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Different Combinations of Genetic/Epigenetic Alterations Inactivate the p53 and pRb Pathways in Invasive Human Bladder Cancers[1]

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ABSTRACT

Inactivation of both the pRb (pRb-cyclin D1/cyclin-dependent kinase 4/6-p16) and p53 (p53-p21^{WAF1}-p14^{ARF}) 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/−p21^{WAF1} (one TCC), and −p16/−p14^{ARF} (two TCCs). These groups include two new combinations (*i.e.*, −p16/−p53 and −p16/−p21^{WAF1}) 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/−p21^{WAF1} and −pRb/−p14^{ARF} were not observed. Only two of eight TCCs with altered p16 had concomitant p14^{ARF} loss, demonstrating that simultaneous inactivation of these two 9p21*INK4a* 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, p21^{WAF1}, or mdm2 or by cell cycle arrest. Only two of four TCCs with wild-type p53 and wild-type pRb (the combination of −p16/−p14^{ARF}) 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/−p21^{WAF1} and one TCC with −p16) failed to show cell cycle arrest in response to radiation. This was attributed to the absence of p21^{WAF1} 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/Cdk[3]4/6-p16) and p53 (p53-p21^{WAF1}-p14^{ARF}) 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 (1). 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* (2). 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 G_1 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*),

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; ARF, alternate reading frame; TCC, transitional cell carcinoma; IHC, immunohistochemistry; HUC, human uroepithelial cell; BrdUrd, bromodeoxyuridine.

DNA repair (*GADD45*), cell cycle arrest ($p21^{WAF1}$/*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 $p21^{WAF1}$/*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 NH_2 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 p16/*CDKN2A* 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 $p19^{ARF}$ in mice (the human homologue is $p14^{ARF}$), 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 $p19^{ARF}$ and $p14^{ARF}$ have the ability to arrest cell proliferation at both G_1 and G_2-M in a p53-dependent manner involving the up-regulation of downstream target $p21^{WAF1}$/*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 $p14^{ARF}$ 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 $p21^{WAF1}$ levels after ionizing or UV irradiation, thus exhibiting an intact p53 checkpoint pathway. Because mouse embryo fibroblast lines (MEFs) that retained $p19^{ARF}$ always sustained mutations in *TP53* and *vice versa*, these studies suggest that the functional losses of either p53 or $p19^{ARF}$ are mutually exclusive events for immortalization (50). Recent evidence also showed that E2F1 could induce $p14^{ARF}$ expression by activating the *ARF* promoter (51, 52). $p14^{ARF}$ stabilizes p53 by binding and promoting the degradation of another oncoprotein, mdm2 (53, 54). $p19^{ARF}$ 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 $p14^{ARF}$ (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, $p14^{ARF}$, or $p21^{WAF1}$) 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 $p14^{ARF}$ can be concomitantly expressed with wild-type p53 in invasive TCCs. Thus, we show that a $p14^{ARF}$ 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, $p21^{WAF1}$, or $p14^{ARF}$. Finally, we show that because of their unique genetic organization, inactivation of both p16 and $p14^{ARF}$ 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 T_1 to T_4 (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, $p21^{WAF1}$, and mdm2. Briefly, $2–5 \times 10^6$ cells growing in late log phage were lysed in ECB buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 100 mM NaF, 0.2 mM Na_2VO_4, 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), $p21^{WAF1}$ (Ab-1), and α-tubulin (Ab-1) were obtained from Oncogene Research Products. Monoclonal antibody to pRb (14001A) was obtained from PharMingen, and monoclonal antibodies to mdm2 (SMP14) and cyclin D1 (HD-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 $p14^{ARF}$ was used (61). Bound antibody was detected using enhanced chemiluminescence (Pierce). Equal loading was verified using an antibody to α-tubulin.

For $p14^{ARF}$ detection, cells were lysed in RIPA buffer [1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, with 10 μl/ml each of phenylmethylsulfonyl fluoride (10 mg/ml) and Na_2VO_4 (100 mM) and 30 μl/ml of aprotinin (10 mg/ml)], and 75 μg protein/lane was run on a 15% SDS-polyacrylamide gel.

For pRb-E2F1 immunoprecipitation studies, $\sim 10^7$ 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 1:1000 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 p14 ^{ARF}/*ARF* deletions or altered methylation in the TCCs. DNA was extracted from early-passage cells using TNE buffer [0.5 M Tris-Cl, 0.1M NaCl, and 20 mM EDTA with 100–200 μg/ml of proteinase K (Sigma)], 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 *Eco*RI and methylation-sensitive *Sac*II 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 islands of the promoter and exon 1α (19) in Rapid-hyb buffer (Amersham Life Sciences). The probe was labeled with [γ-^{32}P]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 p14^{ARF} 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* DNA was used to detect p16 mRNA. The p14^{ARF} 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 p14^{ARF}.

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 ^{137}Cs 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 1α and 2 of p16/*CDKN2A* and exons 1β and 2 of p14 ^{ARF} were PCR amplified, and the PCR products were cloned into a plasmid using the TOPO TA cloning kit (Invitrogen) according to 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 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 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, 35 cycles of 45 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 60 s at 94°C; 30 s each at 68°C, 65°C, 62°C, 60°C, and 58°C; 60 s at 72°C; and a final elongation step of 5 min at 72°C.

Initial identification of TCCs with mutations in exons 5–9 of *TP53* was done by cleavage fragment length polymorphism analyses using the protocol of Third Wave Technologies (Madison, WI.). *TP53* exons 2–11 were then sequenced at early passage in culture ($P < 5$) in all 12 TCCs used in this study by Oncor, now Genelogic (Gaithersburg, MD), according to their published protocol.

