Frequent Alteration of CDKN2 (p16INK4A/MTSI) Expression in Human Primary Prostate Carcinomas

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ABSTRACT

CDKN2 (p16INK4A/MTSI) is found to be mutated in a variety of human tumor types. To explore the involvement of CDKN2 in prostate carcinogenesis, alterations of CDKN2 were examined in 116 human prostate tissues and cell lines and xenografts. Markedly reduced expression of CDKN2 mRNA was found in 43% (26 of 60) of untreated primary carcinomas, whereas no alteration was observed in 10 benign prostatic hyperplasias. In 17 matched sets from individual patients, 41% of cancerous tissues in contrast to 6% of noncancerous tissues expressed low levels of CDKN2 mRNA, supporting the role of CDKN2 as a tumor suppressor in prostate cancer. Alteration of CDKN2 was observed in each prostate tumor cell line, including one with a missense mutation, and in one of three xenograft tumor tissues derived from primary carcinomas. Two cell lines (PC-3 and TSU-Prl) expressed only CDKN2 E1β transcripts, indicating that the expression of CDKN2 E1α and E1β are under separate control in the prostate. A high level of CDKN2 expression was related to abnormal RB1 in one primary tumor and in the DU145 cell line, which expressed the mutated CDKN2 allele. Analysis of genomic DNA indicated that altered CDKN2 expression in primary carcinomas of the prostate was more frequently due to down-regulation of transcription (five of seven) than deletion of the gene (two of seven). Additionally, CDKN2 mRNA was induced in nonexpressor cell lines by treatment with 5-aza-2'-deoxycytidine. This study demonstrates that alteration of CDKN2 is one of the most frequent genetic abnormalities in prostate cancer and may contribute to prostate carcinogenesis.

INTRODUCTION

Chromosome 9p21 is frequently deleted or rearranged in a number of tumor types, including malignant melanoma, and has been shown to be linked to familial melanoma (1, 2). Kamb et al. (3) isolated a putative tumor suppressor gene, MTS1, from this locus, and deletions or mutations of this gene were detected in approximately 75% of melanoma cell lines and 50% of cell lines derived from other tumor types (3). MTS1 encodes a previously identified inhibitor of Cdk4 (4) (p16INK4A) and the gene is now referred to as CDKN2 (4). p16INK4A has been characterized as a potent and specific inhibitor of the G1-to-S-phase progression of the cell cycle, and its ability to arrest the cell cycle correlates with inhibition of cyclin D1/Cdk4 and cyclin D1/Cdk6 kinase activities. It was observed that cell cycle arrest occurred at the G1-S boundary after overexpression of CDKN2 mRNA (5). Several tumor-derived mutant CDKN2 alleles, in comparison to wild-type alleles, show loss of or markedly reduced cell cycle inhibition activity (6).

The CDKN2 protein, p16INK4A, regulates the phosphorylation state of the RB1 protein, which is thought to feed back and control CDKN2 expression (4). Inhibition of cyclin D1/Cdk4 and cyclin D1/Cdk6 complexes by Cdk2 decreases the phosphorylation of Rb1, resulting in a cell cycle checkpoint that limits the G1-to-S transition, ultimately inhibiting cell growth. The presence of functional Rb1 appeared to be necessary to confer the full sensitivity to Cdkn2-mediated cell cycle arrest (7). It has been observed in some cell lines that expression of CDKN2 and RB1 inactivation are inversely correlated such that CDKN2 mRNA accumulates to a high level in cells lacking RB1 function (8-10). This phenomenon was explained by cell culture studies that showed that the cell cycle inhibition activity of CDKN2 was dependent on the wild-type function of RB1 (7). It was also demonstrated that CDKN2 antagonizes a major function of cyclin D1 by blocking E2F-dependent transcription (11). Taken together, these data implicate CDKN2 in playing a critical role in the RB1 growth suppression pathway.

