Different Combinations of Genetic/Epigenetic Alterations Inactivate the p53 and pRb Pathways in Invasive Human Bladder Cancers


ABSTRACT

Inactivation of both the pRb (pRb-cyclin D1/cyclin-dependent kinase 4/p16) and p53 (p53-p21WAF1-p14ARF) pathways is thought to be essential for immortalization in vitro and malignant transformation in vivo. We identified different combinations of pRb and p53 pathway alterations in 12 invasive transitional cell carcinomas (TCCs) and addressed the functional significance of the different combinations observed. Results showed four combinations of alterations including −pRb/−p53 (i.e., pRb inactivated in the pRb pathway and p53 inactivated in the p53 pathway; four TCCs), −p16/−p53 (four TCCs), −p16/−p21WAF1 (one TCC), and −p16/−p14ARF (two TCCs). These groups include two new combinations (i.e., −p16/−p53 and −p16/−p21WAF1) not reported previously for TCCs. An alteration in the key components of the p53 pathway was not detected in one invasive TCC that had inactivated p16. Note that all four TCCs with inactivated pRb had mutant p53; thus, the combinations of −pRb/−p16WAF1 and −pRb/−p14ARF were not observed. Only two of eight TCCs with altered p16 had concomitant p14ARF loss, demonstrating that simultaneous inactivation of these two p21INK4a tumor suppressor genes is not obligatory. To determine the biological phenotypes of TCCs with different combinations of pRb and p53 pathway alterations, their downstream responses to gamma radiation were studied in vitro. As expected, none of eight TCCs with mutant p53 responded to gamma radiation by elevation of p53, p21WAF1, or mdm2 or by cell cycle arrest. Only two of four TCCs with wild-type p53 and wild-type pRb (the combination of −p16/−p14ARF) showed normal downstream responses to gamma radiation and underwent cell cycle arrest. Two TCCs with wild-type pRb and wild-type p53 (the combination of −p16/−p21WAF1 and one TCC with −p16) failed to show cell cycle arrest in response to radiation. This was attributed to the absence of p21WAF1 in one TCC. In summary, these data support a model of invasive bladder cancer pathogenesis in which both the pRb and p53 pathways are usually inactivated and the biology of the tumor is impacted by the mechanism of their inactivations.

INTRODUCTION

Multiple genetic alterations have been identified in invasive human bladder cancers. These alterations are present in different combinations and frequencies in different tumors. Among these alterations are those that inactivate the pRb (pRb-cyclin D1/Cdk4/p16) and p53 (p53-p21WAF1-p14ARF) tumor suppressor checkpoint pathways. We have proposed that alterations in the pRb pathway are required for bypassing senescence and are an essential step in pathogenesis of invasive bladder cancer. Recent studies support a model in which a p53 pathway alteration may also be required for bypassing the senescence checkpoint and for tumorigenesis in vivo. Many studies support a role for p53 in maintaining genome stability after DNA damage. This is thought to occur by p53 activation of DNA repair mechanisms, cell cycle arrest, and/or by apoptosis (3–7). In the current study, we examined the hypothesis that inactivation of the p53, as well as the pRb pathway, is required for progression to invasive human bladder cancer and identified both previously reported and new genetic/epigenetic combinations. We also examined the hypothesis that different combinations of inactivating pRb and p53 pathway alterations might be associated with different tumor phenotypes.

Alterations in the pRb pathway are frequent in human cancers. In this respect, inactivation of p16/CDKN2A is the second most common alteration found across all human cancer types, including bladder cancer (reviewed in Ref. 8). Alterations involving p16/CDKN2A have been identified in ~50% of human bladder cancers (9–13). Several genetic/epigenetic mechanisms, including mutation (14–16), de novo methylation (17–19), and hemizygous and homozygous deletions (10) target the INK4a/p16/CDKN2A locus for inactivation. p16 inhibits cyclin D1-Cdk4/6-dependent phosphorylation of pRb, resulting in the sequestration of the E2F transcription factors and subsequent cell cycle arrest (15, 20, 21). Our group was the first to demonstrate that p16 elevation occurs at senescence in human epithelial cells (uroepithelial and prostate epithelial cells), and thus we proposed that these high p16 levels contribute to G1 senescence cell cycle arrest (22, 23). p16 is elevated at senescence in other cell types as well (21, 24, 25). Alterations in pRb are also frequent in human cancers and are found in ~30% of human bladder cancers (26–28).

Inactivation of different components in the pRb pathway has different biological impacts. For example, either a p16 or pRb inactivation suffices for bypassing senescence (1). However, only pRb inactivation also abrogates p53-dependent cell cycle arrest after DNA damage. Not surprisingly, pRb alterations are associated in vivo with later-stage and higher-grade bladder cancers, as well as with a greater probability of tumor progression and lower overall survival (27, 28). Amplification of CCND1, which codes for cyclin D1, occurs frequently in many cancer types, including bladder cancer (29–31), but its biological impact on bypassing senescence or tumor progression is unclear. Alterations in CDK4 and CDK6 are infrequently observed in human cancer. For example, a mutation in residue 24 of Cdk4 that alters the ability of Cdk4 to bind to or be inhibited by p16 has been identified in melanoma. However, this mutation does not alter its catalytic activity (32). Thus, such mutations, although rare, might suffice for bypassing senescence and impact significantly on tumor progression.

