Thrombospondin-1 Expression in Bladder Cancer: Association With p53 Alterations, Tumor Angiogenesis, and Tumor Progression

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Background: Thrombospondin-1 (TSP) is a 430-kd glycoprotein that is an important component of the extracellular matrix and is known to be a potent inhibitor of angiogenesis (i.e., formation of new blood vessels) both in vitro and in vivo. Several reports suggest that TSP possesses tumor suppressor function, possibly through its ability to inhibit tumor neovascularization. It has recently been shown that TSP expression is enhanced by the product of the p53 gene (also known as TP53). Purpose: We examined the role of TSP expression in tumor recurrence and overall survival in patients with invasive bladder cancer. We also examined the relationship between alterations in p53 protein expression, TSP expression, and tumor angiogenesis. Methods: Tumors from 163 patients (with a median follow-up of 7.7 years) who underwent radical cystectomy for invasive transitional cell carcinoma of the bladder (63 patients with organ-confined disease and no lymph node involvement, 48 patients with extravesical extension of the disease and no lymph node involvement, and 52 patients with metastasis to regional lymph nodes) were examined for TSP expression by immunohistochemistry, utilizing monoclonal antibody MA-II, which recognizes an epitope in the amino-terminal region of TSP. For each tumor, microvessel density counts and p53 protein expression status (via immunohistochemistry) were also determined. TSP expression was graded as low, moderate, or high without knowledge of clinical outcome, p53 status, and microvessel density count; tumors with moderate and high TSP levels were considered as one group. Groups of patients were compared by Kaplan–Meier product limit estimates of overall survival, the complement of cumulative incidence curves for recurrence-free survival, and the stratified logrank test. Reported P values are two-sided. Results: TSP expression was significantly associated with disease recurrence (P = .009) and overall survival (P = .023). Patients with low TSP expression exhibited increased recurrence rates and decreased overall survival. TSP expression was an independent predictor of disease recurrence (P = .002) and overall survival (P = .01) after stratifying for tumor stage, lymph node status, and histologic grade, but it was not independent of p53 status. TSP expression was significantly associated with p53 expression status (P = .001) and microvessel density counts (P = .001). Tumors with p53 alterations were significantly more likely to demonstrate low TSP expression, and tumors with low TSP expression were significantly more likely to demonstrate high microvessel density counts. Results of an analysis of variance were compatible with the hypothesis that p53 affects tumor angiogenesis by regulating the level of TSP expression. Conclusions and Implications: These data support the concept that TSP may possess a tumor-inhibitory function. TSP may act, in part, through the regulation of tumor neovascularity. These results may also provide insight into one mechanism by which p53 exerts its tumor suppressor effects, i.e., through the control of tumor angiogenesis. [J Natl Cancer Inst 1997;89:219-27]

Thrombospondin-1 (TSP) is a 450-kd adhesive glycoprotein that was initially discovered in platelets, where it is sequestered within the platelet α-granule (1). Human TSP is a disulfide-bonded trimer (2) with several different domains, including a heparin-binding amino terminal, a calcium-dependent region, three epidermal growth factor-like repeats, and a carboxy-terminal domain (1). In addition to being involved with hemo- stasis as a component of the platelet α-granule, TSP has been shown to be synthesized and secreted by many normal and transformed cells in culture (3). TSP is now recognized as an endog- enous constituent of the extracellular matrix in many human tissues.

TSP has been implicated in the regulation of cell growth and proliferation (4), cell motility (5,6), cytoskeletal organization (7), inflammation and wound healing (8), and the development and differentiation of cell types (9,8). In vitro, TSP induces the attachment and spreading of human squamous carcinoma cells (10) and melanoma cells (11) and promotes chemotaxis and haptotaxis (i.e., cell migration to a substratum-bound gradient) of human melanoma cells and breast carcinoma cells (5).