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. 1*A*). In contrast, pRb was undetectable in three TCCs that showed elevated levels of p16 (Fig. 1*A*). These latter p16 levels were comparable with the p16 levels seen at senescence in normal HUCs (Fig. 1*A*). 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. 1*A*). 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 1 *Association of TCC phenotypes in vivo and in vitro with combinations of pRb and p53 pathway alterations*

	Tumor phenotypes *in vivo*[a]				Radiation response *in vitro*[b]		
TCC	Stage and grade	No. of recurrences	Progression at recurrence	Metastasis at recurrence	Downstream p53 response	Cell cycle arrest	Pathway combinations
92-1	T_4 III	Terminal[c]	NA	NA	−	−	−pRb/−p53
96-2	T_3 II/III	Terminal	NA	NA	−	−	
97-6	T_3 III	Terminal	NA	NA	−	−	
97-24	T_3 III	Terminal	NA	NA	−	−	
97-7[d]	T_1 II/III	2, Terminal	+ (T_1 II, T_4 III)	+	−	−	−p16/−p53
96-1	T_2 II/III	2	− (T_2 II, T_2 II)	−	−	−	
97-18-I	T_2 III	1 (97-21-M)	+ (T_4 III)	+	−	−	
97-21-M	T_4 III	Terminal	NA	NA	−	−	
94-10	T_3 II/III	2	− (T_a I, T_1 II)	−	+[e]	−	−p16
97-1	T_2 I/II	2	− (T_2 II/III, T_2 I)	−	+[e]	−	−p16/−p21
97-15	T_1 III	1	− (T_1 II)	−	+	+	−p16/−p14^{ARF}
97-29	T_1 I/II	1	− (T_a I)	−	+	+	

[a] The stage and grade of the primary TCCs are reported, followed by patients' subsequent phenotypes of recurrence, progression, and metastasis.
[b] The *in vitro* radiation response was determined using the early-passage TCC cultures from the primary tumors.
[c] Terminal refers to patients who died of metastatic bladder cancer. NA, not applicable.
[d] This TCC has low levels of p16 but was classified as −p16 based on functional studies (see text).
[e] Two TCCs in which p53 and mdm2, but not p21, were induced.

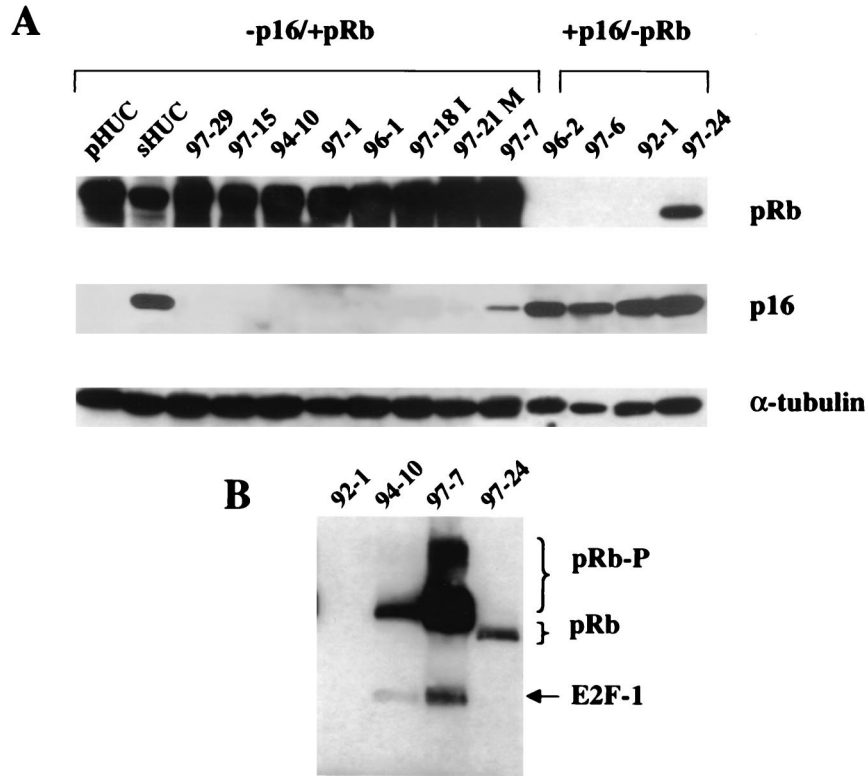

Fig. 1. Western blot analysis of p16 and pRb. In *A*, pRb was undetectable in three TCCs, whereas p16 was lost in seven TCCs. Note that TCC 97-24 and TCC 97-7 have both proteins present. α-Tubulin was used as a loading control. *B*, immunoprecipitation and Western analysis to show the functional status of pRb in TCC 97-7 and TCC 97-24. TCC 92-1 (mutant pRb) and TCC 94-10 (wild-type pRb) were used as controls. pRb is hypophosphorylated with no associated E2F-1 in TCC 97-24, suggesting that it is mutant. pRb is predominantly in the hyperphosphorylated form (*pRb-P*) TCC 97-7, suggesting that the low levels of p16 shown in Fig. 1*A* are not inhibitory.

also showed that pRb did not bind E2F-1 (Fig. 1*B*). 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. 1*B*). 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. 2*A* and Table 2). All other TCCs with mutations show significantly elevated p53 levels (Fig. 2*A*). 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 $p21^{WAF1}$ 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 $p21^{WAF1}$ levels (Fig. 2*A*), 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 $p21^{WAF1}$, except for TCC 97-1, in which no $p21^{WAF1}$ was ever detected. Thus, $p21^{WAF1}$ was considered altered in this tumor, and it was classified as $-p16/-p21^{WAF1}$.