Mutation of the tumor suppressor gene, TP53 is the most frequent alteration among all human cancer types (33, 34). TP53 is thought to play an important role in maintaining genome stability by mechanisms including transactivation of genes involved in p53 stability (MDM2),...
DNA repair (GADD45), cell cycle arrest (p21WAF1/Cip1), and apoptosis (BAX). Mutant p53, which is usually increased or elevated compared with wild type, fails to transactivate these downstream genes or initiate these protective cascades. Inactivating alterations have also been identified in several downstream genes transactivated by p53. For example, a low percentage of bladder tumors contain inactivating mutations in p21WAF1/Cip1, altering the normal cellular downstream cell cycle response after DNA damage (35, 36). MDM2 is found amplified (37) or overexpressed in a variety of tumors (38). Because mdm2 binds to the NH2 terminus of p53, which is required for transactivation, its overexpression inhibits p53-mediated transactivation (39). Thus, elevated mdm2 levels also result in abrogation of p53-mediated cell cycle arrest and apoptosis (40–42). Another study shows that when MDM2 is overexpressed, wild-type p53 is elevated, representing an alternative mechanism for p53 inactivation (43).

Most recently, a new member of the p53 pathway has been identified. Studies with mouse embryo fibroblasts on the INK4a locus that codes for p16CDKNA2A have led to the identification of a second novel INK4a transcript that has a 3’ sequence identical to p16 but with a unique 5’ end (44–47). This transcript, called p19ARF in mice (the human homologue is p14ARF), is derived from a distinct first exon (exon 1β), which is spliced to exon 2 that is shared with p16 but in an ARF (48). Both p19ARF and p14ARF have the ability to arrest cell proliferation at both G1 and G2-M in a p53-dependent manner involving the up-regulation of downstream target p21WAF1/Cip1. This response is abolished in cells lacking p53 (48, 49). However, two ARF-negative cell lines (i.e., A375 in which exon 1β is deleted and MCF7 in which exons 1α, 2, and 3 are deleted) undergo cell cycle arrest when treated with actinomycin D, showing that p14ARF expression is not involved in the response to DNA damage (49). These data have also been confirmed in mice, where ARF-null cells showed elevated p53 and p21WAF1 levels after ionizing or UV irradiation, thus exhibiting an intact p53 checkpoint pathway. Because mouse embryo fibroblasts (MEFs) that retained p19ARF always sustained mutations in TP53 and vice versa, these studies suggest that the functional losses of either p53 or p19ARF are mutually exclusive events for immortalization (50). Recent evidence also showed that E2F1 could induce p14ARF expression by activating the ARF promoter (51, 52).

p14ARF stabilizes p53 by binding and promoting the degradation of another oncoprotein, mdm2 (53, 54). p19ARF can be activated by other oncoproteins including myc, ras, and E1A (55, 56). Thus, deregulated E2F-1 activity, attributable to inactivation of pRb and activation of other oncogenes, initiates abnormal proliferation, resulting in the induction of a p53 response through activation of p14ARF (5, 57–59). This would lead to cell cycle arrest or apoptosis unless a second mutation occurred in either ARF or TP53.

As summarized above, inactivation of both the pRb and p53 pathways is common in human cancers, including bladder cancer. We reported recently that an alteration in the pRb checkpoint pathway appears to be required for progression of superficial bladder tumors to invasive bladder cancer (1). In the present study, we tested the hypothesis that an alteration in the p53 pathway is also required for transformation to invasive human bladder cancer. We then tested the hypothesis that different combinations of pathway alterations might have different impacts on tumor phenotype. We report here the presence of a p53 pathway alteration (p53, p14ARF, or p21WAF1) in 11 of 12 TCCs examined, and we identify four different combinations of pRb and p53 pathway alterations in these 12 invasive human TCCs. Only two of these combinations of alterations have been identified previously (2); thus two combinations are reported herein for the first time. We also report here for the first time that p14ARF can be concomitantly expressed with wild-type p53 in invasive TCCs. Thus, we show that a p14ARF or a p53 alteration is apparently not a required event in the development of invasive bladder cancer. Indeed, one invasive TCC failed to show an alteration of p53, p21WAF1, or p14ARF. Finally, we show that because of their unique genetic organization, inactivation of both p16 and p14ARF can be accomplished by a single alteration, but this was infrequent in our samples.

**MATERIALS AND METHODS**

**TCC Biopsies.** Twelve biopsies of bladder tumors were obtained by transurethral resection of the bladder or at cystectomy from 11 patients at the University of Wisconsin Hospital and Clinics. All of the tumors were classified as TCCs. The TCC stages ranged from T1 to T4 (Tumor-Node-Metastasis classification) and were grades I to III. The TCCs were established in culture using an explant technique on collagen substrates in a supplemented Ham’s F12 medium with 1% fetal bovine serum, exactly as described (60). Epithelial cells grow out from the explants, which can then be removed. These conditions do not support the growth of human fibroblasts and therefore select for uroepithelial cells. All of the TCCs in this study were invasive, and all spontaneously bypassed senescence and formed immortal cell lines. Six of the TCCs (TCC 97-1, TCC 94-10, TCC 96-1, TCC 96-2, TCC 97-6, and TCC 92-1) were used previously in a study of bypassing senescence in human cancer pathogenesis (1). Six additional invasive TCCs were used in the current study (TCC 97-7, TCC 97-15, TCC 97-18-I, TCC 97-21-M, TCC 97-24, and TCC 97-29). Two of these TCCs (TCC 97-18-I and TCC 97-21-M) were from an invasive (I) and a metastatic (M) biopsy taken from the same patient at the time of primary tumor resection and later at the time of tumor recurrence, respectively. The other 10 samples all represent the first tumor biopsy from each patient. All of the patients in this study were followed for 2 years for recurrence and progression of their cancers at the University of Wisconsin Hospital and Clinics and the Veteran’s Administration Hospital in Madison, Wisconsin.