Both supportive and inhibitory roles for TSP in cancer cell proliferation and metastasis have been identified (10-19). TSP injection into mice 5 minutes before the injection of sarcoma cells potentiates lung tumor colony formation, possibly by mediating tumor cell adhesion (12). In NIH 3T3 cells, overexpression of TSP results in cell growth that is serum and anchorage independent (15). In breast cancer, increased levels of TSP expression may promote malignant transformation and support the invasive nature of breast cancer (13,16).
Strong evidence also exists to support an inhibitory role for TSP in cancer cell proliferation and metastasis. TSP expression in the desmoplastic stroma associated with invasive ductal carcinomas of the breast may have a protective effect on tumor invasiveness. TSP is known to promote the adhesion and growth of fibroblasts that may aid in tumor proliferation and metastasis. TSP expression comes from studies in murine melanoma and human lung and breast cancer cell lines, where an inverse correlation has been reported between TSP messenger RNA (mRNA) and protein expression and malignant progression (18). Furthermore, transfection studies in breast cancer cell lines have demonstrated that tumor cell production of TSP exerts an inhibitory effect on tumor progression (19). TSP expression appears to be increased by the nm23-1 tumor suppressor gene, but it is repressed by both the c-jun and ras oncogenes (18,20).

These latter studies strongly suggest that TSP may possess a tumor suppressor function. While the mechanism by which TSP exerts this function is unknown, it is possible that TSP may act, in part, by inhibiting tumor angiogenesis. TSP has been shown to modulate endothelial cell adhesion, motility, and growth and to exert an antiangiogenic effect on cord formation in vitro (4,21). In vivo, human TSP has been found to be a potent inhibitor of neovascularization, leading to the hypothesis that TSP is a tumor suppressor gene-dependent inhibitor of angiogenesis (22).

Recent studies have established a link between TSP expression, tumor angiogenesis, and the p53 tumor suppressor gene (also known as TP53). Experiments in cultured fibroblasts from Li–Fraumeni patients demonstrate that tumor cells switch to an angiogenic phenotype coincident with the loss of the wild-type (wt) allele of the p53 gene and the concomitant decline in TSP expression (23). Transfection assays in these cells revealed that reintroduction of the wt p53 allele stimulates the endogenous TSP gene, thereby again restoring an antiangiogenic phenotype. These studies indicate that, in fibroblasts, wt p53 inhibits angiogenesis through the up-regulation of TSP synthesis.

The purpose of this study was to investigate further the role of TSP expression in tumor progression in patients with bladder cancer by examining the relationship of TSP expression with disease recurrence and overall survival. In addition, the relationship between TSP expression, p53 status, and tumor angiogenesis was investigated to examine the hypothesis that the p53 gene product regulates the level of TSP expression, which in turn influences the degree of tumor neovascularization.

**Materials and Methods**

**Patient Population**

We studied tumor tissue from 163 patients who underwent radical cystectomy, pelvic lymph node dissection, and urinary diversion at the University of Southern California/Kenneth Norris Jr. Comprehensive Cancer Center for bladder cancer during the period from January 1982 through February 1992. Comprehensive follow-up data and samples from the cystectomy specimen were available for each patient studied. All specimens were transitional cell carcinomas; a minority demonstrated glandular or squamous differentiation. We excluded patients with pure adenocarcinoma, squamous cell carcinoma, or small-cell carcinoma of the bladder who were undergoing cystectomy during this interval. The median age of all patients was 66 years (range, 38-87 years); 82% were male, and 18% were female.

**Clinical and Pathologic Evaluation**

The indications for radical cystectomy in this group of patients included tumor invasion of the muscularis propia of the urinary bladder or stroma of the prostate, high-grade superficially invasive tumors associated with carcinoma in situ, carcinoma in situ refractory to intravesical chemotherapy or immunotherapy, and recurrent multifocal disease after conservative therapy. None of the patients received systemic chemotherapy before surgery.