The finding of CDKN2 alterations frequently in cell lines and much less commonly in uncultured primary tumors raised the question of whether CDKN2 alterations reflect a tissue culture selection process (12). However, the recent finding of somatic mutations of CDKN2, particularly in pancreatic adenocarcinomas and esophageal carcinomas, suggests that CDKN2 plays an important role in the carcinogenesis of certain types of cancer (13, 14). Ranade et al. (6) observed that members of familial melanoma have inherited a non-functional CDKN2 allele and subsequently lost the wild-type allele by a secondary

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The abbreviations used are: Cdk, cyclin-dependent kinase; CaP, carcinoma of the prostate; BPH, benign prostatic hyperplasia; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformation polymorphism; CAB, combined androgen blockade.

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event. The melanoma-predisposing mutations were impaired in their ability to inhibit the catalytic activity of the cyclin D1/Cdk4 and cyclin D1/Cdk6 complexes. Overall, these observations strongly support that CDKN2 is a tumor suppressor gene, the alterations of which contribute to carcinogenesis of several types of cancers.

To investigate the involvement of CDKN2 alterations in human prostate carcinogenesis, we evaluated its expression in 123 human prostate specimens including 82 primary CaPs, 24 noncancerous tissues from CaP patients, 10 BPHs, 4 CaP cell lines, and 3 human CaP/nude mouse xenografts.

MATERIALS AND METHODS

Human Cell Lines and Xenografts. The human prostate carcinoma cell lines (LNCaP, DU145, PC-3, and TSU-Pr1) and xenografts (CWR22, CWR31, and CWR91) used in this study have been described previously (15–19). Fourteen human cell lines, derived from lung carcinomas (H522, H661, H209, A549, A427, and Calu1), bladder carcinomas (J82 and T24), breast carcinomas (MCF7 and MDA-MB-231), lymphomas (HL60 and Raji), a promyelocytic leukemia (K562), and a neuroblastoma (SK-N-SH), as well as a human fetal lung fibroblast strain (T3891), were also examined to validate the quantitative RT-PCR assay of CDKN2 expression.

Human Prostate Tissues. One hundred sixteen human prostate tissues were obtained from 89 CaP and 10 BPH patients by surgical resection in the University of California, Davis Medical Center (Sacramento, CA) or in the Catherine McAuley Health System (Ann Arbor, MI). Tissue specimens were snap-frozen in liquid N2 and stored at −70°C until used. Our protocol reported previously was used for the histological determination of cancer in the tissues (20).

Quantitative RT-PCR Analysis of CDKN2 Expression. Total cellular RNA was extracted, and 1 μg of RNA was converted to cDNA by reverse transcription using random hexamer primers as described previously (21). For quantitative evaluation by RT-PCR, PCR was initially performed over a range of 22–40 cycles. CDNA diluted 1:4 (12.5 ng/50 μl of PCR reaction) undergoing 28–36 cycles was observed to be within the logarithmic phase of amplification and yielded reproducible results with all primers used for CDKN2 and an endogenous expression standard gene N-RAS. The CDNA was then subjected to 30–34 cycles of PCR at 95°C (1 min), 61°C (0.5 min), and 72°C (0.75 min) in 2.5 mM MgCl2 and 5% DMSO-containing reaction buffer (PCR buffer II; Perkin-Elmer Corp., Norwalk, CT) using different sets of oligonucleotide primers: SC15 (sense, 5'-ATGGAGCCCTCGCTGACTG-3') and SC12 (antisense, 5'-AGGAAGCCCTCCGGGACGGCTG-3') for exons 1–2 of E1α; E1β (sense, 5'-CCGGAGGTAGGGTTTCTGTTG-3') and SC17 (antisense, 5'-GTTTCTTCTACAGTGCT-3') for SC15 (sense, 5'-ACTCTACCCGACCGGTCAGACG-3') and SC16 (antisense, 5'-TCCCGAGATTCTTCAGAGCC-3') for exons 2–3, which cover the entire coding region of CDKN2 transcript. Twelve μl of RT-PCR products were resolved on 2% agarose gels and subjected to Southern blot analysis to confirm the specificity of amplification using biotinylated internally positioned oligonucleotides as probes (SC18, 5'-GATGATGGGCAGCGCCCGAG-3' for SC15/SC12 PCR products; and SC19, 5'-ATGGTTACTGCCTCCGGTGC-3' for SC13/SC16 PCR products) and chemiluminescence detection. Additionally, restriction endonuclease DdeI digestion of RT-PCR products confirmed the CDKN2 identity of the PCR products. Quantitation of signal was performed by laser densitometric scanning of the X-ray films. Expression levels from each specimen were then compared after adjustment for the expression level of N-RAS. RT-PCR for N-RAS was done with an aliquot of the same cDNA simultaneously with CDKN2 under the same conditions except with no DMSO in reaction buffer. The sequences of oligonucleotides used as RT-PCR primers and the probe for N-RAS (EK265, RS61, and EK221) have been reported previously (20). Two cell lines, LNCaP and DU145, were included in every RT-PCR run of patient specimens as the low and high level CDKN2 expression controls, respectively. Quantitative RT-PCR was repeated at least three times for both genes for each specimen, and the calculated means were obtained. To exclude the possibility that mouse RNA from nude mouse tissues contributed to CDKN2 expression levels in xenograft tumor specimens, two mouse cell lines (NIH3T3 and X1323) were subjected to RT-PCR with the same primer sets. No amplification of mouse CDKN2 products was detected from these mouse cell lines. To examine the induction of CDKN2 mRNA expression in 5-aza-2'-deoxycytidine-treated cells, heminestered PCR was performed for 30 cycles with primers SC15/SC12 using 1 μl of PCR products from an initial PCR reaction (SC15/SC16) as the template. Ten μl of these heminestered PCR products were resolved on 2% agarose gels.