**Protein Analysis.** The status of proteins in the pRb and p53 pathways was examined before passage (P) 5 and again after cell line establishment (after P15) using Western blot analysis as described previously (1). The proteins examined included: pRb, cyclin D1, CDK4, CDK6, p16, p53, p21WAF1, and mdm2. Briefly, 2–5×106 cells growing in late log phase were lysed in EC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 100 mM NaF, 0.2 mM Na3VO4, 0.5% NP-40 with 10 mg/ml each of leupeptin, phenylmethylsulfonyl fluoride, and aprotinin (Sigma)]. The proteins were quantified using the Bradford assay, loaded using 50 µg/lane and run on a 12% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon–P, Millipore) and incubated with the appropriate primary antibody. Monoclonal antibodies to p53 (Ab-2), p16 (Ab-1), p21WAF1 (Ab-1), and α-tubulin (Ab-1) were obtained from Oncogene Research Products. Monoclonal antibody to pRb (1400A1) was obtained from PharMingen, and monoclonal antibodies to mdm2 (SMP14) and cyclin D1 (BD-11) and polyclonal antibodies to CDK6 (C-21) and CDK4 (H-22) were obtained from Santa Cruz Biotechnology. A well-characterized rabbit polyclonal antibody to p14ARF was used (61). Bound antibody was detected using enhanced chemiluminescence (Pierce). Equal loading was verified using an antibody to α-tubulin.

For p14ARF detection, cells were lysed in RIPA buffer [1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, with 10 µg/ml each of phenylmethylsulfonyl fluoride (10 mg/ml) and Na3VO4 (100 mM) and 30 µg/ml of aprotinin (10 mg/ml)], and 75 µg protein/lane was run on a 15% SDS-polyacrylamide gel. For pRb-E2F1 immunoprecipitation studies, ~107 cells were lysed in RIPA buffer, as described above. The cell lysate was incubated with 2 µg of primary antibody at 4°C for 1 h, followed by an overnight incubation with 20 µl of Protein G Plus-Agarose (Oncogene Research Products) with mixing at 4°C. Precipitated proteins were collected by centrifugation, washed with RIPA buffer, boiled for 2 min, separated on 12% SDS-polyacrylamide gels, and immunoblotted as above. Monoclonal antibody to E2F-1 (KH95) was obtained from Santa Cruz Biotechnology.

**IHC.** IHC was used as a second assay to assess loss of wild-type pRb. Cells were grown on chambered glass slides (Nunc) and fixed with glutaraldehyde and formalin (62). The pRb monoclonal antibody described above was used at 1:1,000 dilution to detect wild-type nuclear pRb using the Vectastain ABC kit from Vector Laboratories and following the manufacturer instructions. The DAB substrate kit (Vector Labs) for peroxidase was used for final detection.
Groups without primary pRb antibody and with an irrelevant anti-SV40-T antigen antibody (Santa Cruz Biotechnology) were used as controls. IHC was done at least twice for all samples and was scored using coded samples.

**Southern Analysis.** Southern blot analysis was used to detect p16/CDKN2A and p14ARF deletions or altered methylation in the TCCs. DNA was extracted from early-passage cells using TE buffer containing 0.5 M Tris-Cl (pH 8.0) and 1 mM EDTA, phenol-chloroform extraction, and dissolved in TE buffer [10 mM Tris-Cl (pH 8.0) and 1 mM EDTA, pH 8.0] and quantified. Methylation analysis was done as described (1). Briefly, 10 μg of genomic DNA from each cell line were digested with EcoRI and methylation-sensitive SacII restriction enzymes (Promega Corp., Madison WI), electrophoresed on a 1% agarose gel, and transferred to Hybond N+ membrane (Amersham Life Sciences). The blot was hybridized with a p16/CDKN2A probe that spans the CpG island of the promoter and exon 1a (19) in Rapid-hyb buffer (Amersham Life Sciences). The probe was labeled with [γ-32P]dCTP using the Rediprime kit (Amersham Life Sciences) according to the manufacturer’s instructions. The human prostrate carcinoma cell line TSU-PR1 was used as a positive control for methylation.

**Northern Analysis.** Northern blot analysis was used to detect p16 and p14ARF mRNA in TCC 97-15, TCC 97-18 and TCC 97-29. RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Ten μg of total RNA from each cell line were run on a denaturing 1% formaldehyde-agarose gel and transferred to a Hybond N+ nylon membrane. Hybridization was done overnight at 65°C. A whole-length p16/CDKN2A cDNA was used to detect p16 mRNA. The p14ARF probe was made by reverse transcription of total RNA from normal HUCs using the RNA PCR core kit (Perkin-Elmer). The primers for PCR have been described previously (63). The blots were stripped and hybridized with a β-actin cDNA probe to verify equal loading. The HeLa cell line was used as a positive control; MCF7 and A375 cell lines were used as negative controls because neither of these lines contains p14ARF.

