Relationship of Tumor Angiogenesis and Nuclear p53 Accumulation in Invasive Bladder Cancer

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ABSTRACT

The purpose of this investigation was to evaluate the relationship between tumor angiogenesis and nuclear p53 accumulation in invasive bladder cancer. We studied 161 patients with invasive transitional cell carcinoma of the bladder who had previously undergone radical cystectomy. Analysis was performed to determine the presence of p53 nuclear accumulation and extent of tumor-associated angiogenesis. p53 status identified a group of patients at high risk for tumor progression (p53-altered tumors), and microvessel density determinations added additional prognostic information by identifying a subset of aggressive tumors within the wild-type p53 subgroup. At 5 years, patients with tumors exhibiting no evidence of p53 alterations and low microvessel counts demonstrated 3% recurrence and 88% survival, compared to 43% recurrence and 59% overall survival for patients with intermediate vessel counts and 61% recurrence and 43% overall survival for patients with the highest vessel counts (P < 0.001 and P = 0.003, respectively). Angiogenesis also provides additional prognostic information to patients with tumors that demonstrate p53 alterations. An association between angiogenesis and p53 status did exist (P = 0.05); however, 27% of the tumors that showed no evidence of p53 alterations exhibited high microvessel counts, and 26% of tumors with evidence of p53 alterations had low microvessel counts. Tumor-associated angiogenesis adds additional useful prognostic information to that which is obtained from p53 status in patients with invasive transitional cell carcinoma of the bladder. Although an association between p53 status and the degree of angiogenesis was identified, other factors appear to play a role in the regulation of tumor-induced neovascularization.

INTRODUCTION

Bladder cancer is currently the fourth leading cause of cancer deaths among males in the United States. In 1997, 54,500 new cases of bladder cancer will be diagnosed, and 11,700 people will die of this disease (1). It is estimated that up to 50% of patients presenting with muscle invasive TCC may already have occult metastatic disease (2). Although radical cystectomy and pelvic LN dissection may be effective primary therapy for patients with local-regional disease, patients harboring tumors of greater malignant potential would benefit from a more aggressive, multimodality approach to eradicate occult tumor deposits. Identification of patients at high risk for subsequent tumor progression will allow for more appropriate selection of patients for early cystectomy, while providing a more conservative approach in patients whose tumors are less likely to progress.

Current established indicators of disease progression for patients with invasive bladder cancer include histological grade and pathological stage (LN status, presence of metastatic disease; Refs. 2 and 3). Recently, the association of molecular changes to tumor suppressor genes, particularly p53 (4–7) and Rb (8, 9), have been shown to be useful indicators of disease progression in patients with invasive TCC of the bladder. The p53 status, determined by immunohistochemical identification of p53 nuclear protein accumulation, serves as an independent predictor of disease progression in patients with invasive TCC of the bladder (4, 6).

Angiogenesis, the ability to induce new blood vessel growth, is a tightly regulated cellular activity in normal tissues and plays a central role in several homeostatic processes. A significant accumulation of experimental and clinical data now suggests that tumor growth and the development of metastases is dependent on the tumor’s ability to initiate an angiogenic response (10–12). The prognostic significance of tumor angiogenesis, determined by the microvessel density within and adjacent to a tumor, has been established in a variety of tumor systems, including cutaneous melanoma (13), breast (14), lung (15), and prostate (16, 17). We and others (18–20) have recently identified microvessel density as a highly significant, independent prognostic indicator of disease progression in patients with invasive TCC of the bladder.

Recent studies have shown that tumor suppressor genes,
particularly p53, may play a crucial regulatory role in the control of angiogenesis (21, 22). Dameron et al. (22) found that p53 may control the degree of angiogenesis through regulation of the potent angiogenic inhibitor thrombospondin-1. The relationship between the prognostic information obtained from p53 status determinations and tumor angiogenesis, however, has not been explored. The purpose of the present study was to further evaluate the relationship between p53 alterations and angiogenesis in invasive TCC of the bladder.