Tumor tissue was formalin fixed and paraffin embedded, graded according to the method of Bergkvist et al. (24), and staged according to the tumor–node–metastasis (TNM) classification (25). All tumors were processed in a similar fashion. Briefly, fresh tissue removed from the operating room was immediately sectioned and fixed in 10% neutral buffered formalin at room temperature for at least 4 hours, but for no more than 12 hours. Tissue was then dehydrated through graded alcohol solutions, processed in xylene, and embedded in paraffin. Sections were stained in hematoxylin–eosin and reviewed. Representative sections that displayed both carcinoma and normal urothelium were chosen for immunohistochemical analysis.

Among the 163 tumors, five were classified histologically as grade 2, 94 as grade 3, and 64 as grade 4. There were 63 patients with organ-confined (stage P1, P2, P3a) lymph node-negative disease, 48 patients with extravesical (stage P3b or P4) lymph node-negative disease, and 52 patients were found upon pathologic examination to have metastatic disease in the pelvic lymph nodes (N1-3). None of the patients had known distant metastatic disease at the time of surgery.

The median follow-up for the 163 patients was 7.7 years; 93% of the patients currently alive have at least 3 years of follow-up. Patients were seen at 3-month intervals during the first postoperative year, 4-month intervals during the second postoperative year, and every year thereafter. Follow-up consisted of a biochemical profile, chest radiography, and a physical examination. A computerized tomographic scan or bone scan was performed to confirm suspected recurrences of disease.

**Monoclonal Antibodies and Immunohistochemical Analysis**

Unstained sections (5 μm) of archival, formalin-fixed, paraffin-embedded tissue were cut and mounted on commercially provided, positively charged slides (ProbeOn Plus; Fisher Scientific Co., Pittsburgh, PA). The immunohistochemical procedures for p53, using the PAb 1801 antibody, and for microvessel density counts, using the anti-CD34 antibody HPCA-1, were described elsewhere (26,27). Anti-thrombospondin antibody was a gift from Dr. Jack Lawler (Brigham and Women’s Hospital, Boston, MA). The antibody used was the mouse anti-TSP monoclonal antibody MA-II that recognizes an epitope in the amino-terminal region of TSP-1 (28). A dilution of 1:1000 of antibody was utilized in all experiments. The optimal immunohistochemical staining procedure for TSP involves antigen retrieval pretreatment using a low-pH buffer with high-temperature microwave heating. Briefly, all routinely processed paraffin tissues were deparaffinized with xylene–xylene and rehydrated with 95% and 100% ethanol. Endogenous peroxidase was quenched in 3% hydrogen peroxide–methanol for 20 minutes. Slides were then washed with tap water followed by distilled water and placed in a Coplin jar with Tris–HCl buffer solution at pH 1. Antigen retrieval utilizing microwave heating for 5 minutes two times in a Panasonic microwave oven (model NN-5625A) at the highest power setting was then performed.

After antigen retrieval treatment, slides were cooled for 15 minutes, followed by the immunohistochemical staining procedure. Normal horse serum was used for blocking nonspecific binding in tissue sections for 20 minutes. The primary antibody was incubated overnight at 4 °C. Tissues were then incubated with a biotinylated horse–antimouse secondary antibody, and reactivity was visualized by the Elite avidin–biotin–peroxidase system (Vector Laboratories, Inc., Burlingame, CA) with aminoethyl-carbazol as the chromagen. The positive controls were samples of bladder carcinoma with documented TSP reactivity. The internal negative control was normal urothelium. Only extracellular immunoreactivity was considered positive because cytoplasmic and nuclear reactivities were believed to represent an artifact of the antigen retrieval process (29).