**Irradiation and Cell Cycle Analysis.** Logarithmically growing cell cultures were irradiated with 18 Gy of gamma radiation at a rate of 6 Gy/min using a 137Cs source. Twenty-four h after irradiation, the cell cycle arrest was analyzed in duplicate using standard flow cytometric techniques (64). Briefly, cells were incubated for 2 h with 20 μM BrdUrd (Sigma) and fixed in 70% ethanol. These cells were permeabilized and incubated with a primary antibody against BrdUrd (Caltag), followed by another incubation with a FITC-conjugated secondary antibody against mouse IgG (Sigma). After labeling with 50 μg/ml propidium iodide, these cells were analyzed on a FACScan (Becton Dickinson). Cell cycle distribution was determined using CellQuest software package (Becton Dickinson). Irradiated cells were also analyzed using Western blot analysis as described above.

**DNA Sequencing.** Exons 1a and 2 of p16/CDKN2A and exons 1β and 2 of p14ARF were PCR amplified, and the PCR products were cloned into a pBluescript vector and sequenced. The manufacturer’s instructions and sequenced at the University of Wisconsin Biotechnology Center using an automated DNA sequencer. The primers used to amplify exons 1α, 1β, and exon 2 have been described previously (19, 46, 20). PCR reactions were performed in 50-μl volumes using 250 ng of genomic DNA as template, 200 μM deoxynucleotide triphosphates, 1.5 mM MgCl2, 1 μM of each forward and reverse primers and 5 units of AmpliTaq DNA polymerase. Formamide (5%; Sigma) was added to all PCR reactions for exon 1α. Reagents for PCR were obtained from Perkin-Elmer. The PCR conditions for exon 1α and 1β were: an initial denaturation step of 3 min at 95°C, 30 cycles of 54 s each at 94°C, 62°C, and 72°C, followed by a final elongation step of 5 min at 72°C. The PCR conditions for exon 2 included initial denaturation of 5 min at 95°C, followed by 30 cycles of 65 s at 94°C, 65°C, 62°C, and 72°C; and 58°C, 60 s at 72°C; and a final elongation step of 5 min at 72°C.

**RESULTS**

**pRb Pathway Alterations Identified in Invasive TCCs.** Unequivocal data support a significant role for frequent pRb and p16 alterations in bladder cancer pathogenesis. However, other alterations, such as overexpression of cyclin D1 and/or mutation of CDK4 or CDK6, might provide mechanisms for bypassing the p16-mediated senescence checkpoint. In this study, we examined the status of these pRb pathway regulatory proteins in 12 representative invasive TCCs (Table 1). Western analysis was performed at early passage after establishment of tumor biopsies in vitro. Normal HUCs were used as controls. All experiments were repeated at least twice. Results showed that p16 was undetectable in 7 of 12 TCCs with wild-type levels of pRb (Fig. 1A). In contrast, pRb was undetectable in three TCCs that showed elevated levels of p16 (Fig. 1A). These latter p16 levels were comparable with the p16 levels seen at senescence in normal HUCs (Fig. 1A). One tumor, TCC 97-24, had elevated levels of p16 along with a relatively strong pRb signal. However, the pRb signal was consistent with a hypophosphorylated state (Fig. 1A). The functional status of pRb was determined by immunoprecipitation of pRb and associated E2F-1, followed by Western analysis of the immunoprecipitates with antibodies to pRb and E2F-1. Results confirmed that in TCC 97-24, pRb existed primarily in a hypophosphorylated state but

<table>
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<th>TCC</th>
<th>Stage and grade</th>
<th>No. of recurrences</th>
<th>Tumor phenotypes in vivo</th>
<th>Radiation response in vitro</th>
<th>Pathway combinations</th>
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<td>97-29</td>
<td>T2 III</td>
<td>1</td>
<td>– (T1, T2)</td>
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</table>

*The stage and grade of the primary TCCs are reported, followed by patients' subsequent phenotypes of recurrence, progression, and metastasis.

*aTerminal refers to patients who died of metastatic bladder cancer. NA, not applicable.

*bThis TCC has low levels of p16 but was classified as -p16 based on functional studies (see text).

cTwo TCCs in which p53 and mdm2, but not p21, were induced.
also showed that pRb did not bind E2F-1 (Fig. 1B). Thus, pRb in TCC 97-24 was apparently inactivated. Another TCC, 97-7, showed very low presenescence levels of p16 and wild-type pRb levels, a combination not previously identified in TCCs. In this TCC, immunoprecipitation studies revealed that pRb exists primarily in the hyperphosphorylated state, and the levels of E2F-1 were relatively low (Fig. 1B). Thus, p16 is present in this TCC in low quantities but appears functionally inactivated with regard to inhibiting pRb phosphorylation and cell proliferation.

Immunohistochemistry was also used as an additional method to assess pRb status in all of the TCCs (data not shown). pRb was localized to the nucleus in all nine TCCs that showed a signal on the Western blot. Thus, the antibody did not differentiate between the eight functionally wild-type pRbs and the one nonfunctional pRb identified (see above). Three TCCs, which have a mutant pRb and do not show the protein by Western analysis, did not show any staining by immunohistochemistry.

Next, Cdk4, Cdk6, and cyclin D1 levels were examined by Western analysis (data not shown). Several alterations were identified. These included a diminished signal for Cdk4 in TCC 97-1 and for Cdk6 in TCC 97-29. In all other TCCs, both Cdk4 and Cdk6 signals were comparable with the normal HUC wild-type control. Thus, no TCC in this series lost both Cdk4 and Cdk6, which might have served to inactivate the pRb pathway. The signal for cyclin D1 was diminished compared with normal in TCC 96-2. Cyclin D1 was not found amplified in any of the TCCs in the sample.

