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Advances in Brief

Increased p16 Levels Correlate with pRb Alterations in Human Urothelial Cells[1]

Thomas Yeager,[2] Walter Stadler,[2] Cassandra Belair, Jairaj Puthenveettil, Olufunmilayo Olopade, and Catherine Reznikoff[3]

Department of Human Oncology, University of Wisconsin Medical School, Madison, Wisconsin 53792 [T. Y., C. B., J. P., C. R.], and Department of Hematology/Oncology, University of Chicago, Chicago, Illinois 60637 [W. S., O. O.]

Abstract

The *CDKN2 (MTS1)* gene is located at 9p21; its product, p16, inhibits the cyclin D/CDK4 complex that phosphorylates pRb, thus negatively regulating cell cycle progression [M. Serrano *et al.*, Nature (Lond.), *366:* 704, 1994; A. Kamb *et al.*, Science (Washington DC), *264:* 436, 1994; T. Nobori *et al.*, Nature (Lond.), *368:* 753, 1994]. *CDKN2* mutations are more common in cultured human uroepithelial cells (HUC) than in uncultured bladder cancers. We examined the status of *CDKN2/p16* in early and late passage (P) cultures of HUC. HUC immortalization was not accompanied by p16 loss, even in cells with a hemizygous 9p21–pter deletion, but late passage cultures with a p16 loss showed decreased generation time. Thus, the data do not indicate that *CDKN2* is a candidate for a chromosome 9 senescence gene but suggest that p16 loss may confer a growth advantage *in vitro*. Significant differences in p16 levels were observed among HUC cell lines, but no *CDKN2* mutations were detected. However, an inverse correlation between elevated p16 and loss of pRb function was observed ($P < 10^{-4}$). Ten samples with normal pRb showed low or undetectable p16 levels, while seven samples with known pRb alterations showed abundant p16 but nevertheless grew vigorously in culture. These results support the hypothesis that p16 mediated cell cycle inhibition, as well as p16 regulation, occurs via pRb dependent pathway(s).

Introduction

LOH[4] at 9p21 is common in a wide variety of malignancies, including human bladder cancer (1–3). The *CDKN2 (MTS1)* gene is located in the 9p21 region and encodes a protein, p16, that is an inhibitor of cyclin D/CDK4 activity (4–6). Cyclin D/CDK4 catalyzes the phosphorylation of pRb which releases E2F and results in G_1 to S cell cycle progression. Thus, p16 is thought to negatively regulate the cell cycle (4), and *CDKN2* is a reasonable candidate for a 9p21 tumor suppressor gene. In apparent support of this possibility, homozygous deletions or mutations in *CDKN2* were identified in a high percentage of tumor cell lines (5, 6). However, subsequent examination of uncultured tumors showed a comparatively low percentage of *CDKN2* mutations (7–10). This result suggested that p16 loss might frequently play a role in adaptation of cells to growth *in vitro* but infrequently play a role in tumorigenesis *in vivo*. Our initial goal was to test this hypothesis. For this purpose, we examined the status of 9p21, *CDKN2*, and p16 in cultured and uncultured samples of normal, tumor, and viral transformed HUC (Table 1).

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[2] These authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at K4/562 CSC, 600 Highland Ave., Madison, WI 53792.

[4] The abbreviations used are: LOH, loss of heterozygosity; *MTS1*, multiple tumor suppressor 1; CDK4, cyclin-dependent kinase 4; HUC, human urothelial cells; HPV16, human papilloma virus 16; RT-PCR, reverse transcription-polymerase chain reaction; P, passage.

Materials and Methods

Cell Culture. Cultures of normal HUC were established from normal ureteral uroepithelium, grown in 1% fetal bovine serum-F-12+, a supplemented Ham's F-12 medium (GIBCO, Grand Island, NY), and dispersed for serial passage, as described previously (11, 12). Two established human transitional cell carcinoma cell lines (Scaber and 5637) and one SV40 transformed HUC line, T16, were also used in this study and cultured in 1% fetal bovine serum-F-12+. The establishment and characterization of T16 and the E6-HUC#1, E6-HUC#2, E7-HUC#1, and E7-HUC#2 cell lines have been described (11, 12).