**MATERIALS AND METHODS**

**Tissue.** Tissues from 161 patients with invasive TCC of the bladder who had undergone radical cystectomy, pelvic LN dissection, and urinary diversion at the Kenneth Norris Jr. Comprehensive Cancer Center (Los Angeles, CA) were examined. Patients with pure adenocarcinoma, squamous cell carcinoma, or small cell carcinoma were excluded. Patients with TCC that showed focal areas of squamous or glandular differentiation were included. The indications for cystectomy included muscle or prostatic stromal invasion, high-grade superficially invasive disease, and multifocal disease that recurred after conservative treatment. The histological grade and stage of all tumors were confirmed by one of us (R. J. C.). Histological grading was performed according to the method described by Bergkvist et al. (23), and tumor staging was assigned according to the criteria of the tumor-node-metastasis classification (24).

**Vessel Staining.** Archival formalin-fixed, paraffin-embedded tissue was sectioned at 5-μm intervals and mounted on charged slides (ProbeOn Plus; Fisher Scientific, Pittsburgh, PA). Tissue sections underwent deparaffinization, rehydration, and blocking of endogenous peroxidase. Antigen retrieval techniques were performed as described previously (25). Specimens were blocked with normal horse serum (at 1:200) for 30 min, followed by overnight incubation with the primary antibody. On the basis of our observation that anti-CD34 antibodies recognize small-caliber vessels that are associated with neovascularization in bladder cancer more efficiently than do anti-factor VIII antibodies (26), the anti-CD34 mouse monoclonal antibody HPCA-I was used to immunohistochemically detect endothelial cells (IgG1, at 1:50; Becton Dickinson Immunocytometry Systems, San Jose, CA). Tissue was washed with PBS and incubated with biotinylated horse antimouse antibody (Vector Laboratories, Burlingame, CA). Avidin-biotin conjugation (ABC Elite; Vector) was applied, and slides were developed using 3-amino-9-ethyl-carbazole (Sigma Chemical Co., St. Louis, MO) as the chromogen. Slides were counterstained with hematoxylin. Adjacent sections were stained with H&E and used to localize the exact region of tumor invasion.

**Vessel Density Determinations.** Microvessel density determinations were performed by the technique described by Weidner et al. (27). Briefly, light microscopy was used to identify regions within or immediately adjacent to the tumor that contained the greatest vessel density. These so called “hot spots” were found after scanning the entire section at low power (X40–100). Significant heterogeneity with respect to vessel density was noted in virtually all cases, with the regions of greatest density occurring anywhere within the invasive portion of the tumor or in the immediately adjacent tissues. Areas associated with ulceration and granulation tissue (for example, at a prior transurethral biopsy site), which usually exhibited significant neovascularization, were excluded from consideration as the hot spot. These regions, however, served as internal controls to verify the adequacy of endothelial staining.

Microvessel counts were performed within the designated neovascular hot spot on a X200 field (X10 ocular and X20 objective). Any stained endothelial cell was considered to represent a single vessel if it was separate from adjacent microvessels and other connective tissue elements. Counts were performed independently by two readers (B. H. B. and R. J. C.), with randomly selected sections confirmed by a third reader (N. W.). All readers were “blinded” to clinical outcome and to the results that were obtained by the other readers. Cases that varied significantly between readers were reevaluated to determine a consensus count.

**Immunohistochemical Analysis of p53.** Immunohistochemical procedures for detecting nuclear p53 were used as described previously (28). Briefly, deparaffinized 5-μm sections underwent blocking of endogenous peroxidase. The anti-p53 mouse monoclonal antibody PAb1801 (IgG1 class, at 1:10; Biogenex, San Ramon, CA) was incubated overnight with tissue at 4°C. PAB1801 recognizes the p53 protein at a denaturation-resistant epitope, corresponding to amino acids 32–79. Tissues were then incubated with a biotinylated horse antimouse secondary antibody (Vector Labs, Burlingame, CA), and reactivity was visualized with an avidin-biotin-immunoperoxidase system (Vector) using diaminobenzidine (0.03%) as the chromogen. Only nuclear localization of immunoreactivity was evaluated. The samples demonstrating at least 10% nuclear reactivity were considered to be p53 positive (i.e., to have an alteration in p53). We based this criterion on our demonstration of a strong correlation of mutations in the p53 gene with the accumulation of p53 protein in 10% or more of the tumor cell nuclei (4).