**p53 Pathway Alterations Identified in Invasive TCCs.** Next, *TP53* status was studied in all 12 invasive TCCs. Exons 2–11 of *TP53* were sequenced to investigate the underlying mechanisms for altered p53 levels. The data are summarized in Table 2. Briefly, no mutations in exons 2–11 were identified in four invasive TCCs. Mutations in hot spots (exons 5–8) were found in the other eight TCCs. The mutation in TCC 96-2 was a splice site mutation, resulting in protein loss (Fig. 2A and Table 2). All other TCCs with mutations show significantly elevated p53 levels (Fig. 2A). Two TCC biopsies taken from the same patient at different times including 97-18-I, a biopsy of primary invasive TCC, and TCC 97-21-M, a biopsy of a nodal metastasis, had the same mutation in exon 6, suggesting that metastasis occurred after the p53 mutation event. As reported elsewhere, clinical biopsies of human bladder cancers show major and minor clusters of mutational hot spots in exons 5 and 6 and in exons 7 and 8, respectively (64). Codon 8 mutations are in the helical motif of p53 that is involved in DNA binding, whereas codon 5 mutations are involved in structural integrity (64). As discussed below, all of the TCCs in this study with *TP53* mutations showed functional loss consistent with p53 inactivation.

Other components of the p53 pathway were analyzed next. Western analysis was used to examine the p21WAF1 status in both the TCCs with wild-type and mutant p53. Neither the TCC lacking p53 nor the TCCs in which *TP53* mutations were identified (see above) had detectable p21WAF1 levels (Fig. 2A), consistent with mutational inactivation of p53 function (also see below). This result is consistent with p53 functional inactivation by these mutations (Table 2). All of the TCCs with wild-type p53 showed an apparently wild-type level of p21WAF1, except for TCC 97-1, in which no p21WAF1 was ever detected. Thus, p21WAF1 was considered altered in this tumor, and it was classified as −p16/−p21WAF1.

To further investigate the mechanism for increased levels of both wild-type and mutant p53, the levels of mdm2 were next examined. These were elevated in all TCCs with mutant p53, except TCC 96-2 in which a splice mutation resulted in loss of p53 (Table 2). However, mdm2 levels were also elevated in two TCCs (TCC 94-10 and TCC 97-1) with slightly elevated wild-type p53 (Fig. 2A). Thus, there was not a correlation between p53 levels and mdm2 elevation in TCCs with wild-type *TP53*.

Finally, p14ARF expression was examined by Western analysis. Results reproducibly showed that p14ARF was present in all TCCs, except TCC 97-29 and TCC 97-15 (Fig. 2B). Notably, TCC 97-15 and
TCC 97-29 were two of three TCCs in which a p53 pathway alteration was yet to be identified. Mechanism(s) underlying p14ARF loss in these TCCs are described below in the section on INK4a inactivation.

In summary, a p53 pathway alteration was identified in 11 of 12 invasive TCCs, of which 8 had mutant TP53, 2 had p14ARF inactivation, and 1 lost p21WAF1 (Table 2; Fig. 2).

**Mechanisms of Inactivation of the 9p21 INK4a Locus Genes.**

Molecular analyses were performed to identify mechanisms of p16/CDKN2A and p14ARF/ARF inactivation in the TCCs. First p16/CDKN2A inactivation was examined. No p16 signal was detected in seven TCCs (Fig. 3A). Southern blot analysis (using a probe spanning exon 1α) was used to determine p16/CDKN2A promoter inactivation by methylation (65). Absence of any signal in two TCCs indicated the presence of homozygous deletion of exon 1α (Table 2). TCC 94-10 showed one methylated allele and one normal allele (Fig. 3A). Sequence analysis also revealed a mutation in TCC 94-10 (see below). Because protein was lacking in 94-10, it seems reasonable to hypothesize that one allele is methylated and one is mutated.

DNA sequencing of exons 1α and 2 of p16/CDKN2A was used to detect mutations that could account for the loss of p16 from these TCCs. Results showed that TCC 97-21-M had a frameshift mutation in exon 1α, codon 36. The mechanism of inactivation of the second allele was not identified. TCC 94-10 had a frameshift mutation in exon 2 that caused a premature termination signal. TCC 97-15 had a homozygous deletion of exon 2, as confirmed by PCR using primers spanning exon 2 of p16/CDKN2A (data not shown). In TCC 97-18-I and TCC 97-29, no mutations were detected in exon 1α and exon 2.