Western Blot Analysis. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 0.25 M NaCl, 0.1% NP40, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 1 mM sodium β-vanadate. Twenty units of DNase were added, and the mixture was placed on ice for 30 min with occasional vortexing. The lysate was then cleared by centrifugation, and the supernatant was saved. Proteins were quantitated on a 96-well plate spectrophotometer using a modified Bradford assay (Bio-Rad Laboratories, Richmond, CA). Total soluble cellular proteins (150 μg/lane) were separated by SDS-PAGE using a minigel apparatus (Bio-Rad Laboratories) according to the method of Laemmli with slight modifications as reported previously (22, 23). Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) at a constant 125 V for 1 h (23, 24). Membranes were then stained reversibly with Ponceau S (Sigma Chemical Co., St. Louis, MO) to confirm that equivalent amounts of protein had been transferred in each lane.

Membranes were blocked with the following solution: 5% nonfat dry milk in 1.25 mM Tris-HCl, 1 mM NaCl, 0.02% Tween 20 (TBST) for 1 h. Blots were then incubated at 4°C for 16 h with a 1:1000 dilution of anti-Cdkn2 rabbit polyclonal antibody (PharMingen, San Diego, CA). Blots were washed three times in the blocking solution (see above) and incubated for 1 h at room temperature in a 1:250 dilution of a biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). Blots were rinsed once in the blocking solution, twice in TBST, and finally once in TBS (without Tween 20). Blots were then incubated in TBST with a 1:1000 dilution of streptavidin-horseradish peroxidase for 30 min, followed by a 1-min incubation with chemiluminescence detection reagents (ECL; Amersham Chemical Co., St. Louis, MO) to confirm the equivalent amounts of protein that had been transferred in each lane.

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signal was detected by autoradiography by exposure of membranes to Kodak XAR film for 30 s to 5 min.

### Nonisotopic SSCP and Direct Sequencing of CDKN2

#### RT-PCR Products.
- RT-PCR products within exons 1–2 (248 bp; SC15/SC12) and exons 2–3 (275 bp; SC13/SC16) regions were separately amplified. Nonisotopic RT-PCR-SSCP analysis was performed as described previously (25). Briefly, 20 μl of RT-PCR products were mixed with 7 μl of 0.5 n NaOH, 10 mm EDTA, and 15 μl of denaturing loading buffer (95% formamide, 20 mm EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). After heating at 95°C for 5 min, samples were loaded in gel wells precooled to 4°C. SSCP analysis was performed using 8% nondenaturing acrylamide gels containing 5% glycerol at room temperature. Three DNA fragments (121, 241, and 117 bp) generated by MboI and RsaI digestion of the 479-bp RT-PCR products (SC15/SC16) covering the entire coding sequences of CDKN2 were also analyzed. We used essentially the same protocol for generating single-stranded templates for subsequent sequencing as described previously (23). Sequencing was done in both directions to confirm the findings.