**Statistical Analysis.** Overall survival and time to the first recurrence of bladder cancer were the outcomes analyzed in this study. Survival was calculated as the number of years from cystectomy until death or until the last documented contact with the patient. For patients who recurred, the time to the first recurrence of bladder cancer was calculated as the number of years from cystectomy to the date of first documented recurrence of disease. Patients who died prior to recurrence of disease were counted as deaths by a competing cause; patients who were still alive and had not experienced a bladder cancer recurrence were censored at the date last seen free of disease. Two patients were never disease free; for purposes of analysis, they were counted as recurring 1 day after cystectomy.

Kaplan-Meier product limit estimates of overall survival and the complement of cumulative incidence curves for recurrence-free survival were plotted. SEs for the probability of surviving or not recurring were based on Greenwood’s formula for the Kaplan-Meier estimates (29) and on the delta method for the cumulative incidence curves (30). When analyzing survival or recurrence, the log-rank test and the stratified log-rank test were used to compare groups of patients. The relative risk based on the Pike estimate was used to provide a quantitative summary of effect (31), with 95% confidence intervals based on variances derived from the information matrix (31, 32). To establish whether angiogenesis was associated with outcome, patients...
were grouped according to the number of vessels: ≤64 vessels (53 patients), 65–99 vessels (55 patients), and ≥100 vessels (53 patients). For the purpose of the statistical analysis, nuclear accumulations of p53 were classified as either positive (accumulation in 10% or more of tumor cells) or negative.

The \( \chi^2 \) test for trend in contingency tables (33) was used to test for association between angiogenesis (with the number of vessels grouped as above) or p53 status and the baseline values of stage (P1-P3A and LN−) or p53 status. 

### RESULTS

**Patients.** Among the 161 patients with invasive TCC of the bladder, 113 showed no evidence of tumor involvement of the regional LNs (LN−), and 48 were identified as LN+. Of the LN− patients, 27 were classified as stage P1, 15 as P2, 23 as P3A, 39 as P3B, and 9 as P4. Concerning histological grading, there were 3 grade II, 102 grade III, and 56 grade IV tumors. The median age of the study population was 65 years (range, 38–87 years). The median follow-up for all patients was 67 months (range, 5–104 months), with 90% of patients followed for at least 3 years. One patient received preoperative intravesical chemotherapy, and 33 patients received systemic chemotherapy following surgery.

Fifty-two patients experienced a disease recurrence following cystectomy. Metastatic disease was found in 38 patients (73%), and local recurrence only was noted in 14 patients (27%).

**Association of p53 Nuclear Reactivity and Tumor Angiogenesis.** Analysis of the 161 tumors revealed that microvessel density and p53 status were not associated with histological tumor grade. Microvessel density was further found to be independent of pathological stage and LN status. An association was found between p53 status and pathological stage. We stratified our patient population into three distinct groups based on their pathological stage: organ-confined, LN− disease (P1, P2, and P3A; LN−); extravesical extension of the primary lesion with negative LNs (P3B and P4; LN−); and LN+ patients for statistical analysis (Table 1). When we evaluated all tumors for the presence of an association between microvessel counts and p53 status, a significant relationship was identified (\( P = 0.050; \text{Table 2} \)). Of those tumors that exhibited evidence of p53 alterations, 27% had counts in the lowest third, 32% had intermediate counts, and 42% in the highest counts. In contrast, of the tumors that showed no evidence of p53 alterations, 38% had counts in the lowest third, 35% had intermediate counts, and 27% had counts in the highest third.