Dinucleotide Repeat Analysis. Primers for 9p regions were obtained from Research Genetics or synthesized on an ABI Model 380 DNA synthesizer. One primer from each pair was labeled with 10 μCi [α-^{32}P]dATP with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in 70 mM Tris-HCl (pH 7.6)/10 mM $MgCl_2$/5 mM DTT buffer for 1 h at 37°C. PCR was performed in a 15-μl reaction with 50 ng of template, 1.0 μM primers, 100 mM concentration of deoxynucleotide triphosphates; 1.5 mM $MgCl_2$ and 0.375 unit Taq polymerase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl (pH 8.3)/50 mM KCl/0.1 mg/ml gelatin buffer. Amplification was performed in a Perkin Elmer 9600 thermocycler for 30 cycles with denaturation at 94°C for 15 s, annealing at 56°C for 15 s, and extension at 72°C for 15 s. A final extension for 7 min at 72°C was performed. The reaction mixture was diluted 1:2 with 90% formamide/10 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol and heated to 95°C; 5 μl were loaded onto a 7% acrylamide/urea/formamide gel as described (13); and the gel was exposed for 2–12 h.

Western Analysis. Cells were lysed in EBC buffer with protease inhibitors prepared exactly as described (See Ref. 11), lysates were spun down, and the supernatant was quantified using a Bradford assay. Then, 100 μg of total protein per lane were run on a 15% SDS-PAGE gel for p16 and a 7.5% SDS-PAGE gel for p53 and pRb analyses and then transferred to nitrocellulose (Bio-Rad, Richmond, CA). p16 was observed with the $p16^{ink4}$ polyclonal antibody (Pharmingen, San Diego, CA), a horseradish peroxidase conjugated goat anti-rabbit (Sigma Chemical Co., St. Louis, MO) antibody, and detection by enhanced chemiluminescence (Amersham, Arlington Heights, IL). The blot was exposed to Kodak film. p53 was observed with the p53-Ab-2 monoclonal antibody (Oncogene Science, Uniondale, NY), while pRb was observed with a monoclonal anti-human antibody (Pharmingen). Both p53 and pRb blotting used a horseradish peroxidase conjugated goat anti-mouse antibody (Sigma), followed by detection with enhanced chemiluminescence (Amersham).

RT-PCR. For *CDKN2* exon 3 amplification, 50 ng of template were amplified in a 15-μl reaction as described (3). For RT-PCR, RNA was isolated via the guanidinium isothiocyanate lysis method or via Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH). First strand cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (cDNA cycle kit; InVitrogen, San Diego, CA) using CDK413'F and polydeoxythymidylate as primers (3). *CDKN2* mRNA was amplified with a forward primer from exon 2, nucleotides 406 to 424 as numbered previously (1), and a reverse primer from exon 3, nucleotides 656 to 635 as numbered previously (1). β-Actin mRNA was amplified with primers F:CCTTCCTGGGCATGGAGTCCTG and R:GGAGCAATGATCTTGATCTTC. Cycling parameters were 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Reaction products were separated on a 2% agarose gel and stained in ethidium bromide for visualization.