#### DNA-PCR Analysis of CDKN2.
- Genomic DNA was extracted from the same cells of four cell lines and 10 tissues from the DNA phase after RNA was extracted. Two hundred ng of DNA were used for separate amplifications of exon 2 of CDKN2 and exon 2 of N-RAS for 38 cycles at 95°C (1 min), 60°C (0.5 min), and 72°C (0.75 min). The sequences of primers used for exon 2 are p1625 (sense, 5'-ACACAAGCTTTT-GAGCTC-3') and p1623 (antisense, 5'-CTGAGCTTTTG-GAAAGCCTTC-3'). The primers for N-RAS DNA-PCR (RS60/RS61) were reported previously (20). Twelve μl of PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed under UV light. DNA-PCR of CDKN2 was repeated at least three times for each sample. The CDKN2:N-RAS ratio was assessed.

#### 5-Aza-2'-deoxycytidine Treatment.
- To assess re-expression of CDKN2 mRNA, cells were plated in six-well tissue culture plates 24 h before treatment. 5-Aza-2'-deoxycytidine (Fluka, Switzerland) was added to the fresh medium at 20 μM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol.

### RESULTS

#### Expression of CDKN2 in Characterized Cell Lines: Validation of the RT-PCR Assay.
- To validate the RT-PCR approach to quantitatively discriminate normal from low or normal from high CDKN2 expressors, cell lines characterized for both CDKN2 and RBL1 were used, because deregulated CDKN2 transcription has been correlated with RBL1 inactivation in a number of cell lines. The diploid human fetal lung fibroblast strain (T3891) was chosen as a control for normal expression (23). The Raji lymphoma cell line, characterized to retain the germ-line configuration of CDKN2 alleles, was also included as a diploid control (27). Five cell lines (A427, A549, MCF7, MDA-MB-231, and K562) with homozygous deletions of both CDKN2 alleles were chosen to exemplify nonexpressors (9, 28–31). Two cell lines (J82 and H209), which carry mutations of RBJ, were chosen to exemplify abnormally high expressors (12, 30, 32).

#### Table 1: Genomic status and expression levels of CDKN2 as related to RBL1 status in tumor cell lines and xenografts

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Cell type</th>
<th>CDKN2 DNA*</th>
<th>RBL1 status*</th>
<th>CDKN2 E1α mRNA (N-RAS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3891</td>
<td>Normal FLFb</td>
<td>ND</td>
<td>N</td>
<td>0.91</td>
</tr>
<tr>
<td>Raji</td>
<td>Lymphoma</td>
<td>+</td>
<td>N</td>
<td>1.23</td>
</tr>
<tr>
<td>K562</td>
<td>Leukemia</td>
<td>–</td>
<td>N</td>
<td>0.00</td>
</tr>
<tr>
<td>A427</td>
<td>NSCLC</td>
<td>–</td>
<td>N</td>
<td>0.00</td>
</tr>
<tr>
<td>A549</td>
<td>NSCLC</td>
<td>–</td>
<td>N</td>
<td>0.00</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast CA</td>
<td>–</td>
<td>N</td>
<td>0.00</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast CA</td>
<td>–</td>
<td>?</td>
<td>0.00</td>
</tr>
<tr>
<td>J82</td>
<td>Bladder CA</td>
<td>+</td>
<td>MT</td>
<td>1.84</td>
</tr>
<tr>
<td>H209</td>
<td>SCLC</td>
<td></td>
<td>MT</td>
<td>1.74</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H522</td>
<td>NSCLC</td>
<td>+</td>
<td>N</td>
<td>1.32</td>
</tr>
<tr>
<td>H661</td>
<td>NSCLC</td>
<td>+(MT)</td>
<td>N</td>
<td>0.54</td>
</tr>
<tr>
<td>Calu1</td>
<td>NSCLC</td>
<td>+</td>
<td>N</td>
<td>0.71</td>
</tr>
<tr>
<td>T24</td>
<td>Bladder CA</td>
<td>+</td>
<td>N</td>
<td>1.03</td>
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<tr>
<td>HL60</td>
<td>Leukemia</td>
<td>+</td>
<td>N</td>
<td>0.52</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>Neuroblastoma</td>
<td>+</td>
<td>?</td>
<td>0.00</td>
</tr>
<tr>
<td>Prostate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LNCaP</td>
<td>CaP</td>
<td>+</td>
<td>N</td>
<td>0.32</td>
</tr>
<tr>
<td>DU145</td>
<td>CaP</td>
<td>+ (MT)</td>
<td>MT</td>
<td>1.44</td>
</tr>
<tr>
<td>PC-3</td>
<td>CaP</td>
<td>+</td>
<td>N</td>
<td>0.00*</td>
</tr>
<tr>
<td>TSU-Pr1</td>
<td>CaP</td>
<td>+</td>
<td>N</td>
<td>0.00*</td>
</tr>
<tr>
<td>CWR22</td>
<td>CaP xenograft</td>
<td>ND</td>
<td>N</td>
<td>0.19</td>
</tr>
<tr>
<td>CWR31</td>
<td>CaP xenograft</td>
<td>ND</td>
<td>N</td>
<td>1.53</td>
</tr>
<tr>
<td>CWR91</td>
<td>CaP xenograft</td>
<td>ND</td>
<td>N</td>
<td>1.57</td>
</tr>
</tbody>
</table>

