Elevated and Absent pRb Expression Is Associated with Bladder Cancer Progression and Has Cooperative Effects with p53

Richard J. Cote, Matthew D. Dunn, Sunanda J. Chatterjee, John P. Stein, Shan-Rong Shi, Quoc-Chau Tran, Shi Xue Hu, Hong Ji Xu, Susan Groshen, Clive R. Taylor, Donald G. Skinner, and William F. Benedict

Departments of Pathology [R. J. C., M. D. D., S. J. C., J. P. S., S. R. S., C. R. T.], Preventive Medicine [Q.-C. T., S. G.], and Urology [D. G. S.], University of Southern California School of Medicine/Norris Comprehensive Cancer Center, Los Angeles, California 90033, and the Departments of Hematology [S. X. H., W. F. B.] and Molecular Oncology [H. J. X.], M. D. Anderson Cancer Center, The Woodlands, Texas 77381

Abstract

Rb protein (pRb) expression was evaluated in 185 cases of transitional cell carcinoma of the bladder from patients that underwent radical cystectomy. Tumors were stratified into three categories based on the percentage of nuclei expressing pRb: (a) 0, 0% of tumor cells showing nuclear reactivity; (b) 1+, 1–50% of tumor cells showing nuclear reactivity; and (c) 2+, >50% of tumor cells showing nuclear reactivity. Cases with undetectable (pRb 0) and high (pRb 2+) pRb reactivity had identical rates of recurrence. These cases had significantly higher recurrence (P = 0.0001) and lower survival rates (P = 0.0002) compared to cases with moderate (pRb 1+) pRb reactivity, indicating that high levels of pRb expression may reflect a dysfunctional (altered) Rb pathway. The tumors were also examined for alterations in p53 expression; patients with tumors altered in both p53 and pRb had significantly increased rates of recurrence (P < 0.0001) and survival (P < 0.0001) compared to patients with no alterations in either p53 or pRb; patients with alterations in only one of these proteins had intermediate rates of recurrence and survival. These results suggest that: (a) bladder cancers with high pRb expression do not show the tumor suppressor effects of the protein; and (b) alteration in both p53 and pRb may act in cooperative or synergistic ways to promote tumor progression.

Introduction

Cancer is a multistep genetic process involving alterations in oncogenes and tumor suppressor genes (1). Mutations in the tumor suppressor genes p53 and Rb are common events in human cancer, including bladder cancer (2–6). However, a proportion of tumors showing no alteration in p53 will progress; this is also true for Rb. Both p53 and Rb exert their control on the cell cycle at the G1-S-phase transition through independent but interconnected pathways (7). Furthermore, it has recently been shown that germ-line mutations in p53 and Rb may have cooperative tumorogenic effects in mice (8). We therefore reasoned that alterations in both p53 and Rb may act in a cooperative or synergistic way to promote bladder cancer progression in humans. Whereas p53 gene mutations often result in the abnormal nuclear accumulation of the altered protein (9), mutations of the Rb gene correspond to loss of expression of the pRb (6). Because nuclear expression of pRb has been assumed to be indicative of an intact gene and a functional protein, to date, studies examining the prognostic significance of pRb determined whether or not the protein was expressed (4–5). However, it is possible that in some cases, pRb may have lost functional activity, although it is expressed. We undertook the present study to determine whether a subset of tumors that express pRb shows evidence of loss of pRb function. We also sought to determine whether alterations in both pRb and p53 expression exert a cooperative or synergistic effect in promoting bladder cancer progression.

Materials and Methods

Patient Information. Tumor specimens were obtained from 185 patients who underwent en bloc radical cystectomy, pelvic lymphadenectomy, and urinary reconstruction for invasive transitional cell carcinoma of the bladder. Tumor staging was classified according to the tumor-node-metastasis system (10). Median follow-up for this group was 8.6 years.

