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p53 and RB Expression Predict Progression in T1 Bladder Cancer


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Abstract

The optimal clinical management of minimally invasive (stage T1) bladder cancer is controversial. T1 bladder cancers share characteristics of both noninvasive (T0) papillary cancer and high stage, muscle-invasive bladder cancers. Patients with T1 bladder cancer have a higher risk of cancer progression and death than do patients with Ta bladder cancer. However, this risk is much lower than that of patients with high-stage bladder cancers. Methods of identifying T1 bladder cancer patients at greatest risk for progression may significantly improve clinical management. We retrospectively evaluated two tumor suppressor genes, p53 and RB, as potential prognostic markers for progression in a cohort of 45 patients with pT1 bladder cancer. Median follow-up for these individuals was greater than 3.5 years. Of this group, 58% had altered p53 expression based on positive p53 immunostaining. Three patterns for RB nuclear protein staining were observed: absent, heterogeneous (normal), and strongly homogeneous. Progression-free survival was similar for patients with loss of RB protein expression and those with apparent overexpression of RB protein. Therefore, both staining patterns were considered abnormal. Patients with normal expression of both proteins (i.e., p53 negative and RB heterogeneously positive) had an excellent outcome, with no patient showing disease progression, whereas patients with abnormal expression of either or both proteins had a significant increase in progression (P = 0.04 and P = 0.005, respectively). These data support the stratification of T1 bladder cancer patients based on p53 and RB nuclear protein status and suggest that patients with normal protein expression for both genes can be managed conservatively, whereas patients with alterations in one and particularly both genes require more aggressive treatment.

Introduction

The choice of appropriate therapy for minimally invasive (stage T1) bladder cancer presents a difficult clinical decision. Many patients with T1 bladder cancer do well with local management, including transurethral resection of tumors and treatment with BCG or intravesical chemotherapy, with minimal morbidity and mortality (1–6). However, patients with T1 tumors are at a significant risk for progression to more invasive disease and to metastasis. Although the reported risk has varied widely in different studies (2, 3, 7–11), the potential for progression and tumor-related death is considerable. Progression rather than recurrence has been associated with increased chance of metastasis and death from systemic disease. Because of that risk, some clinicians have suggested that all patients with T1 bladder cancers should undergo immediate cystectomy (12, 13), a treatment that can result in both morbidity and mortality. Because only a fraction of patients with T1 tumors experience tumor progression, biomarkers that could identify either the population that does not progress or the population at greatest risk of progression would contribute significantly to the medical management of patients with T1 bladder cancer.

Two genes that have shown significant promise as potential prognostic markers in other bladder cancer studies are the tumor suppressors p53 and RB (14–21). Furthermore, it has been reported recently in a cohort of patients with either T0 or T1 disease that when abnormalities in both genes occur in the same tumor, the probability for progression is increased with a concomitant decrease in overall survival (22). In our present study, we wished to examine the status of these two genes in a similar manner but included only patients with T1 tumors, because they have a considerably greater chance of progression than do those with T0 disease.

Although p53 was initially identified as a nuclear phosphoprotein with transforming capacities (reviewed in Ref. 23), later studies revealed that the transforming protein initially studied was a mutated product, and that the normal p53 protein actually had tumor suppressor activities (23, 24). The important role of the p53 gene in cancer was further documented when the gene was discovered to be mutated in many colon cancers and subsequently shown to be aberrant in many other tumor types (23, 25). One of the functions of the normal p53 protein is a cell cycle checkpoint regulator, preventing cell cycle progression in cells with damaged DNA (24). The normal p53 protein has a very short half-life and usually cannot be detected by immunohistochemical analysis. In contrast, mutations in the p53 gene frequently result in an increased half-life and persistence of the mutated protein within the nucleus, which can be detected using immunostaining. Fortunately, in bladder cancer there is a high

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4 The abbreviation used is: BCG, bacillus Calmette Guérin.
concordance between p53 nuclear staining and the presence of actual mutations (26, 27); therefore, immunohistochemical analysis can be used to examine the p53 status in bladder cancer, whereas in several other tumor types, the concordance between immunostaining results and mutations is considerably lower.