A consistent classification scheme was previously developed by the authors (26). The slides were evaluated by one investigator (G. D. Grossfeld) and subsequently reviewed by a second investigator (R. J. Cote). Light microscopy was used to evaluate the intensity and localization of the immune reaction. Very few cases showed discordant readings. In all of these cases, the slides were restained and reviewed again. Concordance was reached in all cases. Investigators were
blinded to clinical outcome, p53 status, and microvessel density count. TSP immunostaining was graded as low, moderate, or high on the basis of the extent of extracellular immunoreactivity. Tissue sections were classified as having low TSP expression when they showed no or negligible-equivocal immunoreactivity in the intratumoral or immediate peritumoral areas. Tumors with detectable TSP immunoreactivity were classified as having moderate or high TSP expression but were considered together for the purpose of statistical analysis.

Previously described criteria (26) were used to grade p53 nuclear reactivity as positive or negative. Microvessel density counts were determined by use of the anti-CD34 antibody HPCA-1 as previously reported (27). Briefly, light microscopy was used to identify regions within or immediately adjacent to the tumor that contained the greatest microvessel density ("hotspots"). Microvessel counts were then performed on a 200X microscopic high-power field within the designated hotspot. Any stained endothelial cell was considered to represent a single microvessel if it was isolated from adjacent microvessels and other connective tissue elements. The median microvessel density count for this group of tumors was found to be 79. For the purpose of analysis, tumors were separated into two groups based on microvessel density count as previously described (30): Those tumors with counts below the median (<79 vessels) were defined as having low microvessel density counts, whereas those tumors with counts equal to or above 79 vessels were defined as having high microvessel density counts.

Statistical Analysis

The outcomes analyzed in this study were overall survival and time to first recurrence of bladder cancer. To determine whether TSP expression was associated with outcome, we divided the patient population into two groups based on the degree of TSP staining (low versus moderate and high combined). Survival was calculated as the number of years from cystectomy until death or the last documented contact when the patient was known to be alive. For patients with recurrent disease, the time to first recurrence of bladder cancer was calculated as the number of years from cystectomy to the date of first documented disease recurrence. Patients who died before recurrent disease developed were counted as failing from a competing cause at the time of death. Patients who were alive and had not experienced a bladder cancer recurrence were censored at the date they were last seen free of clinical disease.

Kaplan–Meier product limit estimates (31) of overall survival and the cumulative incidence curves (32) for recurrence-free survival were plotted. Standard errors were calculated on the basis of Greenwood’s formula (34) for Kaplan–Meier curves and on the basis of the delta method for cumulative incidence curves (32). When survival or recurrence was analyzed, the logrank test was used to compare groups of patients. To determine whether TSP expression provided prognostic information beyond that provided by tumor grade, tumor stage, and lymph node status, we used a stratified logrank test. We used contingency tables and the chi-squared test to evaluate the association between TSP expression and lymph node status, TSP expression and the pathologic stage of the primary tumor, and TSP expression and histologic grade (33), as well as to examine the pairwise association between TSP expression and immunohistochemical detection of p53 alteration and TSP expression and microvessel density count (dichotomized at the median). To examine the hypothesis that p53 status influences the level of TSP expression, which in turn affects the level of tumor neovascularity, we performed an analysis of variance in which the microvessel density counts were log transformed and used as the dependent ("outcome") variable. The “predictor” or independent variables were p53 status and level of TSP expression. An interaction term to measure whether the effect of TSP on microvessel density counts was the same for p53+ and p53− tumors was also included in this model. This term was not significant (P = .78) and was omitted from the final analysis.

Sequential sums of squares were used to test whether p53 status influenced microvessel density counts after we accounted for TSP and whether TSP affected microvessel density counts after we adjusted for p53 status. All P values reported are from two-sided tests.

Results

Of the 163 bladder tumors examined for TSP expression, 35 were found to have low levels of TSP expression, 100 were found to have moderate levels, and 28 were found to have high levels. For the purpose of this analysis, tumors with moderate and high TSP levels were considered as one group. The presence of TSP was confirmed by its characteristic extracellular staining pattern (Fig. 1). In each case, the tumor appeared to induce TSP expression in the extracellular matrix, inasmuch as no TSP immunostaining was found in the surrounding areas of normal urothelium. Consequently, the surrounding normal urothelium served as an internal negative control.