Northern analysis was performed to determine whether p16 mRNA was present in these lines. p16 mRNA was detected in TCC 97-18-I (which lacked p16 protein; Fig. 3C), suggesting there might be some posttranscriptional regulation in this line that would account for the loss of the protein. The absence of p16 mRNA in TCC 97-29 was

<table>
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<td>ex 8, cd 275</td>
</tr>
<tr>
<td>97-7</td>
<td>+</td>
<td>HD: ex 1α</td>
<td>+</td>
<td>HD: ex 2</td>
<td>+</td>
<td>ex 5, cd 128</td>
</tr>
<tr>
<td>96-1</td>
<td>+</td>
<td>HD: ex 1α</td>
<td>+</td>
<td>HD: ex 2</td>
<td>+</td>
<td>ex 5, cd 175</td>
</tr>
<tr>
<td>97-18</td>
<td>-</td>
<td>NI (protein down-regulated)</td>
<td>+</td>
<td>NI (mRNA absent)</td>
<td>+</td>
<td>ex 6, cd 220</td>
</tr>
<tr>
<td>97-21</td>
<td>-</td>
<td>FSM: ex 1α, cd 36</td>
<td>+</td>
<td>FSM: ex 2, cd 96</td>
<td>(does not affect p14ARF)</td>
<td></td>
</tr>
<tr>
<td>94-10</td>
<td>-</td>
<td>FSM: ex 2, cd 82; 1 allele methylated</td>
<td>+</td>
<td>FSM: ex 2, cd 96</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>97-1</td>
<td>-</td>
<td>HD: ex 1α</td>
<td>+</td>
<td>HD: ex 2</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>97-15</td>
<td>-</td>
<td>HD: ex 2</td>
<td>-</td>
<td>HD: ex 2</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>97-29</td>
<td>-</td>
<td>NI (mRNA absent)</td>
<td>-</td>
<td>NI (mRNA absent)</td>
<td>+/+</td>
<td>NA</td>
</tr>
</tbody>
</table>

* pHUC, presenescent proliferating HUC; sHUC, senescent HUC; NA, not applicable; ND, not done; NI, not identified; ex, exon; cd, codon; FSM, frameshift mutation; SM, splice mutation resulting in loss of protein; HD, homozygous deletion. +, +, and +/+ indicate protein levels compared with HUCs. TCCs 97-18-I and 97-21-M are invasive (I) and metastatic (M) samples from the same patient. The significance of the p53 mutations is discussed in the text.
consistent with p16 loss; however, a mechanism underlying the loss of mRNA in this line could not be identified. In summary, mechanisms for p16 inactivation were identified in six of seven TCCs that lost p16 (Table 2).

Loss of p14ARF was reproducibly demonstrated in two TCCs, TCC 97-15 and TCC 97-29, using Western analysis (Fig. 3C), but no mutations in exon 1β could be identified in either TCC by sequencing (Table 1). However, loss of p14ARF expression in TCC 97-15 could be explained by a homozygous deletion of exon 2 that is shared between p16/CDKN2A and ARF (described above). Northern analysis was performed to study ARF mRNA in the other TCC lacking p14ARF, i.e., TCC 97-29. HeLa and A375 cells were used as standard positive and negative controls, respectively (61). No signal for ARF mRNA was detected in TCC 97-29 (Fig. 3C). This latter finding is consistent with the lack of p14ARF by Western analysis in this TCC. In contrast, ARF mRNA was identified in representative TCCs in this study that showed protein by Western analysis, i.e., TCC 92-1 and TCC 97-18-I.

In summary, molecular analyses identified mechanisms by which p16 and p14ARF were inactivated in most of the TCCs analyzed in this study. The exception was TCC 97-29 in which a mechanism for the loss of mRNA (and subsequently protein) was not identified (Table 2).

Different Biological Phenotypes Associated with Different Combinations. Several biological phenotypes in response to radiation were studied in TCCs with different combinations of p53 and pRb pathway alterations. Induction of p53 and its downstream targets, mdm2 and p21WAF1, were examined after generation of 18 Gy gamma radiation-induced DNA damage in the four TCCs with wild-type TP53 and in three representative TCCs with mutant TP53. Unirradiated TCCs and normal HUCs were used as controls. Twenty-four h after irradiation, proteins were examined using Western blot analysis (Fig. 4). Briefly, p53 and mdm2 were elevated in TCCs with wild-type TP53 after radiation. However, induction of p21WAF1 was observed in only two TCCs, i.e., TCC 97-29 (Fig. 4) and TCC 97-15 (data not shown). One TCC with wild-type p53 did not contain detectable p21WAF1. Finally, one TCC with wild-type p53, TCC 94-10, contained p21WAF1 but did not show induction after radiation. This was the only tumor in which no p53 alteration had yet been identified. As expected, none of the TCCs with mutant p53 showed induction of and...
elevated levels of p53, mdm2, or p21 WAF1 in response to gamma radiation (Fig. 4).

Cell cycle arrest in response to gamma radiation was studied using flow cytometry. Flow cytometry results and bar graph analysis of data from representative TCCs with wild-type and mutant TP53 are shown (Fig. 5). As expected, TCCs with mutant TP53 failed to undergo cell cycle arrest. The two TCCs (TCC 94-10 and TCC 97-1) with wild-type p53 lacking a p21 WAF1 induction response after irradiation (as described above), also failed to undergo cell cycle arrest. However, two other TCCs, TCC 97-29 and TCC 97-15, with the combination of p16/p14 ARF that showed downstream induction of p21 WAF1, underwent cell cycle arrest as indicated by a reduction in the percentage of S-phase cells after radiation (Fig. 5). Thus, loss of p14 ARF in TCC 97-15 and in TCC 97-29 did not affect their ability to show p53 downstream responses or to undergo cell cycle arrest after radiation exposure.