Single Strand Conformation Polymorphism Analysis. Exons 1 and 2 of *CDKN2* were amplified as described (2), except that 1.0 μCi [α-^{32}P]dCTP was added to each 15-μl reaction. DTT was added to a final concentration of 1 mM, 10 units of *Sac*II were added to the exon 1 amplification mixture, and 5 units

Table 1 *Correlation of p16 overexpression with altered pRb in human urothelial cells*

Cells[a]	Description	9p21 LOH	*CDKN2* mRNA	p16 levels	pRb status	*p53* status
Bladder	Urothelial	ND[b]	+[c]	–	wt	wt
HUC[d]	Cultured	ND	+[c]	–	wt	wt
E6-1 P15	HUC cell line	+	ND	+	wt	Degraded
E6-1 P45	HUC cell line	+	–	–	wt	Degraded
E6-2 P9	HUC cell line	–	ND	–	wt	Degraded
E6-2 P28	HUC cell line	–	+	–	wt	Degraded
E7-1	HUC cell line	–	+	+++	E7/pRb	wt
E7-2	HUC cell line	–	+	+++	E7/pRb	wt
E6/7-1	HUC cell line	ND	ND	+++	E7/pRb	Degraded
E6/7-2	HUC cell line	ND	ND	+++	E7/pRb	Degraded
B-E6-1	HUC cell line	ND	ND	–	wt	Degraded
D-E7-1	HUC cell line	ND	ND	+++	E7/pRb	wt
T16	HUC cell line	ND	ND	+++	SV40/pRb	SV40/p53
Scaber	TCC cell line	–	ND	–	wt	wt
5637	TCC cell line	–	+	+++	Deficient	Mutant

[a] The braced cells are all of isogenic origin (12).

[b] wt, wild type; ND, not determined; TCC, transitional cell carcinoma; E7/pRb, pRb bound by the E7 protein; SV40/pRb, pRb bound by the SV40 large T antigen; SV40/p53, p53 bound by the SV40 large T antigen.

[c] *CDKN2* mRNA was not detected by RT-PCR and ethidium bromide staining of the gel as described in Fig. 3. However, Southern transfer followed by hybridization with *CDKN2* cDNA (provided by D. Beach) did show low levels of transcripts in some experiments.

[d] Three independent HUC samples were examined and all showed similar protein results.

of *Apa*LI were added to the exon 2 amplification mixture; each was allowed to incubate for 1 h at 37°C. The reaction was stopped and the products were denatured by addition of 4 volumes of 90% formamide/10 mM EDTA/0.05% bromophenol blue/0.05% xylene cyanol and heating to 95°C for 10 min. The products were separated on a nondenaturing 5% polyacrylamide gel (containing 2% bisacrylamide) at room temperature. Additional gels run at 4°C and at room temperature with 10% glycerol also failed to reveal altered migration patterns.

Results and Discussion

We first determined if *CDKN2* alterations accompanied HUC immortalization. We previously reported a hemizygous clonal deletion of the 9p21–pter chromosome region in a HPV16 E6 immortalized HUC, E6-HUC#1 (12). Since *CDKN2* is altered in a very high percentage (>50%) of urothelial cell lines (8), and because chromosome 9 is hypothesized to contain a gene for cellular senescence (14), we questioned if *CDKN2* might be a gene the loss of which is specifically associated with immortalization. Initially, we confirmed the cytogenetic 9p21 deletion associated with E6-HUC#1 immortalization using three dinucleotide repeat probes from the 9p21 region (Fig. 1). We then analyzed p16 levels in early passage postcrisis E6-HUC#1 and also in an independent isogeneic cell line, E6-HUC#2, that did not show 9p deletion (Fig. 1) (12). Surprisingly, Western analysis showed a clear signal for p16 in the early passage culture, E6-HUC#1, that showed a 9p LOH, but not in the E6-HUC#2 cell line, which showed no such LOH (Fig. 2, *Lanes 4* and *5*). Thus, 9p21 LOH was not accompanied by p16 loss, and p16 loss was not necessarily associated with 9p21 LOH (Figs. 1 and 2). In addition, while immortalization was sometimes accompanied by 9p21 LOH, this was not associated with p16 loss. Thus, these data do not indicate that *CDKN2* is a candidate for a chromosome 9 senescence gene, as was recently proposed (15). Notably, p16 levels in normal HUC were undetectable by Western analysis (Fig. 2, *Lane 1*). However, *CDKN2* mRNA was detected in uncultured normal bladder epithelium and in precrisis cultured HUC using Southern analysis of the PCR product after hybridization with a *CDKN2* cDNA probe (data not shown). By contrast, cells immortalized using HPV16 E7, but not E6, showed highly abundant p16 levels using Western analysis (Fig. 2, *last two lanes*) as discussed below.