* Data from Refs. 8–9, 12, and 27–35.

b +, present; −, deleted; ?, unknown; MT, mutant type; N, normal; Ab, abnormal; ND, not done; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CA, carcinoma; FLF, fetal lung fibroblast.

' Only E1β transcript was expressed.

### Discussion

#### Expression of CDKN2 E1α and E1β mRNA in Prostate Cell Lines and Xenografts.
- Expression levels of CDKN2 mRNA were determined in four CaP cell lines derived from metastatic prostate cancers (LNCaP, DU145, PC-3, and TSU-Pr1) and three serially transplantable xenografts of human primary CaPs (CWR22, CWR31, and CWR91). All specimens showed the predicted lengths of RT-PCR products within the
Exons 2–3 region (Fig. 1). Markedly reduced levels of expression were observed in cell line LNCaP (0.32) and xenograft CWR22 (0.19). We also examined the exon 1 region of CDKN2 transcripts using several upstream primers, the sequences of which are complementary to the 5' untranslated region in exon 1. In two cell lines, PC-3 and TSU-Prl, we were unable to detect transcription of exon 1. Thus, abnormally low mRNA expression of CDKN2 was observed in three of four (75%) CaP cell lines and in one of three (33%) xenograft tumor specimens derived from primary CaPs, whereas the other specimens each expressed abnormally high levels (Table 1).

The lack of exon 1 sequences in the CDKN2 transcripts from PC-3 and TSU-Prl suggested that truncation due to deletion of a portion of this exon or, based on the recent finding of a second CDKN2 transcript (E1β) containing different exon 1 sequences, that PC-3 and TSU-Prl expressed only this alternative CDKN2 transcript (36, 37). As shown in Fig. 2, PC-3 and TSU-Prl express E1β, and its expression was found in all of the other cell lines and xenografts, with only CWR22 displaying reduced expression.

Expression of Cdkn2 in Prostate Cell Lines and Xenografts. To examine the expression of CDKN2 at the protein level compared to mRNA, expression levels of Cdkn2 were determined in the four prostate cell lines and the three xenografts (Fig. 3). In the cell line LNCaP and xenograft CWR22, protein levels were found to be markedly reduced compared to DU145 and xenografts CWR31 and CWR91. In two cell lines, PC-3 and TSU-Prl, no protein was detected. Thus, the relative levels of protein found in all of the prostate cell lines and xenografts reflected the levels of mRNA detected by RT-PCR.
Mutations in CDKN2 Transcripts in CaP. To characterize the mutational status of CDKN2 in prostate tumors, RT-PCR-SSCP analysis covering the entire coding region of the CDKN2 transcript was performed for 32 CaPs, 8 BPHs, the two expressor CaP cell lines (LNCaP and DU145), and the 3 xenografts. Several sequence-overlapped PCR fragments were generated by use of different primer sets or restriction endonuclease digestions of PCR products. Altered migration of single-stranded RT-PCR products in SSCP analysis, suggestive of a sequence alteration, was found in the DU145 cell line (exclusively abnormal banding, no wild-type pattern) and in 1 of 32 primary carcinoma tissue specimens (specimen PS221, both wild-type and abnormal SSCP patterns; data not shown). A missense mutation in DU145 (GAC to TAC, Asp to Tyr) at codon 84 in exon 2 was found by direct sequencing of the RT-PCR products (data not shown).