The RB gene is also deleted or mutated in many cancers (28). This gene is critical in regulating the cell cycle by interaction with the E2F transcription factor (29), and the RB gene product is inactivated by phosphorylation (30). In contrast to missense mutations of p53, which usually result in strong nuclear staining, mutations in the RB gene usually result in the loss of RB nuclear protein expression as detected by immunostaining; in fact, immunohistochemical analysis has been shown to be the most sensitive method of determining the RB status in a given tumor (31). Although the absence of nuclear staining was considered previously to be indicative of RB functional loss, it has been reported recently that in bladder cancer, overexpression of RB as measured by immunohistochemical staining correlates with progression of disease in patients with advanced bladder cancers (32, 33). Such overexpression may suggest dysregulation of the RB protein by upstream changes in the cell cycle pathway involving RB phosphorylation (inactivation). Therefore, we separated the RB-positive tumors into those with normal, heterogeneous staining and those with homogeneous overexpression to determine whether a similar difference in progression and survival occurred between these two staining patterns in T1 tumors.

Materials and Methods

Patients. The medical records of patients with bladder cancer seen at The University of Texas M. D. Anderson Cancer Center during the period December 1992 through April 1994 were reviewed. H&E-stained sections for each block used were examined microscopically to confirm the stage and grade of each tumor. Fifty-five patients met the following eligibility criteria: (a) a pathological block was available from the original T1 tumors resected at M. D. Anderson for immunohistochemical analysis; and (b) the patient had been followed for at least 6 months. One patient was excluded because the specimen contained too few tumor cells to evaluate. All patients were followed for progression (an increase in stage to ≥T2, N1–3, or M1; American Joint Committee on Cancer, Ed. 4). The study population had a median follow-up of 3.75 years. Early cystectomy was defined as cystectomy within 3 months of transurethral resection documenting the presence of T1 disease.

p53 Immunohistoassay. Tissue sections and a positive control slide of colon sausage (Biogenex, San Ramon, CA) were heated in a 60°C oven for 30 min, deparaffinized with xylene, and rehydrated through graded ethanol solutions. Endogenous peroxidase activity was blocked using 0.1% hydrogen peroxide in 50% methanol:50% PBS. Sections were incubated in PBS containing 20% horse serum for 30 min and then with the monoclonal antibody DO1 (Oncogene Science, Cambridge, MA) at 1:100 in NCS/PBS (5% newborn calf serum in PBS) or with purified mouse immunoglobulin at 5 μg/ml in NCS/PBS as a negative control for 1 h. Sections were washed with PBS and developed with the avidin-biotin complex method (ABC kit; Vector Laboratories, Burlingame, CA). The slides were counterstained with Mayer’s hematoxylin. p53 staining was considered abnormal when more than 10% of the nuclei were stained (Fig. 1A).

RB Immunohistoassay. Tissue sections were deparaffinized with xylene and rehydrated through graded ethanol solutions. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide in absolute methanol for 30 min, and the sections were washed in distilled water.

Sections were incubated for 15 min in 10 mM citric acid buffer (pH 6.0) at 95°C, washed in PBS with 100 mM citric acid buffer, and incubated in 100 mM citric acid buffer containing 0.5% nonfat dry milk and 1.5% normal goat serum for 6 h. The slides were removed from buffer and incubated overnight with the rabbit polyclonal anti-RB antibody RB-WL-1 used at 1:628 dilution in 100 mM citric acid buffer containing 0.5% nonfat dry milk, 1.45% goat serum, and 0.02% Triton X-100. The slides were then washed with PBS and incubated for 3 h with 1:400 diluted biotinylated goat anti-rabbit antibody in PBS containing 0.5% nonfat dry milk, 1.5% goat serum, and 0.02% Triton X-100. Slides were washed in PBS and developed using an avidin-biotin complex kit (ABC kit; Vector Laboratories) following the manufacturer’s instructions. The slides were counterstained with Mayer’s hematoxylin. All tumors had normal cells showing nuclear RB staining in blood vessels and stroma as an internal positive control. Three categories of nuclear staining in the tumor cells were scored: (a) absent; (b) normal heterogeneous; or (c) homogeneous (Fig. 1, B, C, and D, respectively).