We next sought to determine if alterations in p16 or *CDKN2* occurred after extended passage of HPV16 E6 and/or E7 immortalized HUC *in vitro*. Examination of p16 levels in late passage E6-HUC#1 demonstrated such a loss (Fig. 2, compare *Lanes 2* and *3*). No such loss was observed in late passage E7-HUCs (Fig. 2). *CDKN2* exon 3 genomic DNA was detected in E6-HUC#1 P23 (Fig. 3*A*, *Lane 1*), but *CDKN2* mRNA was absent (Fig. 3*B*, *Lane 2*), consistent with the p16 loss. The RT-PCR product for *CKDN2* mRNA was seen using ethidium bromide staining in E6-HUC#2 (Fig. 3*B*, *Lane 3*), a cell line that produced no detectable p16 at either late or early passage (Fig. 2, *Lanes 4* and *5*). Single strand conformation polymorphism for *CDKN2* exons 1 and 2 (Fig. 4) and DNA sequence analysis (data not shown) failed to reveal mutations in the *CDKN2* coding regions that could explain p16 loss in the E6-HUC lines. However, these analyses do not rule out mutations in the *CDKN2* promoter or enhancer that could also

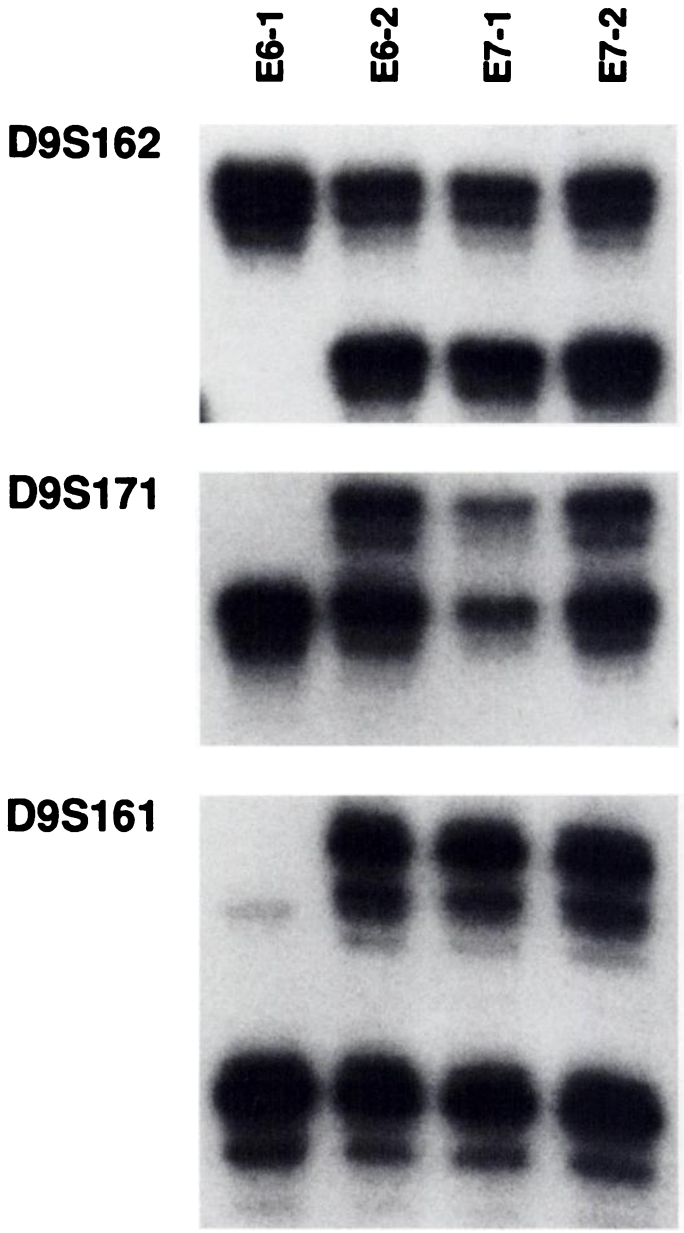

Fig. 1. 