High CDKN2 mRNA Expression and Mutational Status of RB1. To determine whether abnormally high levels of CDKN2 expression in prostate cancers are related to RB1 inactivation, we examined RB1 transcripts in DU145, CWR31, CWR91, and a patient specimen (PS290). The RB1 transcripts in DU145 showed the deletion of exon 21 as reported previously (33). Similarly, the only CaP patient specimen to show high levels of CDKN2 expression (PS290: 1.56) was identified to express abnormal RB1 transcripts missing exon 22 sequences (38). The entire coding region of the xenograft RB1 transcripts were amplified using five different primer sets, and the products were subjected to restriction endonuclease digestion to generate optimally sized fragments for SSCP analysis. No RB1 abnormalities were detected in CWR31 or CWR91.

Genomic Status of the CDKN2 Gene in CaPs. To characterize the genomic status of CDKN2 and its correlation with mRNA expression levels in CaPs, PCR analysis was performed on genomic DNA for CDKN2 in the four CaP cell lines and seven prostate tissue specimens. As shown in Fig. 6, all four cell lines showed strong signals for the exon 2 sequences of the CDKN2 gene, despite the lack of or the low mRNA expression seen in three of these. Two specimens whose mRNA expressions were very low showed weak genomic signals for CDKN2 but strong signals for N-RAS, indicating loss of the gene (PS206 and PS213; Fig. 6). Strong CDKN2 DNA signals were found in the five other low CDKN2 expressors, indicating presence of the gene; four are shown in Fig. 6.

Induction of CDKN2 mRNA Expression by Treatment with 5-Aza-2'-deoxycytidine. To assess whether frequent loss or altered expression of CDKN2 mRNA in CaP is due to gene silencing caused by de novo methylation, we treated two CDKN2 nonexpressor cell lines, PC-3 and TSU-Pr1, with the demethylating agent, 5-aza-2'-deoxycytidine. The expressor cell line, LNCaP, was also subjected to treatment as the control. As shown in Fig. 7, induction of CDKN2 mRNA expression was observed in PC-3 and TSU-Pr1 after treatment at both 0.5 and 1.0 μM. TSU-Pr1 was treated for 3 days, and PC-3 was treated for 6 days. No profound effects of 5-aza-2'-deoxycytidine on cell morphology or cell number were observed with LNCaP or TSU-Pr1. PC-3 did show a marked reduction of cell growth during treatment (data not shown).

Table 2 Expression levels of CDKN2 mRNA in human prostate tissuesa

<table>
<thead>
<tr>
<th>Expression levelsb</th>
<th>BPH</th>
<th>Primary CaP</th>
<th>Noncancerousc</th>
<th>Primary CaP</th>
<th>Noncancerous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (&lt;0.55)</td>
<td>0 (0)</td>
<td>26 (43)</td>
<td>3 (17)</td>
<td>13 (59)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Normal (≥0.56)</td>
<td>10 (100)</td>
<td>34 (57)</td>
<td>15 (83)</td>
<td>9 (41)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>60</td>
<td>18</td>
<td>22</td>
<td>6</td>
</tr>
</tbody>
</table>

a Numbers in parentheses are percentage of samples.
b Specimens showing less than 50% expression of BPH mean (CDKN2:N-RAS, 1.09) were classified as low expressors (<0.55). Normal expressors: 0.56 ± 1.40.
c Noncancerous tissue from a CaP patient.