DISCUSSION

Different combinations of genetic/epigenetic alterations inactivate the pRb and p53 tumor suppressor pathways (pRb pathway/p53 pathway) in invasive human bladder cancers. In this study, four different genetic/epigenetic combinations were identified: −pRb/−p53, −p16/−p53, −p16/p21 WAF1, and −p16/p14 ARF. Two of these combinations (−p16/p21 WAF1 and −p16/−p53) have not been reported previously. One invasive TCC with inactivated p16 did not fit any of these groups, because a p53 pathway alteration was not detected. Two TCCs had both wild-type p53 and p14 ARF, questioning the require-
ment for loss of either of these p53 pathway components for malignant transformation. We also present the first report in human bladder cancers correlating different p53 and pRb alterations with downstream response to gamma radiation.

This is the first study to our knowledge showing that both wild-type p53 and wild-type p14ARF can be expressed simultaneously in malignant tumors. Earlier models proposed that a negative feedback loop exists between p53 and p14ARF (49). Consistent with this model, it was reported that tumor cell lines that expressed wild-type p53 did not express p14ARF (49). In contrast, the data presented herein show that p14ARF and wild-type p53 can be present together in invasive human cancers. The difference may be accounted for by the use of early-stage cultures of tumor biopsies in the current study rather than established cell lines. It has been shown that p14ARF stabilizes both p53 and mdm2 by binding and inhibiting the mdm2-induced turnover of p53 (49). Our studies are somewhat consistent with this model. We showed that 10 of 12 TCCs that expressed p14ARF also had elevated levels of mdm2 and p53 compared with normal. However, two TCCs that did not express p14ARF showed comparatively lower elevations of p53 and mdm2 (Figs. 2A and 4). Thus, the correlation is not perfect in this study.

Our data differ from results obtained in a recent analysis of bladder cancer cell lines showing that bladder cancers with wild-type TP53 or TP53 alterations in exons 1–4 (which are required for transactivation) always show alterations in p16/CDKN2A and ARF (2). In fact, in the present study, TCC 97-1 and TCC 94-10, both with wild-type TP53, did not show any alterations in ARF at either the molecular or protein levels. p21WAF1 was absent in one of these tumors, but no p53 pathway alteration was detected in the other TCCs.

p21WAF1 is clearly a critical component of the downstream p53 pathway. Nonetheless, mutations in p21WAF1 are infrequently reported in cancers, including bladder tumors. However, in the small percentage of tumors that do harbor such mutations, no coexisting TP53 mutations have been identified (35), suggesting that when p21WAF1 mutations occur, they are powerful enough to inactivate the p53 pathway. In our panel, 1 of 12 invasive TCCs, TCC 97-1, failed to show any signal for p21WAF1 by Western blot analysis and failed to undergo cell cycle arrest after radiation exposure. Thus, we assume that p21WAF1 is altered in this one TCC. Notably, there was one invasive TCC in which the p53 pathway alteration was detected. In summary, our study shows that most, but not all, invasive TCCs contain an inactivating alteration in p53, p21WAF1, or p14ARF.

We reported previously that invasive TCCs invariably have a pRb pathway alteration by inactivation of either p16 or pRb. Consistent with this previous observation, inactivation of the pRb pathway was observed in all TCCs in the current study. Loss of p16 was the most common pRb pathway alteration identified. Inactivation of pRb was identified in 4 of 12 samples. Interestingly, one TCC with wild-type pRb in the present study showed significantly decreased p16, rather than absent p16, as did the other TCC with pRb loss. We did not identify a mechanism for this apparent p16 down-regulation, but we did demonstrate that these low presenescent levels of p16 were insufficient to block pRb phosphorylation or cell proliferation in this TCC. Thus, most studies, including our own, show that a pRb or p16 alteration in the pRb pathway is almost always present in invasive human cancers. Nevertheless, the possibility of cancers with neither alteration still exists. Mutations in other critical components of the pRb pathway, such as CCND1, CDK4, or CDK6, could provide alternate mechanisms for bypassing the p16-mediated G1 senescence checkpoint, as discussed in the “Introduction.” However, the relative importance of alterations in these other components of the pRb pathway and whether they can substitute in a small percentage of TCCs for a p16 or pRb alteration have not been determined.

As discussed above, much data support a model in which inactivation of the p9p21 p16/CDKN2A tumor suppressor gene plays a critical role in tumorigenesis in vivo and immortalization in vitro of human cells. However, the more recent identification of a second putative tumor suppressor gene at the INK4a locus, ARF, led to the hypothesis that p14ARF may be the more critical p9p21 tumor suppressor gene and/or that both genes may be frequently inactivated concomitantly. Indeed, recent studies demonstrate that p16 and p14ARF losses can occur concomitantly by a single alteration at the p9p21 INK4a locus. We observed this phenomenon in our TCC samples. For example, in TCC 97-15 (and possibly TCC 97-29), a single mutation at the INK4a locus targets p16 and p14ARF, altering both pRb and p53 pathways. However, such concomitant inactivation is clearly not obligatory, as has been proposed. Specifically in our study, loss of p16 but not p14ARF was observed in six of eight TCCs. Furthermore, our study shows that homozygous deletions can inactivate one INK4a gene but not the other. For example, TCC 97-1 and TCC 96-1 lost p16 because of homozygous deletion of exon 1α but expressed p14ARF. An even more powerful example in this regard is TCC 94-10. In this TCC, p16 was inactivated by two different mechanisms (promoter methylation and an exon 2 frameshift). Because there is no p16 in this tumor, it is reasonable to propose that each of these alterations is on a separate allele. Notably, neither the exon 2 frameshift mutation nor the p16/CDKN2A promoter methylation would affect p14ARF (65). p16/CDKN2A- and ARF-specific mRNA were absent in TCC 97-29. However, no mutations were identified in exons 1α, 1β, or 2 of either p16/CDKN2A or ARF. Thus, a mechanism for the loss of p16 and p14ARF mRNAs in this TCC was not identified. However, such loss could be attributable to several other mechanisms, such as a mutation in the poly(A) site. Thus, these results elucidate both the importance and complexity of the INK4a locus in human bladder cancer pathogenesis.