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. 2*A*). Thus, there was not a correlation between p53 levels and mdm2 elevation in TCCs with wild-type *TP53*.

Finally, $p14^{ARF}$ expression was examined by Western analysis. Results reproducibly showed that $p14^{ARF}$ was present in all TCCs, except TCC 97-29 and TCC 97-15 (Fig. 2*B*). Notably, TCC 97-15 and

Table 2 *Mechanisms of inactivation of p16/CDKN2A, p14^{ARF}/ARF, and p53/TP53 in invasive TCCs*

	p16/*CDKN2A*		p14^{ARF}/*ARF*		p53/*TP53*	
Samples	Protein status	Mechanism(s) of alterations	Protein status	Mechanism(s) of alterations	Protein status	Alterations
pHUC[a]	−	NA	+	NA	+	NA
sHUC	+++	NA	ND	NA	+	NA
92-1	+++	NA	+	NA	+++	ex 5, cd 158; ex 6, cd 162; ex 7, cd 228; ex 8, cd 280, and cd 294
96-2	+++	NA	+	NA	−	ex 7, cd 261 (SM)
97-6	+++	NA	+	NA	++	ex 5, cd 162
97-24	+++	NA	+	NA	+++	ex 8, cd 275
97-7	+	NI (protein down-regulated)	+	NA	+++	ex 5, cd 128
96-1	−	HD: ex 1α	+	NA	+++	ex 5, cd 175
97-18 I	−	NI (mRNA present)	+	NA	+++	ex 6, cd 220
97-21 M	−	FSM: ex 1α, cd 36	+	NA	+++	ex 6, cd 220
94-10	−	FSM: ex 2, cd 82; 1 allele methylated	+	FSM: ex 2, cd 96 (does not affect p14^{ARF})	++	NA
97-1	−	HD: ex 1α	+	NA	++	NA
97-15	−	HD: ex 2	−	HD: ex 2	++	NA
97-29	−	NI (mRNA absent)	−	NI (mRNA absent)	+/++	NA

[a] 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 +++, 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.

TCC 97-29 were two of three TCCs in which a p53 pathway alteration was yet to be identified. Mechanism(s) underlying p14^{ARF} 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 p14^{ARF} inactivation, and 1 lost p21^{WAF1} (Table 2; Fig. 2).

Mechanisms of Inactivation of the 9p21 *INK4a* Locus Genes. Molecular analyses were performed to identify mechanisms of p16/*CDKN2A* and p14^{ARF}/*ARF* inactivation in the TCCs. First p16/*CDKN2A* inactivation was examined. No p16 signal was detected in seven TCCs (Fig. 3*A*). 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. 3*A*). 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. 3*C*), 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

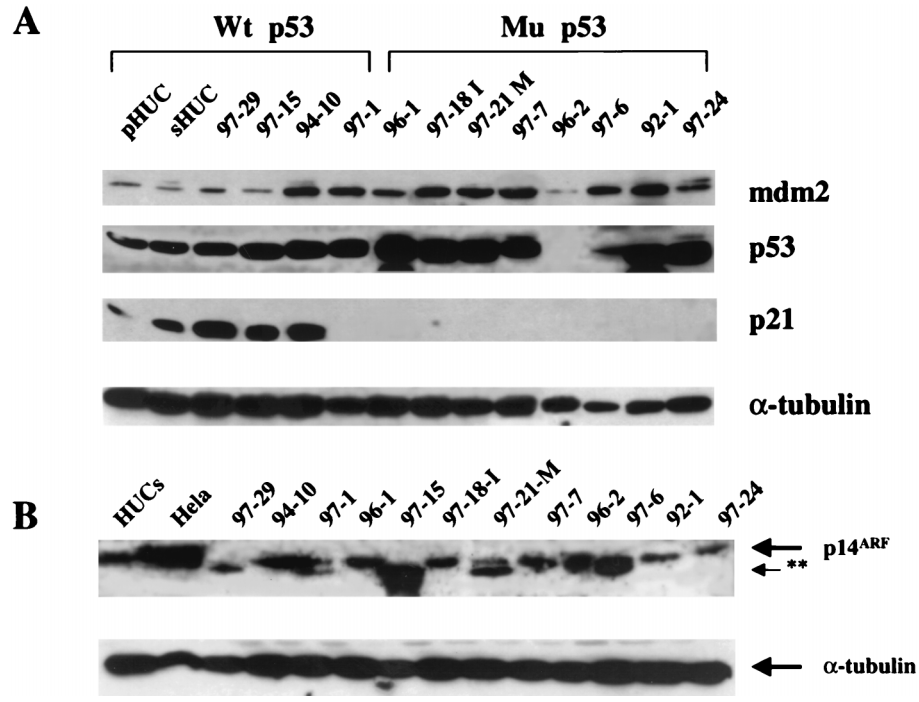

Fig. 2. Analysis of p53 pathway alterations in TCCs. See Table 1 for *TP53* mutation data. In *A*, Western blot analysis of p53, p21^{WAF1}, and mdm2 is shown. p53 levels were consistently elevated in eight TCCs, absent in one (TCC 96-2), and slightly elevated in four TCCs compared with normal proliferating HUCs (*pHUC*) and senescent HUCs (*sHUC*). p21^{WAF1} was detected in three of four TCCs with wild-type (*Wt*) *TP53* but not in TCC 97-1. mdm2 was consistently elevated in all TCCs with mutant (*Mut*) p53, except TCC 96-2, in which no p53 was detected. mdm2 was also elevated in two of four TCCs with wild-type p53. In *B*, Western blot analysis of p14^{ARF} in TCCs is shown. p14^{ARF} was detected in HUCs as well as all TCCs, except TCC 97-29 and TCC 97-15. HeLa was used as a positive control. The upper band corresponds to p14^{ARF}. The lower band (*asterisk*) is a nonspecific band always seen with this antibody. α-Tubulin was used as a loading control in both experiments.