**Association of p53 Nuclear Reactivity, Tumor Angiogenesis, and Prognosis.** Among the tumors with no evidence of p53 nuclear reactivity (\( n = 93 \)), microvessel density was able to identify a subset of patients at increased risk for disease recurrence (\( P < 0.0001 \)) and decreased overall survival (\( P = 0.0034; \text{Fig. 1} \)). At 5 years, patients with no detectable nuclear p53 reactivity and low microvessel counts (\( ≤64 \) vessels/X200 hpf) had a 3% recurrence rate and 88% survival, compared to 43% recurrence and 59% overall survival for patients with the highest vessel counts (\( ≥100 \) vessels/hpf) and 61% recurrence and 43% overall survival for patients with intermediate vessel counts (\( 65–99 \) vessels/hpf); Table 3). When the p53-negative group was stratified by pathological stage (i.e., organ-confined, extravesical extension, and LN+ disease) microvessel density was able to identify a subset of patients with a significantly increased risk of disease recurrence and decreased survival (Table 3). A patient with a p53-negative tumor and elevated vessel count (\( ≥100\) hpf) demonstrated a 30.6-fold increased risk of experiencing a disease recurrence and 4.19-fold increased risk of dying at 5 years compared to patients with p53-negative tumors and low microvessel counts (\( ≤64\) hpf; Table 4).

Table 3 depicts our analysis of those tumors that demonstrated p53 nuclear reactivity (\( n = 68 \)). A similar, significantly increased risk of disease recurrence with increasing microvessel counts was identified in patients whose tumors demonstrated evidence of p53 alterations. At 5 years, patients with detectable nuclear p53 reactivity and low microvessel counts (\( ≤64 \) vessels/×200 hpf) had a 34% recurrence rate, compared to 68% for patients with intermediate vessel counts and 96% for patients with the highest vessel counts (\( P = 0.017 \)). The relative risk of a patient with a p53-altered tumor experiencing a disease recurrence at 5 years was 3.34-fold greater if the tumor demonstrated vessel counts \( ≥100\) hpf, compared to tumors with counts of \( ≤64\) hpf (Table 4). When overall patient survival was evaluated in the p53-altered group, patients exhibited 34, 21, and 21% 5-year survival (\( P = 0.63 \)) for low, intermediate, and high vessel counts, respectively (Fig. 2).
DISCUSSION

The current study demonstrates that the status of tumor angiogenesis, as determined by microvessel density, can further stratify patients with tumors demonstrating the wild-type or altered p53 phenotypes, thus providing additional, useful prognostic information to that which was obtained from the tumor's p53 status. Patients whose tumors exhibit no evidence of p53 nuclear reactivity and low microvessel counts have a significantly lower recurrence and increased survival rate compared to patients with moderate or high microvessel counts. This was seen for the overall group of patients, as well as patients stratified by pathological stage and LN status. Patients whose tumors did demonstrate evidence of an altered p53 protein and elevated microvessel counts also exhibited a significantly increased risk of tumor recurrence. Although a statistically significant difference in overall survival was not found in the p53-altered group when stratified by microvessel counts, a trend toward decreasing survival with increasing vessel counts was noted.

We also demonstrated a significant association between the p53 status and the degree of tumor neovascularity. Tumors that
Table 3  Association of microvessel counts with risk of disease recurrence and overall patient survival in 161 patients with invasive TCC of the bladder