Association Between TSP Expression and Tumor Grade, Pathologic Stage, and Lymph Node Status

Analysis of the group of 163 patients demonstrated that TSP expression was not associated with histologic grade (P = .62) or pathologic stage (P = .59) (Table 1). No statistically significant association was seen between TSP expression and increasing depth of tumor invasion or the presence of lymph node metastases. Twenty-two percent of patients with organ-confined disease (P1, P2, and P3a) were found to have low TSP expression compared with 17% of patients with extravesical disease extension (P3b and P4) and 25% of patients with lymph node involvement.

Association Between TSP Expression and Tumor Recurrence and Overall Survival

Analysis of the entire group of 163 patients demonstrated that low TSP expression was significantly associated with an increased probability of disease recurrence (P = .009) and a decreased probability of overall survival (P = .023) (Table 2) (Figs. 2 and 3). The estimated 5-year probability of disease recurrence was 76% (95% confidence interval [CI] = 67%-85%) for patients with low levels of TSP expression and 42% (95% CI = 37%-47%) for patients with moderate or high levels. The estimated probability of surviving 5 years was 29% (95% CI = 21%-37%) for patients with low levels of TSP expression and 49% (95% CI = 45%-54%) for patients with moderate or high levels.

When stratifying by tumor stage, TSP expression continued to be a significant predictor for both disease recurrence (P = .001) and overall survival (P = .003) in this cohort of 163 patients. Sixty-three patients had pathologically confirmed organ-confined disease (P1, P2, and P3a; lymph node negative). Analysis of this subgroup of patients demonstrated that low TSP expression was significantly associated with an increased probability of disease recurrence (P = .0002) (Table 2) (Fig. 4). The 5-year probability of disease recurrence in these patients with organ-confined disease was 60% (95% CI = 47%-74%) for patients with low levels of TSP expression and only 11% (95% CI = 6%-15%) for patients with moderate or high levels. This association did not reach statistical significance for the 48 patients with extravesical disease extension only (P3b and P4; lymph node negative) or for the 52 patients with lymph node metastases. However, it is important to note that 100% of patients with low levels of TSP expression and lymph node metastases demonstrated disease recurrence by 5 years (Table 2).

TSP expression was significantly associated with overall survival in the entire group of 163 patients after stratifying by tumor...
stage ($P = .003$). This association, however, did not reach statistical significance when we considered only those patients with organ-confined disease (P1, P2, and P3a; lymph node negative) or only those patients with extravesical disease extension (P3b and P4; lymph node negative) (Table 2). When we considered those patients with lymph node involvement, TSP expression was significantly associated with overall survival ($P = .01$). It is again important to note that no patient with low levels of TSP expression and either extravesical disease extension or lymph node metastases survived beyond 5 years (Table 2).

In a multivariable analysis accounting for histologic grade, tumor stage, and lymph node status, TSP expression was found to be an independent predictor of both disease recurrence and overall survival in this cohort of patients with invasive bladder cancer ($P = .002$ for recurrence and $P = .01$ for overall survival). Therefore, TSP expression adds prognostic information above that which is obtained from the established prognostic indicators for patients with bladder cancer, including histologic grade, tumor stage, and lymph node status. TSP expression, however, was not independent of p53 status in predicting disease recurrence ($P = .47$) or overall survival ($P = .88$).

Association Between TSP Expression and p53 Status

TSP expression was significantly associated with p53 status ($P = .001$) (Table 3). Of 90 tumors with wt p53 protein (p53$^-$), 86 (96%) exhibited a moderate or high level of TSP expression, whereas only four (4%) demonstrated a low level. Of 73 tumors with altered p53 protein (p53$^+$), 31 (42%) exhibited a low level of TSP expression, whereas 42 (58%) demonstrated either moderate or high levels.