9p LOH in early passage immortal HUCs. LOH was observed at 9p21 in E6-HUC#1 (*Lane 1*) but not in E6-HUC#2, E7-HUC#1, or E7-HUC#2 (*Lanes 2–4*, respectively). The *D9S162* probe maps to 9p22, telemeric to *CDKN2*. *D9S171* and *D9S161* map to 9p21 centromeric to *CDKN2*. All samples were homozygous at the *IFNA* locus (data not shown). The same results were obtained in late passage cell lines.

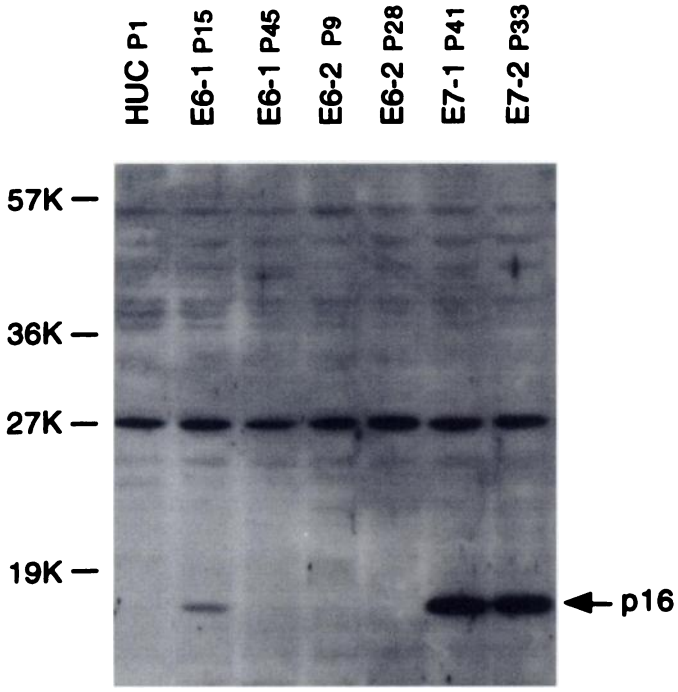

Fig. 2. Western blot analysis of p16 proteins in HUC samples. The E7-HUC cell lines clearly overexpress p16 as compared to cultures of nonestablished HUC. Because p16 levels were reproducibly undetected by Western using either cultured or uncultured HUC, quantitation was not possible. Loss of p16 after long term culture was observed in E6-HUC#1. *K*, thousands.

result in p16 loss. Another interpretation of the data is one in which p16 levels may be controlled by posttranscriptional mechanisms. Further studies are needed to distinguish between these possibilities. To test if decreased p16 levels might be associated with a growth advantage for HUC in culture, we compared the generation times of E6-HUC#1 at P20 and P45. A decrease in generation time from 34 h to 17 h accompanied p16 loss at late passage in E6-HUC#1, supporting the above hypothesis. No such decrease in generation time at late passage was observed in E6-HUC#2 which also showed no alteration in p16 status at late passage.