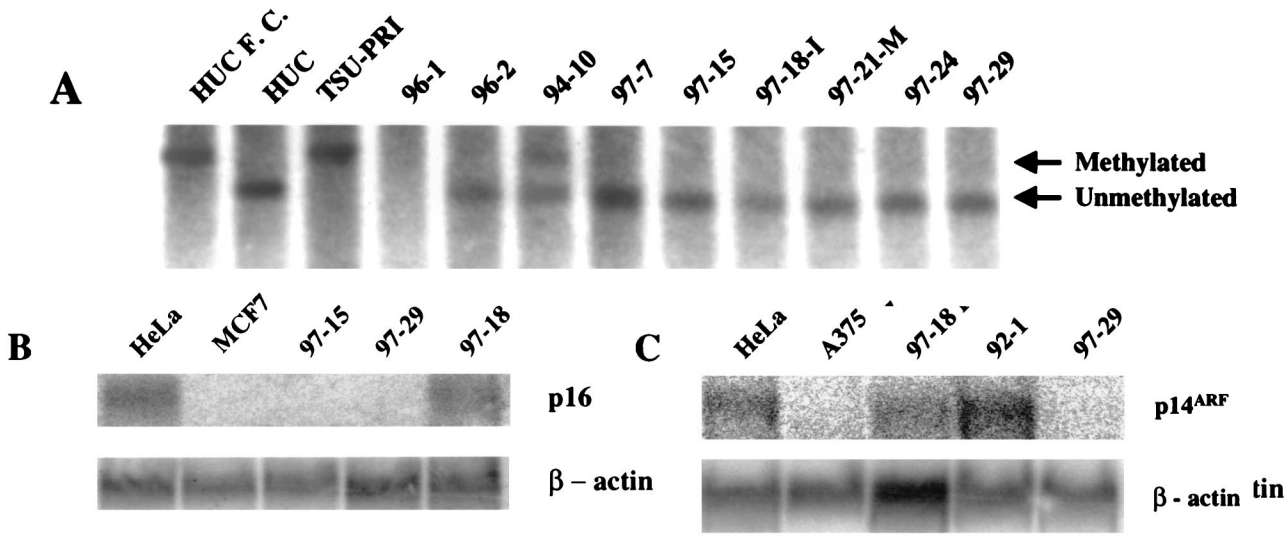

Fig. 3. Analysis of alterations in the *INK4a* locus in TCCs. *A*, Southern blot analysis for p16/*CDKN2A* methylation is shown. TCCs were digested with *Eco*RI and *Sac*II restriction enzymes. One HUC sample was digested with *Eco*RI only and served as the flanking cut (*F. C.*) control. TSU-PRI, a prostate cancer cell line in which both p16/*CDKN2A* alleles are methylated, served as the positive control. TCC 96-1 shows a homozygous deletion for exon 1α, and TCC 94-10 shows one allele methylated and one unmethylated (see Table 2). No methylated alleles were observed in any of the other TCCs in this study. *B*, Northern blot analysis for p16 mRNA in TCCs is shown. HeLa and MCF7 were used as positive and negative controls, respectively. p16 mRNA was detected only in TCC 97-18. Sequencing was done for p16/*CDKN2A* to account for loss of p16 in TCC 97-15 and TCC 97-29 as discussed in the text. *C*, Northern blot analysis for p14*ARF* mRNA is shown. $p14^{ARF}$ was detected in TCC 92-1 and TCC 97-18-I but not in TCC 97-29. HeLa and A375 were used as positive and negative controls, respectively.

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 $p14^{ARF}$ was reproducibly demonstrated in two TCCs, TCC 97-15 and TCC 97-29, using Western analysis (Fig. 3*C*), but no mutations in exon 1β could be identified in either TCC by sequencing (Table 1). However, loss of $p14^{ARF}$ 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 $p14^{ARF}$, *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. 3*C*). This latter finding is consistent with the lack of $p14^{ARF}$ 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 $p14^{ARF}$ 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 $p21^{WAF1}$, 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 $p21^{WAF1}$ 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 $p21^{WAF1}$. Finally, one TCC with wild-type p53, TCC 94-10, contained $p21^{WAF1}$ 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

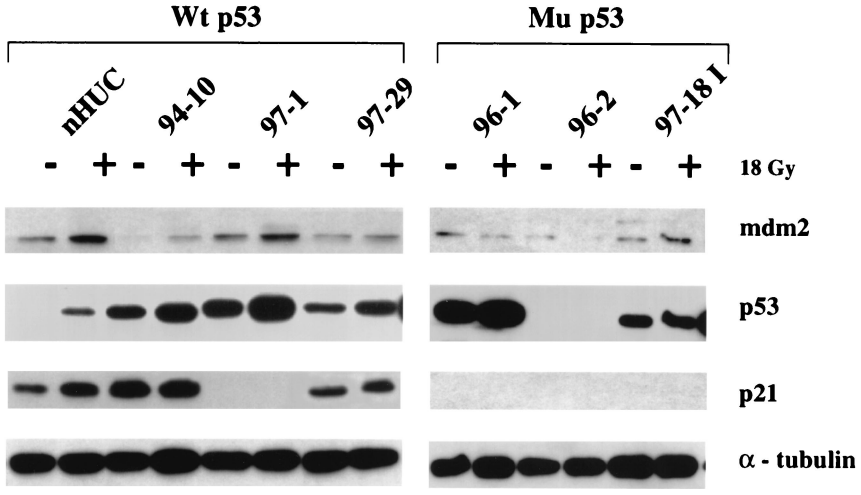

Fig. 4. Induction of p53, $p21^{WAF1}$, and mdm2 in response to gamma radiation. Logarithmically growing cells from representative samples of TCCs were exposed to 18 Gy gamma radiation, and total cell lysates were collected from irradiated and unirradiated TCCs and used for Western analysis. Induction of p53 and mdm2 was observed in normal HUCs and all TCCs with wild-type (*Wt*) *TP53* in response to gamma radiation but not in TCCs with mutant (*Mu*) p53. Note that p53 induction in TCC 96-1 was not seen in three other blots. However, p53, p12, and mdm2 induction (albeit low) in TCC 97-29 was seen in three other blots. α-Tubulin was used as a loading control.