Association Between TSP Expression and Microvessel Density Counts

Microvessel density counts were available for 160 of the 163 patients included in this study. The median microvessel density count in this population was 79 vessels per 200× high-power field. For the purpose of analysis, microvessel density count was
classified as above or below the sample median. TSP expression was found to be significantly associated with microvessel density count (Table 4). Of 34 patients with low levels of TSP expression, 27 (79%) were found to have high microvessel density counts (≥79), whereas only seven (21%) were found to have low microvessel density counts (<79). Of 126 patients with moderate or high levels of TSP expression, 72 (57%) were found to have low microvessel density counts, whereas 54 (43%) were found to have high microvessel density counts. This association was highly significant (P = .001).

To further evaluate the association between TSP expression and tumor angiogenesis, patients were placed into three equal groups based on microvessel density count as previously reported (27). Microvessel density count of 64 or lower, 65-95, or more than 95. A highly significant association was found to be expressed in the desmoplastic stroma associated with invasive ductal carcinomas of the breast, where it may have a protective effect on tumor invasiveness and metastasis (17). In melanoma and lung and breast carcinoma cell lines, the level of TSP mRNA and protein expression exhibits an inverse correlation with malignant progression (18). Direct evidence of the tumor-inhibitory effects of TSP comes from transfection experi-

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>No. of patients with low TSP expression (%)</th>
<th>No. of patients with moderate/high TSP expression (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0 (0)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>20 (21)</td>
<td>74 (79)</td>
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<td>4</td>
<td>64</td>
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<td>49 (77)</td>
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<td>Stage†</td>
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<tr>
<td>Lymph node negative</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ-confined</td>
<td>(P1, P2, and P3a)</td>
<td>63</td>
<td>14 (22)</td>
<td>49 (78)</td>
</tr>
<tr>
<td>Extravascular</td>
<td>(P3b and P4)</td>
<td>48</td>
<td>8 (17)</td>
<td>40 (83)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td></td>
<td>52</td>
<td>13 (25)</td>
<td>39 (75)</td>
</tr>
<tr>
<td>Total No. of patients</td>
<td></td>
<td>163</td>
<td>35</td>
<td>128</td>
</tr>
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</table>

* According to the method of Bergkvist et al. (24).
† Based on a chi-squared test for a 2 x 2 table comparing grade 2 and 3 with grade 4.
‡ Based on the tumor–node–metastasis system (25).
§ Based on a chi-squared test for a 2 x 3 table comparing organ-confined disease (P1, P2, and P3a), extravasal disease (P3b and P4), and lymph node metastases.

Table 2. Estimated rates of recurrence and survival at 5 years in 163 patients with invasive bladder cancer, according to thrombospondin-1 (TSP) expression and pathologic stage

<table>
<thead>
<tr>
<th>Group and stage</th>
<th>No. of patients</th>
<th>Low TSP expression (n = 35)</th>
<th>Moderate/high TSP expression (n = 128)</th>
<th>P</th>
<th>Low TSP expression (n = 35)</th>
<th>Moderate/high TSP expression (n = 128)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>163</td>
<td>76 (67-85)</td>
<td>42 (37-47)</td>
<td>.009</td>
<td>29 (21-37)</td>
<td>49 (45-54)</td>
<td>.023</td>
</tr>
<tr>
<td>Lymph node negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ-confined</td>
<td>(P1, P2, and P3a)</td>
<td>63</td>
<td>60 (47-74)</td>
<td>11 (6-15)</td>
<td>.0002</td>
<td>64 (52-77)</td>
<td>.35</td>
</tr>
<tr>
<td>Extravascular</td>
<td>(P3b and P4)</td>
<td>48</td>
<td>71 (54-89)</td>
<td>53 (45-62)</td>
<td>.123</td>
<td>0</td>
<td>.10</td>
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<tr>
<td>Lymph node positive</td>
<td></td>
<td>52</td>
<td>100</td>
<td>76 (68-84)</td>
<td>.43</td>
<td>0</td>
<td>.01</td>
</tr>
</tbody>
</table>

*Percent recurrence is based on the cumulative incidence estimate (32) with the corresponding 95% confidence intervals (CIs).
†Percent survival from all causes (overall survival) is based on the Kaplan–Meier estimate (31) with the corresponding 95% CIs.