Although most TCCs appear to have some alteration in both the pRb and p53 pathways (Ref. 2 and our data), the impact of the different possible combinations on tumor phenotype in vivo (Table 1) and biological phenotypes in vitro (as discussed below) could differ and has not been reported. Many studies have hypothesized that p53 status is the most useful prognostic indicator to assess potential biological phenotypes in vivo, including tumor recurrence, progression and metastases, and patient survival. Significant data show that assessment of pRb status can alone serve as an excellent prognostic indicator (66–68). Finally, more recent studies suggest that alterations in pRb and p53 exert a cooperative, synergistic effect on survival rates and bladder cancer progression, and both markers should be used in screening assessments (28). Interestingly, our data show that all TCCs with altered pRb have altered p53 (Table 1). The combination of mutant pRb and wild-type p53 was not observed by us, nor to our knowledge has it been reported by others for human bladder cancer. Thus, our data support studies showing that TCCs with altered pRb have a poor prognosis but attribute this to the combination of altered p53 and pRb. Supporting this hypothesis in another human epithelial cell type are data showing that patients with early-stage, non-small cell lung carcinoma with altered pRb and mutant p53 have relatively poor clinical prognoses (69). Furthermore, mice mutant for pRb and p53 have reduced viability and exhibit increased tumor burden and metastasis (70). In contrast to the TCCs with p53 alterations, TCCs with wild-type p53 and an alteration of another p53 pathway component (p14ARF or p21WAF1) showed more favorable prognosis, even in our small sample, consistent with the above clinical observations. These data support the importance of a large clinical study to test the statistical significance of this hypothesis.

We also examined some underlying mechanisms that might help to explain the different biological phenotypes of the TCCs with different
pRb and p53 pathway alterations. Specifically, we studied the response of the 12 TCCs in our study to the DNA-damaging effects of gamma radiation. Our control normal HUCs showed downstream induction of p53, p21WAF1, and G1 cell cycle arrest in response to radiation damage. As expected, none of these p53-dependent responses were seen in TCCs with mutant p53 (Table 2). Loss of the cell cycle arrest component was also seen in TCC 97-1 in which p21WAF1 was never detected. Cell cycle arrest repeatedly did not occur in TCC 94-10 in which p53 and mdm2, but not p21WAF1, were elevated after radiation exposure. The reason for this result is being investigated. On the basis of many studies, loss of the cell cycle arrest response to DNA damage with failure to repair DNA damage and the potential for the accumulation of genetic alterations needed for progression. Consistent with that model, even in this small set of 12 TCCs, 2 that retained the p53 downstream responses stand out as the least aggressive. The apoptotic responses and cell kill of this group of tumors after exposure to gamma radiation would be relevant and of interest to planning chemotherapy and radiation therapy.

A significant new finding in this study is that the TCCs with wild-type p53 and pRb and with the genotype of p16−/−p14ARF were phenotypically similar to normal HUCs in their downstream induction of p53, mdm2, p21WAF1, and G1 cell cycle arrest in response to radiation damage in vitro. Thus, there was an alteration in the p53 pathway in these TCCs, but the pathway was not inactivated by the criteria used in our study. However, wild-type TP53 alone was not always sufficient to predict downstream p53 functions of cell cycle arrest in response to DNA damage. One TCC, TCC 94-10 with wild-type p53, failed to show a p21WAF1 elevation after radiation. Studies on breast cancer show that overexpression of mdm2 increases the de novo resistance of cells to cytotoxic agents like doxorubicin. Therefore, mdm2 level is considered to be a novel marker for predicting lack of response to doxorubicin treatment in breast cancer patients (42). A second tumor with wild-type p53, TCC 97-1, in which p21WAF1 was absent showed p53 and mdm2 induction and also failed to undergo cell cycle arrest in response to radiation. Thus, in these latter two tumors, some of the protective functions of p53 are lost.

In summary, our data contribute to the understanding of the genetic requirements for bladder cancer pathogenesis. We have identified two pRb and p53 pathway combinations not yet reported for human bladder cancers, i.e., p16−/−p14ARF and p16−/−p53. We showed that some TCCs have both wild-type p53 and wild-type p14ARF. Thus, we show that all TCCs do not have either a p53 or p14ARF alteration, as had been hypothesized previously for some tumor types. We identified a TCC with wild-type p53 but with p21WAF1 loss in which downstream responses to radiation were lost. We show that in one TCC, a common p53 pathway alteration (i.e., p53, p21WAF1, or p14ARF) was not present. Finally, we show for the first time in TCCs that invasive cancers with p53 pathway inactivation by p14ARF alteration retain downstream responses to gamma radiation. Thus, although p14ARF loss apparently fulfills the requirements for a p53 pathway alteration, the components of the p53 damage response pathway are intact. These new findings have potential impact on the use of pRb and p53 pathway alterations for diagnosis, prognosis, and treatment of human bladder cancers.

REFERENCES

Different Combinations of Genetic/Epigenetic Alterations Inactivate the p53 and pRb Pathways in Invasive Human Bladder Cancers

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