Finally, we examined the status of p16 in cells with altered pRb. We had observed that two independent HPV16 E7 immortalized cells lines, E7-HUC#1 and E7-HUC#2 showed very high levels of p16, while levels were low or undetectable in two isogeneic companion cell lines immortalized by HPV16 E6 (Fig. 2). Western analysis showed the expected loss of detectable p53 resulting from ubiquitin degradation in the E6 immortal HUCs, along with a strong signal for pRb (Fig. 5). In contrast, Western analysis showed a clear signal for p53 in the E7 immortalized HUCs, but low pRb levels (Fig. 5). This was confirmed in an independent set of E6 *versus* E7 immortalized HUCs (Table 1, see B-E6-1 and D-E7-1). At first, we interpreted the low p16 levels in E6-HUC as indicative of *CDKN2* down-regulation. However, repeated examination of cultured and uncultured samples of normal HUC showed that p16 and *CDKN2* mRNA levles are normally low in this cell type, as discussed above (Table 1; Fig. 2). The E7-HUC lines both express the *E7* gene (12). Since E7 binds and alters pRb, we questioned whether altered pRb function resulting from binding to a DNA tumor viral oncoprotein might correlate with increased p16 levels. We thus tested p16 levels in an SV40 immortalized HUC line, T16, in which we had previously demonstrated T antigen/pRb binding (11). Levels of p16 were abundant in T16 (Table 1). Finally, in order to exclude the possibility that these findings were due to E7 or T antigen inactivation of proteins other than pRB, we compared the status of p16 in two established bladder cancer cell lines (*i.e.*, Scaber and 5637), one of which showed altered pRb (Table 1). Results showed increased levels of p16 only in the cell ine, 5643, lacking wild type pRb. The p53 status of all the cell lines used in this study was also assessed using Western analysis. No correlation was found between p16 levels and p53 status (Fig. 5; Table 1). We interpret this result to indicate that increased p16 levels are associated with loss of normal pRb function, whether caused by RB mutational inactivation or by pRb binding to a viral oncoprotein.

Previous studies identified mutations of *CDKN2* located at 9p21 in a higher percentage of cultured human bladder cancer cells than in uncultured bladder cancers (7, 8). These data did not strongly support