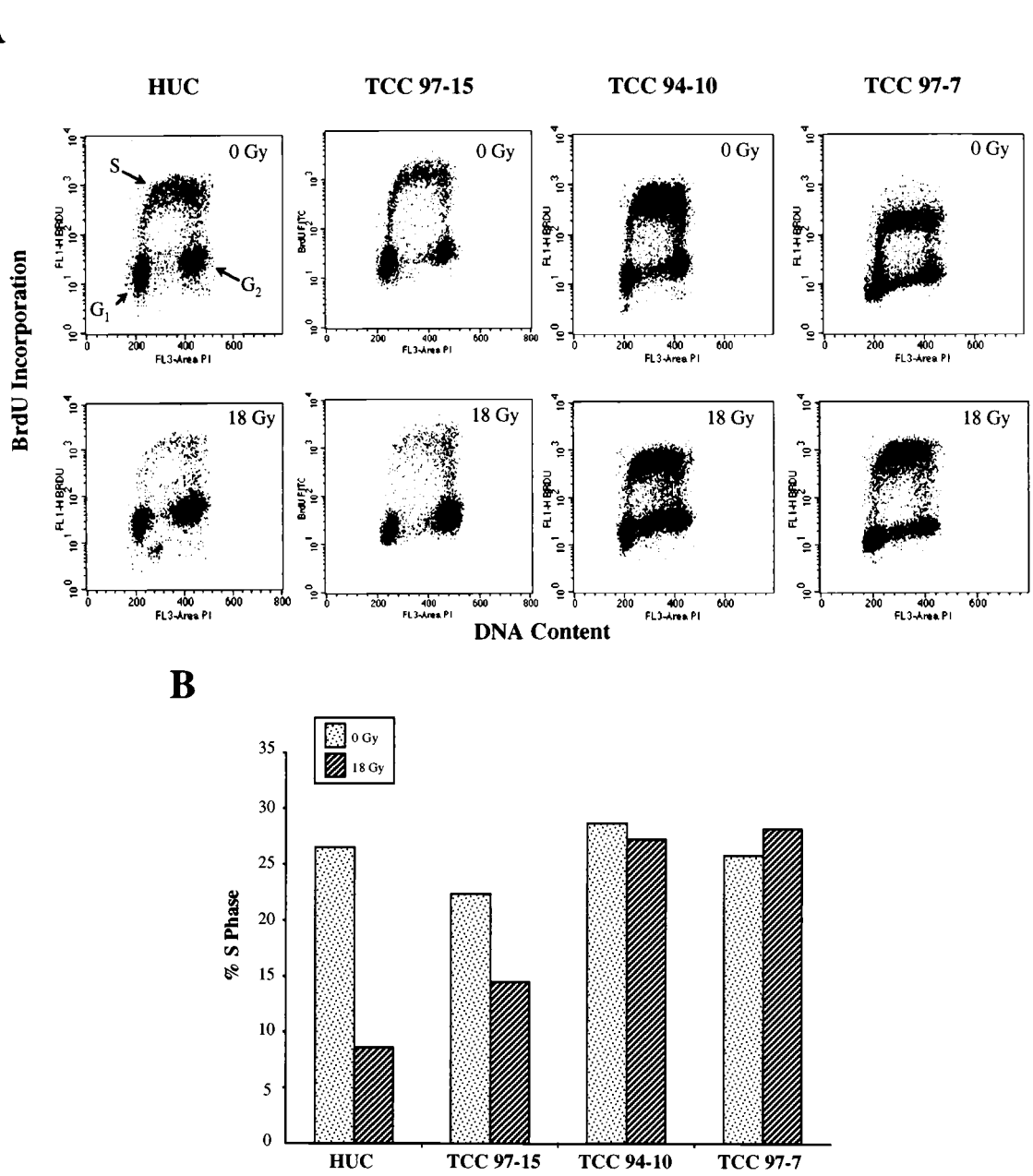

Fig. 5. Cell cycle arrest in response to gamma radiation. *A*, representative dot blots from three independent flow cytometric analyses of normal control HUCs and TCCs with wild-type (example shown is TCC 9-15) or mutant *TP53* (example shown is TCC 97-7). Cells were irradiated with 18 Gy gamma or 0 Gy (controls). HUCs and TCC 97-15 (with wild-type *TP53*) underwent cell cycle arrest, whereas TCC 94-10 (with no p53 alteration) and TCC 97-7 (with mutant *TP53*) failed to do so. BrdUrd indicates DNA synthesis. *B*, analyses of cell cycle response data from the above samples.

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 $p14^{ARF}$ can be expressed simultaneously in malignant tumors. Earlier models proposed that a negative feedback loop exists between p53 and $p14^{ARF}$ (49). Consistent with this model, it was reported that tumor cell lines that expressed wild-type p53 did not express $p14^{ARF}$ (49). In contrast, the data presented herein show that $p14^{ARF}$ and wild-type p53 can be present together in invasive human cancers. The difference may be accounted for by the use of early-passage cultures of tumor biopsies in the current study rather than established cell lines. It has been shown that $p14^{ARF}$ 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 $p14^{ARF}$ also had elevated levels of mdm2 and p53 compared with normal. However, two TCCs that did not express $p14^{ARF}$ showed comparatively lower elevations of p53 and mdm2 (Figs. 2*A* 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. $p21^{WAF1}$ was absent in one of these tumors, but no p53 pathway alteration was detected in the other TCCs.