Statistics. Survival statistics were calculated using Kaplan-Meier analysis with a log rank test (SPSS for Windows, release 7.5.1; SPSS, Inc., Chicago, IL).

Results

As is typical for T1 bladder cancers, all tumors were relatively high grade, either grade 2 or 3. Tumor grade did not correlate with either recurrence or progression. Patients who experienced tumor recurrence without progression did not differ significantly from those who remained tumor free (data not shown). Bladder cancer progression was a strong indicator of both overall survival (P = 0.0005, log-rank test) and disease-specific survival (P < 0.0001; log-rank test). Just as disease-specific survival is a more sensitive indicator of the impact of bladder cancer on patient outcome than overall survival, eliminating the patients who had early cystectomy yields a clearer picture of the prognostic value of tumor markers on disease outcome. This occurs because of the beneficial effect of early cystectomy on outcome. None of the 9 patients treated with early cystectomy experienced bladder cancer progression. The remainder of the reported analyses are limited to the 45 patients who did not have cystectomy within 90 days of the documentation of T1 bladder cancer.

Alterations in p53 and RB protein expression were observed frequently. Twenty-six of the 45 patients not having early cystectomy (58%) showed abnormal expression of p53. In evaluating the RB protein status, three patterns of expression were observed: normal heterogenous RB protein expression in 26 patients (58%); loss of RB expression in 9 patients (20%); and increased RB expression in 10 patients (22%) as shown in Fig. 1. For each case in which RB nuclear staining was absent in the tumor cells, numerous RB-positive normal cells were found as...
Fig. 1 Immunohistochemical staining showing p53-positive (A) and RB-negative (B) T1 bladder tumors. RB-positive tumors with heterogeneous and homogeneous staining patterns are seen in C and D, respectively. The RB-positive cells in B are normal endothelial cells, which are examples of the positive internal control cells required to score a given tumor as RB negative.

internal controls (e.g., Fig. 1B). Kaplan-Meier analysis indicated similar progression-free survival rates in patients with tumors not expressing RB protein (P = 0.009 compared with wild-type RB, log-rank test) and patients with tumors overexpressing RB protein (P = 0.017 compared with wild-type RB, log-rank test; Fig. 2A). We therefore subsequently categorized the tumors overexpressing RB protein along with those not expressing RB as having abnormal RB protein expression. Patients whose tumors exhibited altered RB protein had a significantly decreased disease specific survival (P = 0.011, log-rank test; Fig. 2B). A trend approaching significance was observed for progression-free survival when p53 was considered abnormal (P = 0.076, log-rank test; Fig. 3). Combining RB and p53 status strikingly demonstrated that patients with tumors exhibiting normal p53 and RB expression, as determined by immunohistochemical analysis, showed no disease progression, resulting in a 100% progression-free survival (Fig. 4). Compared with the patients with normal p53 and RB protein status, patients with an abnormality in either p53 or RB had a worse prognosis (P = 0.04, log-rank test), and in patients whose tumor had alterations in both p53 and RB, the decrease in progression free-survival was even greater (P = 0.005, log-rank test).

Discussion

This study clearly supports the stratification of patients with T1 bladder cancer based on p53 and RB protein expression, because no patient with normal p53 and RB nuclear staining
patterns had progression of their disease. These results suggest that patients with normal p53 and RB protein expression can be followed with a conservative bladder-preserving strategy. Although these patients may have multiple recurrences, the risk of progression in this population appears to be very small. In contrast, patients with tumors showing abnormalities in either p53 or RB protein expression, and particularly in both, are at a much higher risk of progression with an associated poorer overall survival. However, this risk is not absolute. In our series, a number of patients had abnormalities in either p53 or RB expression or both in the absence of progression. Therefore, a significant number of these patients are probably not well served by immediate cystectomy, with its associated morbidity and mortality, but may respond positively to aggressive chemotherapy, immunotherapy, or perhaps in the future to other modalities of therapy. Nevertheless, aggressive therapy appears particularly warranted in patients whose tumors show both p53 and RB abnormalities.