Antibodies and Immunohistochemistry. The immunohistochemical procedure for the rabbit polyclonal anti-pRb antibody, RB-WL-1, including the antigen retrieval protocol, has been previously described by us (11). The extent of pRb reactivity was classified into three categories based on the percentage of tumor cells showing nuclear reactivity: (a) 0, 0% of tumor cells showing nuclear reactivity; (b) 1+, 1–50% of tumor cells showing nuclear reactivity; and (c) 2+, >50% of tumor cells showing nuclear reactivity (Fig. 1). The normal bladder urothelium demonstrated pRb 1+ nuclear reactivity in all cases. In most cases, tumors showing 2+ pRb nuclear reactivity demonstrated intense staining of more than 80% of tumor cells (Fig. 1). All slides were read independently at two different institutions (by R. J. C., M. D. D., S. R. S., and J. P. S. and by W. F. B. and H. J. X.); concordance was reached in nearly all cases. In the discordant cases, the tissue was retested. In all of these cases, concordance was reached.

The immunohistochemical procedure for p53 has been described previously by us (2). The level of p53 nuclear reactivity was classified into two categories: (a) p53−, 0–9% of tumor cell nuclei were positive for p53 nuclear reactivity; and (b) p53+, 10–100% of tumor cell nuclei were positive for p53 nuclear reactivity (2). Based on our previous studies comparing p53 nuclear reactivity with p53 gene mutations, only tumors showing at least 10% nuclear reactivity were considered to be p53-altered (p53+; Ref. 9).

Statistical Analysis. The clinical outcomes analyzed were time to first recurrence of bladder cancer and overall survival. Time to recurrence was calculated as days from cystectomy to the date of the first documented clinical recurrence or last follow-up visit, if the patient had not recurred; patients who died before recurrence were censored at the time of death; deaths from any cause were included. Survival was calculated as days from cystectomy to death. For the purpose of statistical analysis, pRb status was initially classified into two groups, negative (pRb 0) and positive (pRb 1+ and 2+), and then analyzed in relation to probability of recurrence and survival. Classification into three groups (pRb status 0, 1+, and 2+) was then performed and analyzed in a similar manner. pRb status was then combined with p53 status and analyzed in relation to recurrence and survival. Contingency tables and Pearson’s χ² test (12) were used to evaluate the association of Rb reactivity with pathological stage and lymph node status of the tumor. Kaplan-Meier plots (13) and log-rank tests were produced to evaluate the association of these prognostic variables with Rb status alone and combined with p53 status in regard to survival and recurrence. SEs of these estimates were calculated using Greenwood’s formula (14).

Results

Rb Status. No pRb nuclear reactivity (pRb 0) was noted in 54 tumors (29%), 83 tumors (45%) had 1+ pRb reactivity, and 48 tumors
(26%) had 2+ pRb reactivity. Normal bladder urothelium showed heterogeneous (1+) pRb nuclear reactivity (Fig. 1). When pRb status was stratified into two groups based on the presence or absence of nuclear reactivity [pRb+ (pRb 1+ and 2+) versus pRb− (pRb 0)], a significant association with stage of disease was noted (P = 0.002); tumors with pRb+ nuclear reactivity tended to be of lower stage than pRb− tumors. The pRb 0 group showed increased recurrence (P = 0.013) and decreased overall survival (P = 0.09) compared with the pRb 1+/2+ group. The estimated 5-year survival rates were 33 and 54%, respectively.

When stratified into three categories based on the level of pRb nuclear reactivity (pRb 0 versus 1+ versus 2+), a significant association was seen with stage of disease (P = 0.001; Table 1). However, this analysis showed that tumors with no (pRb 0) or high (pRb 2+) expression of pRb were similar with regard to association with disease stage and were significantly different from tumors that showed moderate (pRb 1+) levels of pRb expression. Tumors with moderate (pRb 1+) pRb expression tended to be of lower stage than pRb 0 or 2+ tumors.

Among these three groups (pRb 0 versus 1+ versus 2+), a significant difference in overall rates of recurrence and survival was observed (Fig. 2). Patients with tumors demonstrating homogeneous nuclear reactivity (pRb 2+) had nearly identical rates of recurrence and survival compared to patients with tumors showing no detectable nuclear reactivity (pRb 0). These patients had significantly higher rates of recurrence (P < 0.0001) and decreased survival (P = 0.0002) compared to patients with tumors demonstrating moderate nuclear reactivity (pRb 1+). The 5-year recurrence rates for patients with pRb 0, 1+, and 2+ bladder tumors were 60, 29, and 61%, respectively (P = 0.0001). The 5-year survival rates for these same groups were 33, 66, and 33%, respectively (P = 0.0002).