Fig. 3 Cdkn2 (p16INK4A) expression in prostate carcinoma cell lines and xenografts. Total soluble cell protein lysates (150 μg per lane) were analyzed by SDS-PAGE and Western blotted using an anti-p16 antibody. Two human lung carcinoma cell lines were used as positive and negative controls for p16 expression (H209 and Calu-1, respectively). Human prostate cell lines DU145 and LNCaP and the xenografts CWR22, CWR31, and CWR91 all express p16, with high levels found in DU145, CWR31, and CWR91 reflecting the RNA results shown in Fig. 1. Lower levels of expression are seen in LNCaP and CWR22, both of which expressed abnormally low levels of CDKN2 mRNA. No p16 protein expression was detected in PC-3 or TSU-Pr1, reflecting the lack of detection of CDKN2 E1α transcripts. Following transfer of the proteins to the membrane, reversible Ponceau S staining was done to confirm that equivalent amounts of protein had been transferred in each lane.
Abnormalities in Primary Human Prostate Cancer

1894 CDKN2 Abnormalities in Primary Human Prostate Cancer

DISCUSSION

Here we report that CDKN2 mRNA expression is frequently altered in primary CaPs. Twenty-six of 60 (43%) untreated primary CaPs were found to express markedly reduced levels of CDKN2 mRNA. No BPHs (0 of 10) and only 13% (3 of 24) of noncancerous tissues from cancer patients showed decreased expression. Furthermore, abnormally low CDKN2 expression was observed in 41% of cancerous areas in 17 matched specimens from individual patients. In addition, all four cell lines and one of three xenografts showed no expression, abnormally low expression, or expression of mutated CDKN2 transcripts. In those specimens, the levels of mRNA as measured by RT-PCR were reflected in the levels of protein detected by Western blotting. The data reported here on the CaP specimens represent the total expression levels of the E1α plus E1β transcripts, which resulted from the use of exon 2 and 3 oligonucleotides that prime amplification of both transcripts. All CaP cell lines and xenografts (except CWR22) expressed abundant levels of E1β transcripts, as has been observed in other cell types (36, 37). However, at this time, the biological significance of E1β expression is not well understood. Homozygous deletion of CDKN2 alleles is the most frequently found genetic mechanism, leading to the inactivation of this gene in a variety of established cell lines and tumor types (3, 13–14, 27–32). In a recent loss of heterozygosity study, it was reported that 16% of prostate cancers showed loss of chromosome 9p sequences (39). We examined the genomic status of CDKN2 in the cell lines and some tissue specimens using genomic DNA extracted from the same cells used for the RNA analysis. Two of seven tissue specimens, which were identified to express low or nearly undetectable levels of mRNA, showed low or no amplification of CDKN2 genomic sequences. Thus, in these specimens, abnormal CDKN2 mRNA expression likely results from deletion of the gene. Because five of seven abnormally low expressors retain genomic copies of the gene, it appears that the altered CDKN2 expression in CaPs results from not only allelic deletion of the gene but more frequently from down-regulation of transcription. In all three cell lines with abnormal levels of CDKN2 expression (LNCaP, PC-3, and TSU-Prl), strong amplification of the gene was also found. It is worth noting that these three lines express substantial levels of the CDKN2 E1β, indicating that CDKN2 E1α and E1β transcriptions are under separate control.

A missense mutation in CDKN2 transcripts from the cell line DU145 was detected (codon 84, GAC to TAC, Asp to Tyr). This is the same mutation found in DU145 at the DNA level reported recently by Tamimi et al. (40). Here it is demonstrated that DU145 expresses only the mutant allele at both the RNA and protein levels. Only 1 of 32 CaPs displayed an abnormal SSCP migration pattern; we were unable to sequence that abnormality due to a lack of sample. Regardless, these results are similar to the genomic DNA study mentioned above, where 2 of 20 CaPs showed SSCP abnormalities, with one missense mutation found and another where no mutations were found in the DNA from American CaPs (40, 41). Although we cannot exclude the possibility that our SSCP conditions failed to detect some mutations, it appears unlikely that point mutations in coding sequences are a predominant mechanism leading to inactivation of the CDKN2 gene in prostate carcinogenesis.