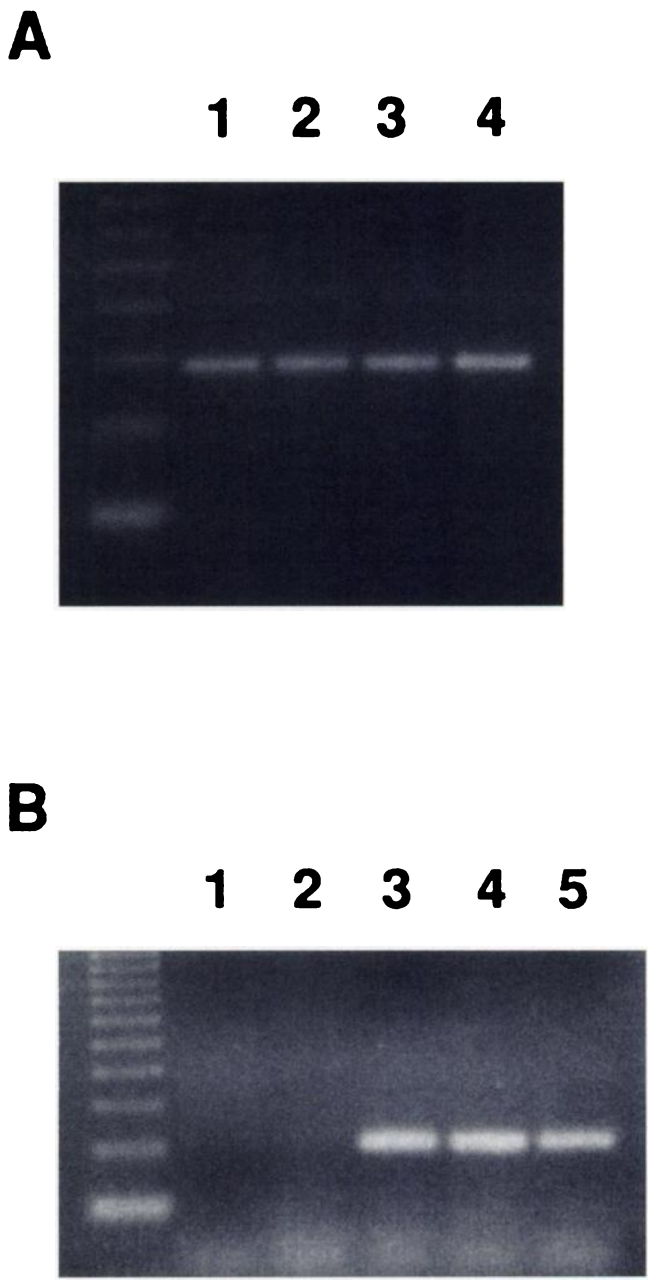

Fig. 3. *CDKN2* DNA and mRNA analyses. In *A*, *CDKN2* exon 3 genomic DNA is present in all transformed HUC samples: *Lane 1*, E6-HUC#1 P23; *Lane 2*, E6-HUC#2 P33; *Lane 3*, E7-HUC#1 P15; *Lane 4*, E7-HUC#2 P30. In *B*, *CDKN2* mRNA was examined with RT-PCR primers for exon 3 and showed that mRNA was missing in E6-HUC#1 P23: *Lane 1*, normal bladder HUC; *Lane 2*, E6-HUC#1 P23; *Lane 3*, E6-HUC#2 P33; *Lane 4*, E7-HUC#1 P15; *Lane 5*, E7-HUC#2 P30. β-Actin expression was detected at equal levels in all samples (data not shown).

the hypothesis that *CDKN2* is the putative 9p21 tumor suppressor gene associated with 9p LOH in many tumors but suggested that p16 loss may give cells a selective growth advantage *in vitro*. Since chromosome 9 is thought to house a gene for cellular senescence (14), the findings also suggested that *CDKN2* might be such a gene (15). This paper addresses these questions and reports three new findings concerning the role of CDKN2/p16 alteration in HUC transformation. (*a*) Neither p16 loss nor detectable *CDKN2* alterations accompanied HUC immortalization, even in cells showing 9p21 LOH. Thus, our data do not indicate that *CDKN2* is a candidate for the putative chromosome 9 senescence gene. However, our data do suggest a growth advantage for cells with p16 loss. (*b*) p16 loss was observed in HUC cell lines in which neither mutations in the *CDKN2* coding regions nor loss of *CDKN2* mRNA were detected, a finding consistent with the possibility of posttranscriptional regulation of p16 levels. A recent report showed that CDK4 overexpression or gene amplification are also alternate mechanisms to p16 loss in growth deregulation (16). Thus, analysis of tumor cells for *CDKN2* mutations may underestimate the role of p16 alteration in tumorigenesis. Finally, (*c*) our data provide strong evidence that increased p16 levels correlate with loss of pRb function ($P < 10^{-4}$), as hypothesized previously (4). However, high levels of p16 are not associated with cell cycle inhibition in the absence of normal pRb. This latter result is consistent with the observation that the normal cyclin D/p21/CDK4/proliferation cell nuclear antigen quaternary complex is disrupted in pRb altered cell lines and, instead, CDK4 associates exclusively with p16 (4, 17). This is particularly exciting because it provides evidence that p16 levels are regulated through a pathway involving pRb. We hypothesize that these elevated p16 levels contribute to the dissolution of the usual cyclin D/CDK4 complex, thus inhibiting its kinase function. Such a feedback loop, although requiring experimental verification, could potentially provide exquisite regulation of pRB phosphorylation.