When stratified into three categories based on the level of expression, the pRb status was independent of tumor grade, stage, and lymph node status in predicting recurrence and survival in a multivariable analysis (P = 0.012 and 0.010 for recurrence and survival, respectively).

p53 Status. Of the 185 tumors studied, 109 (61%) demonstrated no nuclear accumulation of p53 (p53 wt tumors), and 76 (39%) demonstrated nuclear accumulation of p53 protein (p53-altered tumors), confirming our previous results (2). Patients with p53 wt tumors had significantly lower rates of recurrence and better overall survival than patients with p53-altered tumors. The 5-year recurrence rates for patients with p53 wt tumors versus p53-altered tumors were 31 versus 69% (P < 0.0001). The 5-year survival rates for patients with p53 wt tumors versus p53-altered tumors were 63 versus 28% (P < 0.0001).

Combined Rb and p53 Status. The combined effects of p53 and pRb status on tumor progression (relationship to stage, recurrence, and survival) in patients with invasive bladder cancer are summarized in Table 1 and Fig. 3. In this analysis, those tumors demonstrating no (pRb 0) or high levels (pRb 2+) of nuclear reactivity were con-
considered pRb-altered, because their recurrence and survival rates were nearly identical. Tumors with moderate levels of pRb expression (pRb 1+) were considered to have a pRb wt phenotype. The combined p53/pRb status was significantly associated with tumor stage (Table 1); low-stage tumors tended to have fewer alterations than did high-stage tumors. Patients with p53 wt/pRb wt bladder tumors had significantly decreased recurrence and increased survival rates when compared to patients with tumors showing alterations in either p53 or pRb (p53 wt/pRb-altered tumors or p53-altered/pRb wt tumors; P < 0.0001 and 0.0001, respectively). Patients with alterations in either p53 or pRb expression demonstrated decreased recurrence and increased survival rates compared to patients with tumors showing altered expression of both p53 and pRb (p53-altered/pRb-altered tumors; Fig. 3). The 5-year recurrence rates for patients with tumors demonstrating p53 wt/pRb wt versus p53 wt/pRb-altered or p53-altered/pRb wt versus p53-altered/pRb-altered expression were 22 versus 42 versus 79%, respectively (P < 0.0001); the 5-year survival rates for these groups were 71 versus 51 versus 16%, respectively (P < 0.0001). In patients with organ-confined (P1-P3a) lymph node-negative disease, the estimated 5-year recurrence rates for patients with tumors demonstrating p53 wt/pRb wt versus p53 wt/pRb-altered or p53-altered/pRb wt versus p53-altered/pRb-altered expression were 21 versus 27 versus 78%, respectively (P < 0.0001); the 5-year survival rates for these groups were 82 versus 75 versus 27%, respectively (P < 0.0001).

Discussion

Our results strongly suggest that at least in the case of bladder cancer, high levels of pRb expression may reflect an altered Rb pathway. In our study, patients with high levels of pRb nuclear reactivity in their tumors had essentially identical recurrence and survival rates as did patients with tumors showing no detectable pRb expression. Both of these groups of patients had significantly increased recurrence and decreased survival rates compared to patients with tumors demonstrating heterogeneous patterns of pRb reactivity. These findings suggest that both loss of pRb reactivity and overexpression of pRb involve perturbation of normal Rb function. It is known that absent pRb reactivity is indicative of loss of gene expression (generally through mutation; Ref. 6). We hypothesize that pRb overexpression reflects an alteration in the Rb pathway resulting in loss of tumor suppressor function.