Abnormally low CDKN2 expression can be caused by several mechanisms, such as alterations in the promoter region, aberrant methylation of the gene, or reduced mRNA stability. Decreased expression, rather than mutations in CDKN2, was observed to play a role in the development of nasopharyngeal carcinomas and gliomas (42, 43). Recently, methylation of the 5′ CpG island of CDKN2 was found in cell lines with structurally unaltered CDKN2 and in approximately 20% of various primary neoplasms but not in normal tissues (31, 44). Hypermethylation of a G:C-rich region within exon 1 of CDKN2 was observed in 100% of lung cancer cell lines, which showed no detectable CDKN2 mutation and wild-type RB1 expression (45).
Table 3  Expression levels of CDKN2 in untreated human primary CaPs related to pathological tumor stage and Gleason score

<table>
<thead>
<tr>
<th>Expression levels</th>
<th>Stage</th>
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<tbody>
<tr>
<td></td>
<td>B</td>
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<tr>
<td>Low (≤0.55)</td>
<td>18 (55)</td>
</tr>
<tr>
<td>Normal (0.56–0.59)</td>
<td>15 (45)</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
</tr>
</tbody>
</table>

*Specimens showing less than 50% expression of BPH mean (CDKN2:N-RAS, 1.09) were classified as low expressors: ≤0.55. Normal expressors: 0.56 ≤ 1.40.

*Numbers in parentheses are percentage of samples.

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Gonzalez-Zulueta et al. (46) reported that CDKN2 was inactivated in 67% of primary transitional cell carcinoma of the bladder by de novo methylation of its 5′ CpG island. These findings of a distinct methylation pattern associated with a complete transcriptional block suggest that methylation of the 5′ CpG island is a common mechanism for CDKN2 inactivation in human cancers. In this study, analysis of genomic DNA indicated that altered CDKN2 expression in primary CaPs was more frequently due to down-regulation (five of seven) than deletion of the gene (two of seven). Furthermore, we observed induction of CDKN2 transcripts in two nonexpressors PC-3 and TSU-Pr11 after treatment with the demethylating agent, 5-aza-2′-deoxycytidine. These results are consistent with the observation of methylation of the 5′ CpG island of CDKN2 in these two cell lines (31). These data show that de novo methylation of the CDKN2 gene is one mechanism for the functional inactivation of CDKN2 in human prostate cancer cell lines. However, hypermethylation of the 5′ CpG island should be examined in human CaPs to determine whether this is the mechanism of CDKN2 silencing in those specimens that retain the gene but do not express the mRNA.

We discovered no association between low CDKN2 expression and pathological stage or Gleason score; however, it is worth noting that a substantial fraction of low-stage (55% of stage B) and low-grade (43% of grades 3–6) tumors showed abnormally low CDKN2 mRNA expression. In addition, the high frequency of alterations in CaP tissues, but not in BPH or noncancerous prostate tissues, suggests that the inactivation of CDKN2 may be an important genetic event in the process of prostate cancer development. Thirteen of 22 (59%) primary CaP specimens retrieved following treatment with CAB showed abnormally low CDKN2 mRNA. The cause of this increased frequency compared to untreated CaP is unknown. It is possible that abnormally low expression of CDKN2 prevents the appropriate apoptotic response of prostate cells to hormone withdrawal and, thus, may contribute to prostate cancer progression.

A correlation of high CDKN2 expression levels with RB1 alterations has been found in several types of cell lines (8–11, 47). Li et al. (10) demonstrated that transcription of CDKN2 is repressed by Rb1, providing evidence for a regulatory feedback loop. In the present study, we found that the DU145 cell line (with abnormal RB1) expressed high levels of CDKN2 mRNA, as did the one CaP patient specimen (PS290) with a truncated RB1 transcript. Two of the three CaP xenografts (CWR31 and CWR91) showed similarly high levels of CDKN2 expression; however, no mutations have yet been detected in their RB1 transcripts; thus, the cause for the high levels of expression is unknown. The presence of both abnormal CDKN2 and RB1 transcripts in the DU145 cell line indicates that the mutational inactivation of CDKN2 and RB1 are not mutually exclusive
events. Although this type of double knock-out in the RB pathway is unique and may reflect tissue culture selection, this particular mutation in CDKN2 (codon 84, Asp to Tyr) has not yet been tested in a functional assay (48).

Further studies are required to determine the biological role of CDKN2 as a putative tumor suppressor in the carcinogenesis of human prostate cells. It will also be important to examine whether CDKN2 abnormalities are useful as a prognostic marker for hormone ablation failure or as a target for the design of improved therapies for CaP patients, such as DNA demethylating agents.

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