<table>
<thead>
<tr>
<th>p53 status</th>
<th>No. of patients</th>
<th>Microvessel counts/×200 hpf</th>
<th>Estimated probability of recurrence at 5 yearsa (%)</th>
<th>Log-rank P</th>
<th>Microvessel counts/×200 hpf</th>
<th>Estimated probability of survival to 5 yearsb (%)</th>
<th>Log-rank P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤64</td>
<td>65–99</td>
<td>≥100</td>
<td></td>
<td>≤64</td>
<td>65–99</td>
</tr>
<tr>
<td>Negative</td>
<td>93</td>
<td>3 (3)</td>
<td>43 (9)</td>
<td>61 (10)</td>
<td>&lt;0.001</td>
<td>88 (5)</td>
<td>59 (9)</td>
</tr>
<tr>
<td>Positive</td>
<td>68</td>
<td>34 (11)</td>
<td>68 (10)</td>
<td>96 (4)</td>
<td>0.02</td>
<td>34 (12)</td>
<td>21 (9)</td>
</tr>
<tr>
<td>P53−</td>
<td>44</td>
<td>0</td>
<td>7 (7)</td>
<td>27 (13)</td>
<td>0.05</td>
<td>90 (7)</td>
<td>86 (9)</td>
</tr>
<tr>
<td>P1/P2/P3A; LN−</td>
<td>27</td>
<td>0</td>
<td>60 (16)</td>
<td>N.A.c</td>
<td>0.002</td>
<td>89 (11)</td>
<td>58 (16)</td>
</tr>
<tr>
<td>LN+</td>
<td>22</td>
<td>33 (19)</td>
<td>58 (22)</td>
<td>N.A.c</td>
<td>0.006</td>
<td>71 (17)</td>
<td>11 (11)</td>
</tr>
</tbody>
</table>

a Percent recurrence is based on the cumulative incidence estimate (30), with the estimate of SE (shown in parentheses) calculated by the delta method (29).

b Percent survival from all causes (overall survival) is based on the Kaplan-Meier estimate (29). The corresponding estimate of the SE (shown in parentheses) is calculated with Greenwood’s formula (29).

c No patients were evaluable at 5 years. All patients had recurred or were censored prior to 5 years.

Table 4  Relative risk of disease recurrence and overall survival stratified by microvessel density in 161 patients with TCC of the bladder

<table>
<thead>
<tr>
<th>p53 status</th>
<th>No. of patients</th>
<th>Microvessel counts/×200 hpf</th>
<th>Relative riska</th>
<th>log-rank P</th>
<th>Microvessel counts/×200 hpf</th>
<th>Relative riska</th>
<th>Log-rank P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤64</td>
<td>65–99</td>
<td>≥100</td>
<td></td>
<td>≤64</td>
<td>65–99</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>1.00</td>
<td>4.46</td>
<td>6.38</td>
<td>&lt;0.001</td>
<td>1.00</td>
<td>1.85</td>
</tr>
<tr>
<td>Negative</td>
<td>93</td>
<td>1.00</td>
<td>21.0</td>
<td>30.6</td>
<td>&lt;0.001</td>
<td>1.00</td>
<td>2.93</td>
</tr>
<tr>
<td>Positive</td>
<td>68</td>
<td>1.00</td>
<td>2.18</td>
<td>3.34</td>
<td>0.017</td>
<td>1.00</td>
<td>1.26</td>
</tr>
<tr>
<td>Overallc</td>
<td></td>
<td>1.00</td>
<td>4.25</td>
<td>5.21</td>
<td>&lt;0.001</td>
<td>1.00</td>
<td>1.69</td>
</tr>
</tbody>
</table>

a A relative risk of 1 indicates that the hazard of failing is the same as that of patients with microvessel count ≤64. The group of patients with the lowest microvessel count (≤64) was designated as the reference group for risk comparison. A relative risk >1 indicates that the hazard of failing is greater than the reference group.

b P based on log-rank test, stratified by stage, grade, and p53 status.

c Overall P based on stratified log-rank test; all other Ps are based on unstratified log-rank test.

have the wild-type p53 phenotype tend to have low microvessel counts. In contrast, tumors demonstrating p53 alterations tend to exhibit high microvessel counts. This provides further evidence for the idea that p53 may play a role in the regulation of tumor angiogenesis.