Although the mechanism for increased pRb expression and possible alterations is not clear, a number of possibilities exist. The overexpressed pRb may represent a hyperphosphorylated form of the protein. Rb acts to control cell proliferation through regulation of the cell cycle at the G1-S-phase transition. In its underphosphorylated state, pRb inhibits this transition by sequestering the transcription factor E2F (7). However, when pRb is phosphorylated by the cyclin-dependent kinase complexes (15), E2F is released, and the cell can initiate DNA synthesis (16). Hyperphosphorylation of pRb has been demonstrated in acute myeloid leukemias, in which it is associated with poor prognosis (17). Phosphorylation of pRb is accomplished by cyclin-dependent kinase complexes; these complexes are inhibited by several cyclin-dependent kinase inhibitors, including p21, p27, and p16 (18, 19). It has been shown that loss of expression of these inhibitors may be associated with tumor progression (7). It is therefore tempting to speculate that abnormalities of one or more upstream regulators of pRb phosphorylation might result in constitutive hyperphosphorylation (and inactivation) of pRb. Alternatively, it is possible that some mutations of the Rb gene might lead to expression of a functionally inactive aberrantly expressed protein, although this is not consistent with the present data, because mutations in the Rb gene in the presence of expressed protein would be expected to be rare (6). Finally, binding of pRb to other proteins such as MDM2 or certain DNA viral oncoproteins may also abrogate the function of pRb while preserving its expression. Whatever its basis, our results indicate that the pattern of pRb reactivity must be taken into account when evaluating the Rb status in bladder tumors. It is likely that this will be the case for other tumor types as well.

Whereas alterations in p53 and pRb have been shown to be independent predictors of bladder cancer progression, our results demonstrate that concomitant alterations in both p53 and pRb have further negative effects on recurrence and survival compared to alterations in either one alone. Furthermore, when neither p53 nor pRb is altered, patients demonstrate very low rates of recurrence and increased survival, no matter what the stage of disease. Gordon-Cardo et al. (20) have recently reported this for patients with superficial bladder tumors. Of note is the extremely low rate of recurrence in patients with organ-confined tumors that show neither p53 nor pRb alterations. It is of interest that patients with organ-confined tumors that show neither p53 nor pRb alterations. It is of interest that patients with organ-confined tumors showing alterations in either p53 or pRb (but not both) also have low rates of recurrence and higher rates of survival; however, those with tumors showing alterations in both p53 and pRb have significantly increased recurrence and decreased survival rates. These results indicate that alterations in both p53 and Rb are important in the progression of early stage bladder cancer.
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tumors. Thus, knowledge of both the p53 and pRb status provides more specific information about the most likely outcome for the patient.

That alterations in p53 and pRb act in cooperative or synergistic ways to promote bladder cancer progression is not entirely unexpected. Cancer is a multistep genetic process; the accumulation of multiple alterations produces the malignant phenotype (1). Furthermore, p53 and pRb have effects in the same phase of the cell cycle and act in separate but interconnected pathways. Finally, studies in mice have demonstrated that germ-line mutations in both p53 and Rb produce tumors in mice at higher rates than if the mice had germ-line mutations in either p53 or Rb, but not both (7). Thus alterations in both p53 and Rb might be expected to be more deleterious than alterations in one system alone.

In summary, our results indicate that the level of pRb expression, and not simply its presence or absence, should be determined when assessing the impact of Rb on bladder cancer progression. The basis for this is an important issue and is the topic of ongoing research. Furthermore, determination of alterations in more than one tumor suppressor pathway or within the same pathway can provide increased information regarding prognosis. These findings may be relevant in other tumors as well. Finally, determining the status of a tumor suppressor pathway may be important in making specific treatment decisions for patients with bladder cancer, a subject that can be further addressed by large prospective clinical trials that incorporate p53 and pRb status into the treatment protocol.

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References


Fig. 2. Probability of (A) remaining relapse-free and (B) survival in 185 patients with invasive transitional cell carcinoma of the bladder according to pRb status. pRb 0, no detectable nuclear reactivity; pRb 1+, 1–50% nuclear reactivity; pRb 2+, >50% nuclear reactivity.

Fig. 3. Probability of (A) remaining relapse-free and (B) survival in 185 patients with invasive transitional cell carcinoma of the bladder according to combined pRb/p53 status. Altered pRb (pRbalit) tumors include those with pRb 0 and 2+ nuclear reactivity, and wt pRb (pRbwt) tumors were those with pRb 1+ nuclear reactivity. Altered p53 (p53alt) tumors were those with ≥10% nuclear reactivity, and wt p53 (p53wt) tumors were those with <10% nuclear reactivity.
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