To further examine the interaction between p53 status, TSP expression, and tumor neovascularity, we conducted an analysis of variance. When considered first, both p53 status and level of TSP expression were significantly associated with microvessel density counts (P = .015 for p53 status and P = .0006 for level of TSP expression). However, after we adjusted for TSP level, p53 was no longer significantly associated with microvessel density counts (P = .35), whereas TSP expression remained significantly associated with microvessel density counts after we adjusted for p53 (P = .008). These results are compatible with the hypothesis that p53 influences the level of TSP expression and that TSP expression has an impact on microvessel density counts or tumor angiogenesis.

**Discussion**

We found that the level of TSP expression, as determined by immunohistochemistry, can provide prognostic information with respect to disease recurrence and overall survival in patients with invasive bladder cancer. Patients with low TSP expression exhibit a significantly increased risk of disease recurrence and a significantly decreased overall survival when compared with patients with moderate or high TSP expression. The association between TSP expression and prognosis was independent of tumor stage, histologic grade, and lymph node status, and this association was strongest in those patients with organ-confined disease, where patients with low levels of TSP expression were more likely to have disease recurrence than patients with moderate or high levels. In a multivariable analysis, the level of TSP expression was found to be an independent prognostic indicator when considered along with the historically important indicators of disease progression, including tumor grade, tumor stage, and lymph node status.

These data are consistent with the hypothesis that TSP possesses tumor suppressor (or inhibitor) function. TSP has been found to be expressed in the desmoplastic stroma associated with invasive ductal carcinomas of the breast, where it may have a protective effect on tumor invasiveness and metastasis (17). In melanoma and lung and breast carcinoma cell lines, the level of TSP mRNA and protein expression exhibits an inverse correlation with malignant progression (18). Direct evidence of the tumor-inhibitory effects of TSP comes from transfection experi-
ments in human breast carcinoma cell lines (19). In these experiments, TSP overexpression in clones transfected with TSP complementary DNA injected into the mammary fat pad of nude mice resulted in a dose-dependent inhibition of primary tumor size and an inhibition of spontaneous pulmonary metastases when compared with controls with low TSP expression. TSP expression is stimulated by the nm23-1 tumor suppressor gene, but it appears to be suppressed by the ras and c-jun oncogenes (18,20).

One possible mechanism by which TSP exerts this tumor-inhibitory function is through its inhibition of tumor neovascularization. TSP has been shown to exert an inhibitory effect on cord formation by endothelial cells in vitro (21), thereby inhibiting neovascularization. In vivo, TSP has been shown to be a potent inhibitor of angiogenesis, and it is through this function that TSP may influence the ability of a tumor cell to invade and metastasize (22,34). Experiments on breast carcinoma cell lines have demonstrated that tumors formed from clones overexpressing TSP exhibited a reduction in capillary density when compared with control tumors with a lower level of TSP expression (19). It should be noted that some studies (35,36) have suggested that TSP possesses angiogenic properties. Our data, however, are not consistent with that conclusion.

TSP expression has also been linked to the p53 tumor suppressor gene. p53 is a transcriptional activator of the TSP gene, and it has been shown that increased expression of the wt p53 protein results in increased expression of TSP (23). Conversely, the mutant p53 gene product (or no p53 expression) results in significantly decreased TSP expression. Thus, one way by which wt p53 may exert its tumor suppressor function is through the TSP-dependent inhibition of tumor angiogenesis. Because p53 gene mutations have been shown to occur in a high proportion of

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**Fig. 2.** Probability of remaining relapse free in 163 patients with invasive transitional cell carcinoma of the bladder with low and moderate/high levels of thrombospondin-1 expression. Each tick mark represents a patient who had no evidence of disease recurrence at the time of last follow-up. Open symbols indicate patients who died before recurrence of bladder cancer. 95% confidence intervals are given at 3, 6, and 9 years.