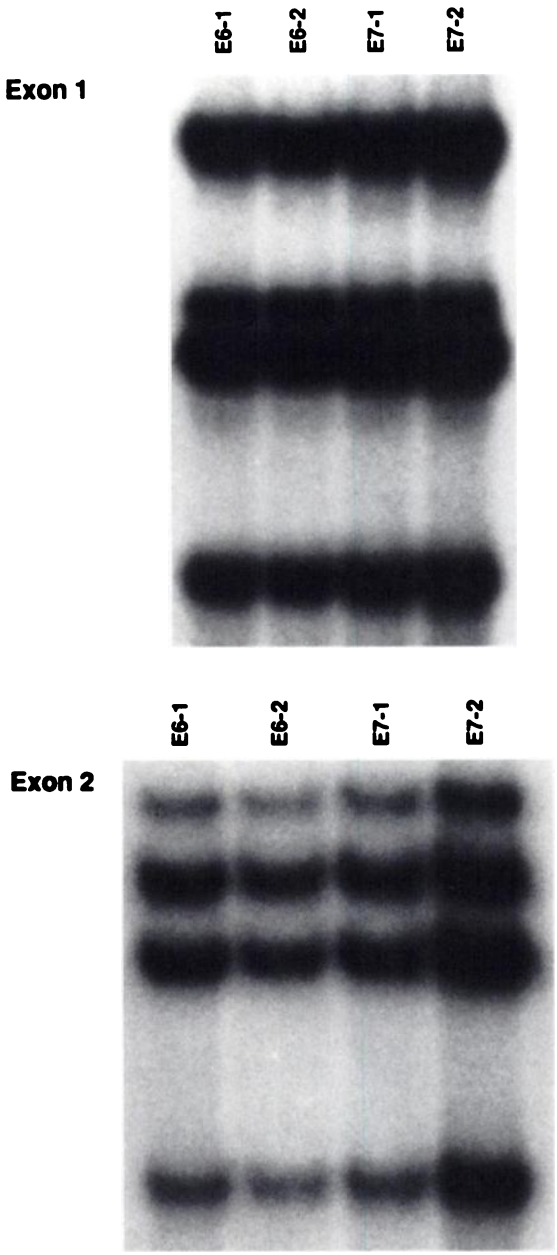

Fig. 4. Single strand conformation polymorphism of *CDKN2* exons 1 and 2 in E6 and E7 HUC cell lines. DNA from E6 and E7 transformed lines migrated at the same position as normal HUC DNA indicative of the absence of mutations. DNA sequencing also failed to reveal mutations in the *CDKN2* coding regions consistent with this result (data not shown).

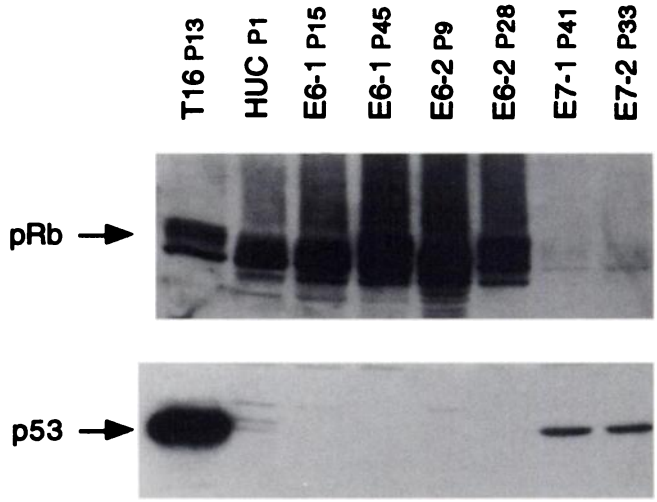

Fig. 5. Western blot analysis of pRb and p53 in HUC cells. *Top*: pRb levels were decreased in E7-HUC lines in comparison with control HUC and E6-HUC lines. *Bottom*: p53 is absent in the E6 transformed HUC lines, stabilized in the SV40 transformed T16 line, and present at slightly elevated levels in the E7 transformed lines. Western analysis was reproduced multiple times with the same results.

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