$p21^{WAF1}$ is clearly a critical component of the downstream p53 pathway. Nonetheless, mutations in $p21^{WAF1}$ 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 $p21^{WAF1}$ 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 $p21^{WAF1}$ by Western blot analysis and failed to undergo cell cycle arrest after radiation exposure. Thus, we assume that $p21^{WAF1}$ is altered in this one TCC. Notably, there was one invasive TCC in which no p53 pathway alteration was detected. In summary, our study shows that most, but not all, invasive TCCs contain an inactivating alteration in p53, $p21^{WAF1}$, or $p14^{ARF}$.

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 G_1 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 9p21 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 $p14^{ARF}$ may be the more critical 9p21 tumor suppressor gene and/or that both genes may be frequently inactivated concomitantly. Indeed, recent studies demonstrate that p16 and $p14^{ARFl}$ losses can occur concomitantly by a single alteration at the 9p21 *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 $p14^{ARF}$, 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 $p14^{ARF}$ 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 $p14^{ARF}$. 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 $p14^{ARF}$ (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 $p14^{ARF}$ 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 ($p14^{ARF}$ or $p21^{WAF1}$) 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, $p21^{WAF1}$, and G_1 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 $p21^{WAF1}$ was never detected. Cell cycle arrest repeatedly did not occur in TCC 94-10 in which p53 and mdm2, but not $p21^{WAF1}$, 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 is associated 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 $-p16/-p14^{ARF}$ were phenotypically similar to normal HUCs in their downstream induction of p53, mdm2, $p21^{WAF1}$, and G_1 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 $p21^{WAF1}$ 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 $p21^{WAF1}$ 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.*, $-p16/-p21^{WAF1}$ and $-p16/-p53$. We showed that some TCCs have both wild-type p53 and wild-type $p14^{ARF}$. Thus, we show that all TCCs do not have either a p53 or $p14^{ARF}$ alteration, as had been hypothesized previously for some tumor types. We identified a TCC with wild-type p53 but with $p21^{WAF1}$ loss in which downstream responses to radiation were lost. We show that in one TCC, a common p53 pathway alteration (*i.e.*, p53, $p21^{WAF1}$, or $p14^{ARF}$) was not present. Finally, we show for the first time in TCCs that invasive cancers with p53 pathway inactivation by $p14^{ARF}$ alteration retain downstream responses to gamma radiation. Thus, although $p14^{ARF}$ loss apparently fulfils 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