Several cellular and molecular markers have been identified as potential indicators of disease progression in invasive bladder cancer. Among these are histological tumor grade, pathological stage (2), p53 (4–7) and/or Rb (8, 9) tumor suppressor gene alterations, and, more recently, tumor-associated angiogenesis (18–20). This study was performed to further evaluate the biological and prognostic relationship between the p53 tumor suppressor gene and tumor-associated angiogenesis in patients with invasive TCC of the bladder. We have previously shown that both p53 status, as determined by nuclear protein accumulation (4), and tumor-associated angiogenesis (18) are significant, independent predictors of disease progression as determined by univariate and multivariate analyses. Increased p53 nuclear accumulation and elevated microvessel counts identified subsets of patients at high risk for tumor recurrence and decreased overall survival.

Alterations in the p53 gene have been established as a common event in the development of tumors in a variety of organ systems (34). The presence of p53 genetic alterations and p53 nuclear accumulation has been frequently identified in high-grade, invasive human bladder cancer and rarely associated with low-grade, superficial tumors (28, 35). Genetic mutations leading to alterations in p53 protein conformation and prolonged mutant protein half-life are the proposed basis by which current immunohistochemical techniques were developed to identify increased nuclear p53 protein accumulation. Recently, the association between p53 gene alterations and nuclear accumulation has been established by immunohistochemical methods in patients with invasive TCC of the bladder (28).

The dependence of tumor growth and metastatic potential on a tumor’s ability to induce a neovascular response is based on a significant accumulation of experimental data (10–12). Tumor angiogenesis represents one of the many necessary interactions between the tumor cell and normal surrounding stroma that enhances a tumor cell’s ability to overcome the normal barriers preventing invasion and metastasis formation. The ability of microvessel density determinations to serve as a useful predictor of tumor behavior has been demonstrated in several tumor systems, including breast (10–12), melanoma (13), prostate (16, 17), and lung (15) cancers. Studies have demonstrated a signifi-
significant correlation between disease progression and microvessel counts in human bladder cancer (18–20). The lack of significant angiogenic activity within most normal tissues suggests a tightly controlled regulatory mechanism for the growth of new vessels. Although several mechanisms have been proposed to explain how tumor endothelial cells interact, the underlying regulatory controls of this process remain unknown. Evidence suggests that various tumor suppressor genes may play an important role in the regulation of new vessel formation (21, 22). Dameron et al. (22) have reported that the p53 tumor suppressor gene can regulate the degree of tumor angiogenesis through its ability to control the production of thrombospondin-1, a potent inhibitor of endothelial cell growth. Thrombospondin-1 was found to be up-regulated by the wild-type p53 gene product in fibroblasts of Li-Fraumeni patients. Loss of the wild-type p53 gene was associated with the development of an angiogenic phenotype by the tumors and subsequent increased aggressive behavior. We have shown that p53 status is associated with thrombospondin-1 expression, which in turn is associated with the degree of...
angiogenic activity within the tumor (36). This observation further substantiates the link between p53 and the angiogenic status of the tumor.

The findings of p53 alterations in a small but substantial proportion of tumors that demonstrated the lowest degree of angiogenic activity and in a similar proportion of p53 wild-type tumors that displayed high degrees of angiogenic activity suggest that factors other than p53 play a significant role in the regulation of new vessel formation. The redundancy of the angiogenic pathway is highlighted by the identification of many substances produced by mammalian cells that demonstrate angiogenic and angiostatic activity in vivo. Thus, although p53 mutations may play a role in the initiation of a tumor-induced neovascular response, the presence of a variety of angiogenic and inhibitory substances suggests a complex and redundant system in which other factors clearly play a role in the regulation of tumor angiogenesis.

We have shown that p53 nuclear accumulation and tumor angiogenesis are independent predictors of disease progression in patients with invasive TCC of the bladder. We have found that microvessel density determinations added additional, useful prognostic information to that which is obtained from p53 determinations, as well as from histological grade and pathological stage. This study also demonstrates that, although p53 status is associated with the degree of tumor neoangiogenicity, it appears to be one of several possible factors that regulate tumor angiogenesis.

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