**Fig. 3.** Probability of survival in 163 patients with invasive transitional cell carcinoma of the bladder with low and moderate/high levels of thrombospondin-1 expression. Each tick mark represents a patient who was alive at last follow-up. 95% confidence intervals are given at 3, 6, and 9 years.
bladder cancer (37,38), this mechanism may be important in determining the biologic aggressiveness of an individual bladder tumor. In fact, p53 nuclear accumulation, which significantly correlates with mutation in the p53 gene, is an important prognostic indicator with regard to disease recurrence and overall survival in patients with invasive transitional cell carcinoma of the bladder (26,38,39).

Our results indicate that TSP expression was not independent of p53 status in predicting disease recurrence or overall survival in this cohort of patients with bladder cancer. This is not surprising, in view of our hypothesis that the p53 status of the tumor influences the level of TSP expression. In fact, our data demonstrate a highly significant association between TSP expression and p53 nuclear accumulation. Tumors with altered p53 were significantly more likely to express low levels of TSP. Conversely, tumors with wt p53 were significantly more likely to express moderate or high levels of TSP. Furthermore, there was a significant association between TSP expression and microvessel density count. Tumors with low TSP expression were significantly more likely to demonstrate high microvessel density counts, whereas tumors with moderate or high TSP expression were significantly more likely to demonstrate low microvessel density counts. The sequence of these events was examined by use of an analysis of variance, which demonstrated a significant interaction between p53, TSP expression, and microvessel density counts and supports the hypothesis that the p53 gene exerts its influence on tumor angiogenesis through the regulation of TSP.

It is interesting to note that the majority of tumors with low TSP expression were found to express altered p53 protein. However, approximately one third of tumors with moderate or high TSP expression also expressed altered p53 protein. This result can be explained by the fact that TSP expression is known to be influenced by factors other than p53 (18,20), and these factors may stimulate TSP expression in the presence of altered p53. Alternatively, it has been demonstrated that p53 mutations may differ in their effects on gene expression (40,41). This does not appear to be the case with TSP expression. We have analyzed TSP expression as it relates to the site of mutation in the p53 gene, and neither the exon nor the site of mutation appears to affect TSP expression (unpublished data). Conversely, when p53 is not altered (wt p53 is expressed), TSP expression is almost always present. With regard to microvessel density counts, the majority of tumors with low levels of TSP expression demonstrated high microvessel density counts. However, more than 40% of tumors with moderate or high levels of TSP expression also exhibited high microvessel density counts. TSP is one of many factors that are known to regulate angiogenesis, and some of these factors may stimulate tumor angiogenesis despite high levels of TSP (42). Thus, while the pathway linking p53, TSP, and tumor angiogenesis provides an important mechanism by which tumor cells acquire the ability to invade and metastasize,
it appears to be only one pathway of a complex series of events contributing to the regulation of tumor progression. The choice of which patients should receive postoperative adjuvant chemotherapy for bladder cancer currently depends predominantly on the depth of tumor invasion in the cystectomy specimen. The level of TSP expression adds prognostic information above that obtained from tumor stage, histologic grade, and lymph node status, especially in patients with organ-confined disease. Therefore, TSP expression may assist the clinician in deciding which patients with organ-confined disease should receive adjuvant treatment, with its potential risks and benefits, as well as which patients should be spared the toxicity of chemotherapy. The role of TSP in bladder cancer, however, may not be limited to that of prognostic marker. TSP functions to inhibit angiogenesis, and increased levels of TSP expression are associated with a lower probability of disease progression and an increased probability of survival. Therefore, if TSP can be delivered directly to the extracellular matrix surrounding tumor cells, it may be possible to inhibit tumor neovascularity and thereby decrease the probability of tumor invasion and metastasis, thus opening up the possibility of new therapeutic interventions.

References

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Notes

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