1. Yeager, T. R., DeVries, S., Jarrard, D. F., Chinghai, K., Nakada, S. Y., Moon, T. D., and Bruskewitz, R. Overcoming cellular senescence in human cancer pathogenesis. Genes Dev., *12:* 163–174, 1998.
2. Markl, I. D., and Jones, P. A. Presence and location of TP53 mutation determines pattern of CDKN2A/ARF pathway inactivation in bladder cancer. Cancer Res., *58:* 5348–5353, 1998.
3. Fritsche, M., Haessler, C., and Brandner, G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene, *8:* 307–318, 1993.
4. Kaufmann, W. K., and Kaufmann, D. G. Cell cycle control. DNA repair and initiation of carcinogenesis. FASEB J., *7:* 1188–1191, 1993.
5. Lowe, S. W., and Ruley, H. E. Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev., *7:* 535–545, 1993.
6. Caelles, C., Helmberg, A., and Karin, M. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature (Lond.), *370:* 220–223, 1994.
7. Smith, M. L., Chen, I. T., Zhan, Q., O'Connor, P. M., and Fornace, A. J., Jr. Involvement of the p53 tumor suppressor in repair of U.V.-type DNA damage. Oncogene, *10:* 1053–1059, 1995.
8. Ruas, M., and Peters, G. The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim. Biophys. Acta, *1378:* 115–177, 1998.
9. Cairns, P., Mao, L., Merlo, A., Lee, D. J., Schwab, D., Eby, Y., Tokino, K., van der Riet, P., Blaugrund, J. E., and Sidransky, D. Rates of p16 (MTS1) mutations in primary tumors with 9p loss. Science (Washington DC), *265:* 415–417,1994.
10. Cairns, P., Polascik, T. J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., *et al.* Frequency of homozygous deletion at p16/CDKN2 in primary human tumors. Nat. Genet., *11:* 210–212, 1995.
11. Stadler, W. M., Sherman, J., Bohlander, S. K., Roulston, D., Dreyling, M., Rukstalis, D., and Olopade, O. I. Homozygous deletions within chromosomal bands 9p21-22 in bladder cancer. Cancer Res., *54:* 2060–2063, 1994.
12. Stadler, W. M., and Olopade, O. I. The 9p21 region in bladder cancer cell lines: large homozygous deletion inactivate the *CDKN2A*, *CDKN2B* and *MTAP* genes. Urol. Res., *24:* 239–244, 1996.
13. Williamson, M. P., Elder, P. A., Shaw, M. E., Devlin, J., and Knowles, M. A. p16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. Hum. Mol. Genet., *4:* 1569–1577, 1995.
14. Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S., and Nakamura, Y. Frequent somatic mutation of the *MTS1/CDK4I* (*multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor*) gene in esophageal squamous cell carcinoma. Cancer Res., *54:* 3396–3397, 1994.
15. Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. Deletions of the *cyclin-dependent kinase-4 inhibitor* gene in multiple human cancers. Nature (Lond.), *368:* 753–756, 1994.
16. Liu, Q., Neuhausen, S., McClure, M., Frye, C., Weaver-Feldhaus, J., Gruis, N. A., Eddington, K., Allalunis-Turner, M. J., Skolnick, M. H., Fujimura, F. K., *et al.* *CDKN2* (*MTS1*) tumor suppressor gene mutations in human tumor cell lines. Oncogene, *10:* 1061–1067, 1995.
17. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones, P. A. Methylation of the 5′ CpG island of the *p16/CDKN2* tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.
18. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res., *55:* 4525–4530, 1997.
19. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.
20. Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. A cell cycle regulator potentially involved in genesis of many tumor types. Science (Washington DC), *264:* 436–440, 1994.
21. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic *ras* provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell, *88:* 593–602, 1997.
22. Reznikoff, C. A., Yeager, T. R., Belair, C. D., Savelieva, E., Puthenveettil, J. A., and Stadler, W. M. Elevated p16 at senescence and loss of p16 at immortalization in human papillomavirus 16 E6, but not E7, transformed human uroepithelial cells. Cancer Res., *56:* 2886–2890, 1996.
23. Jarrard, D. F., Sarkar, S., Shi, Y., Magrane, G., Kinoshita, H., Yeager, T. R., Nassif, N., Meisner, L., Waldman, F. M., and Reznikoff, C. A. p16/pRb alterations are required for overcoming senescence in prostate epithelial cells. Cancer Res., *59:* 2957–2964, 1999.
24. Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. Regulation of p16/CDKN2 expression and its implications for cell immortalization and senescence. Mol. Cell. Biol., *16:* 859–867, 1996.
25. Icorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Proc. Natl. Acad. Sci. USA, *93:* 13742–13747, 1996.
26. Ishikawa, J., Xu, H. J., Hu, S. X., Yandell, D. W., Maeda, S., Kamidono, S., Benedict, W. F., and Takahashi, R. Inactivation of the retinoblastoma gene in human bladder and renal cell carcinomas. Cancer Res., *51:* 5736–5743, 1991.
27. Xu, H. J., Cairns, P., Hu, S. X., Knowles, M. A., and Benedict, W. F. Loss of RB protein expression in primary bladder cancer correlates with loss of heterozygosity at the *RB* locus and tumor progression. Int. J. Cancer, *53:* 781–784, 1998.
28. Cordon-Cardo, C., Zhang, Z. F., Dalbagni, G., Drobnjak, M., Charytonowicz, E., Hu, S. X., Xu, H. J., Reuter, V. E., and Benedict, W. F. Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors. Cancer Res., *57:* 1217–1221, 1997.
29. Shin, K. Y., Kong, G., Kim, W. S., Lee, T. Y., Woo, Y. N., and Lee, J. D. Overexpression of cyclin D1 correlates with early recurrence in superficial bladder cancers. Br. J. Cancer, *75:* 1788–1792, 1997.