Our data are consistent with other reports in the literature. It has been shown previously that p53 immunostaining could be used to stratify patients for outcome independently of other variables associated with progression and survival (e.g., stage, grade, multiplicity of tumors, age, sex, and vascular invasion; Refs. 14–17, 34, and 35). Similarly, loss of RB function by immunohistochemical analysis has been reported to be an independent marker of worse prognosis in more advanced disease (18, 19).

Our results now extend those of the recent report, which included both T\textsubscript{a} and T\textsubscript{1} bladder tumors, that showed that patients with abnormalities of both RB and p53 protein expression have significantly higher progression and associated lower survival rates than do those with normal expression of both genes (22). In the earlier study, patients with defects in only one

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**Fig. 2** A. Kaplan-Meier progression-free survival in 45 patients who did not have early cystectomy. RB is characterized as wild type, negative, and overexpressed. B. Kaplan-Meier disease-specific survival in 45 patients who did not have early cystectomy. RB is characterized as wild type or altered. Altered RB cases included tumors in which RB staining was either negative or overexpressed.

**Fig. 3** Kaplan-Meier progression-free survival in 45 patients who did not have early cystectomy characterized by p53 expression.

**Fig. 4** Kaplan-Meier progression-free survival in 45 patients who did not have early cystectomy characterized by p53 and RB protein expression.
gene also had an intermediate risk of progression and survival (22), a conclusion that is similar to the findings from our study.

All of the previous immunohistochemical analysis studies to determine RB function have used absence of nuclear protein staining in all tumor cells as the criterion for scoring a given tumor as having loss of RB function. A recent report, however, in which two of us (W. F. B. and S-X. H.) participated has suggested in bladder tumors specifically that an overexpression of RB measured by strongly homogeneous staining is also indicative of dysfunctional RB status (32, 33). That study involved patients with higher stage tumors, all of whom had cystectomies. Those patients with cancers having RB overexpression showed as marked an increase in tumor progression and decrease in overall survival as did patients whose tumors lacked RB protein expression. The results from our study are consistent with that report and suggest, at least in bladder tumors, that such overexpression of RB protein may indicate dysfunctional RB regulation. Nevertheless, the specific basis for this staining pattern in bladder cancer is presently unknown. However, hyperphosphorylated RB protein has been associated with a worse overall prognosis in acute myelogenous leukemia (36), and it is tempting to speculate that an upstream regulating gene is defective in such situations, resulting in a disproportional level of the inactive phosphorylated RB protein form(s).

Why are p53 and RB protein expression such powerful predictors of outcome in bladder cancer? Although the role of these proteins in cell cycle regulation has been well studied, it is likely that there are other effects of alterations in these genes that contribute to the invasive potential of tumors. For example, p53 response elements have been found on the promoter for the genes that encode epidermal growth factor receptor and thrombospondin (37, 38), two proteins that contribute significantly to growth and invasion of bladder cancers. Furthermore, RB expression has been shown to inhibit bladder tumor cell invasion in vitro (39). Therefore, the biological effects of alteration in p53 and RB protein expression may have much more impact on tumor progression than is simply due to cell cycle dysregulation associated with aberrant expression of either or both of these genes.

Given the above observations, appropriate treatments for patients stratified by p53 and RB protein expression must be identified. We have initiated a clinical trial based on the findings in this report. Despite abnormal p53 expression, intravesical therapy with BCG can still be used because p53 expression does not predict response to BCG (21, 40–41). However, p53 persistence after BCG therapy is an ominous finding associated with a high incidence of disease progression. New therapeutic combinations, possibly including angiogenesis inhibitors or tyrosine kinase inhibitors, are needed to effectively treat patients with tumors refractory to BCG that exhibit altered p53 and/or RB protein function (42–43).

References


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