF protein expression. Our study confirms these data and adds further information on the important role of p53 mutations in tumor angiogenesis control. Nineteen of the 24 cases analyzed showed p53 mutations in exons 5–8, and these mutations are probably responsible for the loss of suppressive activity of wt p53 on the expression of the VEGF, allowing an increase of VEGF synthesis with increased angiogenesis. These data find strong support in some experimental evidence reported by various groups, who have underlined interesting interactions between the p53 gene and VEGF (18, 19), although in a recent paper Agani et al. (27) have demonstrated that wt p53 does not repress the hypoxia-induced transcription of the VEGF in the Hep3B cell line. However, other recent observations in human colon cancer highlight that p53 overexpression, vessel count, and VEGF are strictly associated (28). In our study, no correlation was found between TSP and vascular count (as a measure of tumor angiogenesis); however, an association was noticed between p53 alterations, VEGF protein expression, and microvascular density; therefore, we can hypothesize that in NSCLC, neoangiogenesis can be regulated by p53 differently than in other human cancers. As a significant association was demonstrated between p53 gene mutations and VEGF expression, the p53 regulation of neoangiogenesis in NSCLC could be mediated through VEGF instead of TSP. However, additional and larger analyses will be necessary both in in vitro and in in vivo models to clarify the intrinsic mechanisms and interactions that govern the gene regulation of tumoral angiogenesis.

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