30. Dickson, C., Fantl, V., Gillett, C., Brookes, S., Bartek, J., Smith, R., Fisher, C., Barnes, D., and Peters, G. Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. Cancer Lett., *90:* 43–50, 1995.
31. Hinds, P. W., Dowdy, S. F., Eaton, E. N., Arnold, A., and Weinberg, R. A. Function of a human *cyclin* gene as an oncogene. Proc. Natl. Acad. Sci. USA, *91:* 709–713, 1994.
32. Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H., and Beach, D. A p16INK4a insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science (Washington DC), *269:* 1281–1284, 1995.
33. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. p53 mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
34. Levine, A. J., Momand, J., and Finlay, C. A. The *p53* tumor suppressor gene. Nature (Lond.), *351:* 453–456, 1991.
35. Lacombe, L., Orlow, I., Silver, D., Gerald, W. L., Fair, W. R., Reuter, V. E., and Cordon-Cardo, C. Analysis of p21WAF1/CIP1 in primary bladder tumors. Oncol. Res., *8:* 409–414, 1996.
36. Stein, J. P., Ginsberg, D. A., Grossfeld, G. D., Chatterjee, S. J., Esrig, D., Dickinson, M. G., Groshen, S., Taylor, C. R., Jones, P. A., Skinner, D. G., and Cote, R. J. Effect of p21WAF1/CIP1 expression on tumor progression in bladder cancer. J. Natl. Cancer Inst., *90:* 1072–1079, 1998.
37. Oliner, D. J., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature (Lond.), *358:* 80–83, 1992.
38. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature (Lond.), *387:* 296–299, 1997.
39. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The *mdm2* oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell, *69:* 1237–1245, 1992.
40. Chen, J., Wu, X., Lin, J., and Levine, A. J. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol. Cell. Biol., *16:* 2445–2452, 1996.
41. Chen, C. Y., Oliner, J. D., Zhan, Q., Fornace, A. J., Jr., Vogelstein, B., and Kastan, M. B. Interactions between p53 and MDM2 in a mammalian cell cycle checkpoint pathway. Proc. Natl. Acad. Sci. USA, *91:* 2684–2688, 1994.
42. Suzuki, A., Toi, M., Yamamoto, Y., Saji, S., Muta, M., and Tominaga, T. Role of MDM2 overexpression in doxorubicin resistance of breast carcinoma. Jpn. J. Cancer Res., *89:* 221–227, 1998.
43. Keleti, J., Quezado, M. M., Abaza, M. M., Raffeld, M., and Tsokos, M. The MDM2 oncoprotein is overexpressed in rhabdomyosarcoma cell lines and stabilizes wild-type p53 protein. Am. J. Pathol., *149:* 143–151, 1996.
44. Duro, D., Bernard, O., Della Valle, V., Berger, R., and Larsen, C. J. A new type of *p16/INK4a/MTS1* gene transcript expressed in B-cell malignancies. Oncogene, *11:* 21–29, 1995.
45. Haber, D. A. Splicing into senescence: the curious case of p16 and p19ARF (Review). Cell, *91:* 555–558, 1997.
46. Mao, L., Merlo, A., Bedi, G., Shapiro, G. I., Edwards, C. D., Rollins, B. J., and Sidransky, D. A novel p16INK4A transcript. Cancer Res., *55:* 2995–2997, 1995.
47. Stone, S., Jiang, P., Dayananth, P., Tavtigian, S. V., Katcher, H., Parry, D., Peters, G., and Kamb, A. Complex structure and regulation of the *p16* (*MTS1*) locus. Cancer Res., *55:* 2988–2994, 1995.
48. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. Alternative reading frames of the *INK4a* tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, *83:* 993–1000, 1995.
49. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. The alternative product from the human *CDKN2A* locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. EMBO J., *17:* 5001–5014, 1998.
50. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G., and Sherr, C. J. Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product $p19^{ARF}$. Cell, *91:* 649–659, 1997.
51. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. p14ARF links the tumour suppressors RB and p53. Nature (Lond.), *395:* 124–125, 1998.
52. Robertson, K. D., and Jones, P. A. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down regulated by wild-type p53. Mol. Cell. Biol., *18:* 6457–6473, 1998.
53. Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H., Cordon-Cardos, C., and DePinho, R. A. The *Ink4a* tumor suppressor gene product, p19, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell, *92:* 713–723, 1998.
54. Zhang, Y., Xiong, Y., and Yarbrough, W. G. ARF promotes MDM2 degradation and stabilizes p53: *ARF-INK4a* locus deletion impairs both Rb and p53 tumor suppression pathways. Cell, *92:* 725–734, 1998.
55. Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev., *12:* 2424–2433, 1998.
56. de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S. Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J., and Lowe, S. W. E1A signaling to p53 involves the p19(ARF) tumor suppressor. Genes Dev., *12:* 2434–2442, 1998.
57. Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens [see comments]. Nature (Lond.), *371:* 72–74, 1994.
58. Hermeking, H., and Eick, D. Mediation of c-Myc-induced apoptosis by p53. Science (Washington DC), *265:* 2091–2093, 1994.
59. Kowalik, T. F., DeGregori, J., Leone, G., Jakoi, L., and Nevins, J. R. E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2. Cell Growth Differ., *9:* 113–118, 1998.
60. Yeager, T. R., Jarrard, D. F., and Reznikoff, C. A. An *in vitro* model for human bladder cancer pathogenesis studies. *In*: K. Adolph (ed.), Human Genome Methods, pp. 159–178. Boca Raton, FL: CRC Press, Inc., 1998.
61. Della Valle, V., Duro, D., Bernard, O., and Larsen, C. J. The human protein p19ARF is not detected in hemopoietic human cell lines that abundantly express the alternative β transcript of the p16INK4a/MTS1. Oncogene, *15:* 2475–2481, 1997.
62. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., *et al.* A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc. Natl. Acad. Sci. USA, *92:* 9363–9367, 1995.
63. Gardie, B., Cayuela, J. M., Martini, S., and Sigaux, F. Genomic alterations of the p19ARF encoding exons in T-cell acute lymphoblastic leukemia. Blood, *91:* 1016–1020, 1998.
64. Swaminathan, S., and Reznikoff, C. A. Biochemical and molecular carcinogenesis. *In*: N. J. Vogelzang, P. T. Scardino, W. U. Shipley, and D. S. Coffey (eds.), Comprehensive Textbook of Genitourinary Oncology, pp. 305–314, 1996.
65. Gonzalgo, M. L., Hayashida, T., Bender, C. M., Pao, M. M., Tsai, Y. C., Gonzales, F. A., Nguyen, H. D., Nguyen, T. T., and Jones, P. A. The role of DNA methylation in expression of the *p16/p19* locus in human bladder cancer cell lines. Cancer Res., *58:* 1245–1252, 1998.
66. Cote, R. J., Dunn, M. D., Chatterjee, S. J., Stein, J. P., Shi, S. R., Tran, Q. C., Hu, S. X., Xu, H. J., Groshen, S., Taylor, C. R., Skinner, D. G., and Benedict, W. F. Elevated and absent pRb expression is associated with bladder cancer progression and has cooperative effects with p53. Cancer Res., *58:* 1090–1094, 1998.
67. Grossman, H. B., Liebert, M., Antelo, M., Dinney, C. P., Hu, S. X., Palmer, J. L., and Benedict, W. F. p53 and RB expression predict progression in T1 bladder cancer. Clin. Cancer Res., *4:* 829–834, 1998.
68. Xu, H. J., Quinlan, D. C., Davidson, A. G., Hu, S. X., Summers, C. L., Li, J., and Benedict, W. F. Altered retinoblastoma protein expression and prognosis in early-stage non-small cell lung carcinoma. J. Natl. Cancer Inst., *86:* 695–699, 1994.
69. Xu, H. J., Cagle, P. T., Hu, S. X., Li, J., and Benedict, W. F. Altered retinoblastoma and p53 protein status in non-small cell carcinoma of the lung: potential synergistic effects on prognosis. Clin. Cancer Res., *2:* 1169–1176, 1996.
70. Williams, B. O., Remington, L., Albert, D. M., Mukai, S., Bronson, R. T., and Jacks, T. Tumorigenic effects of germline mutations in Rb and p53. Nat. Genet., *7:* 480–484, 1994.