Comprehensive Analysis of CDKN2A Status in Microdissected Urothelial Cell Carcinoma Reveals Potential Haploinsufficiency, a High Frequency of Homozygous Co-deletion and Associations with Clinical Phenotype

Emma J. Chapman, Patricia Harnden, Philip Chambers, Colin Johnston, and Margaret A. Knowles

Abstract

Purpose: There are significant differences in reported frequencies, modes of inactivation, and clinical significance of CDKN2A in urothelial cell carcinoma (UCC). We aimed to address these issues by investigating all possible modes of inactivation and clinicopathologic variables in a single tumor panel.

Experimental Design: Fifty microdissected UCCs were examined. CDKN2A gene dosage (quantitative real-time PCR), allelic status (microsatellite analysis), hypermethylation (methylation-specific PCR), mutation status (denaturing high-performance liquid chromatography and sequencing), protein expression (immunohistochemistry), and clinicopathologic variables (stage, grade, and disease recurrence during follow-up) were assessed.

Results: Exon 2 was underrepresented in 20 of 46 (43%) and exon 1β in 21 of 46 (46%) of cases. Underrepresentation of exon 2 was accompanied by loss of heterozygosity (LOH) of 9p in 6 of 18 (30%) and of exon 1β in 11 of 19 assessable cases (58%). Overall, LOH of 9p was identified in 15/41 (37%). Homozygous deletion of exons 2 and 1β was detected in 16 of 46 (35%) and 10 of 46 tumors (22%), respectively. Co-deletion was most common, but exon 2–specific homozygous deletion was also detected. In tumors without homozygous deletion, p16 promoter hypermethylation was detected in 1 of 18 (6%). Hypermethylation of the p14ARF promoter or mutations in CDKN2A were not observed. Homozygous deletion of exon 2 or LOH on 9p were associated with invasion. Homozygous deletion of exon 2 or exon 1β was associated with recurrent disease.

Conclusions: These results confirm CDKN2A as a clinically relevant target for inactivation in UCC and show that the true frequency of alteration is only revealed by comprehensive analysis. Our results suggest that CDKN2A may be haploinsufficient in human cancer.

Loss of heterozygosity (LOH) of chromosome 9 is one of the most common events in urothelial cell carcinoma (UCC) of the bladder. Using multiple polymorphic markers, key regions of deletion have been identified on 9p and 9q. On 9p, the critical region includes the CDKN2A locus at 9p21 (1). CDKN2A encodes two proteins, p16 and p14ARF (p19ARF in mice), which are translated from alternatively spliced mRNA and each regulated by a unique promoter. p16 is encoded by exons 1α, 2, and 3 and p14ARF by an alternative exon 1, 1β, and the same exons 2 and 3 in an alternative reading frame (Fig. 1).

Inactivation of this locus is one of the most frequent genetic changes seen in human solid tumors and can occur by deletion, methylation, or mutation.

The importance of inactivation of CDKN2A in cancer development stems from the fact that its products are involved in the Rb and p53 tumor suppressor pathways. As its products are encoded by shared exons, inactivation of the locus has the potential to inactivate both pathways simultaneously. p16 inhibits the phosphorylation of Rb by cyclin D-CDK4/6 complexes and thus inhibits cell cycle progression in G1. p16 has a role in mediating senescence in human urothelial cells (2) and its loss is necessary for immortalization of some urothelial cancer cells in culture (3). In addition, p16 has other potential tumor suppressor effects, in modulating anchorage-independent growth (4), anoikis (5), angiogenesis (6), and tumor cell invasion (7). p14ARF inhibits the degradation of p53 by binding to hdm2 to help mediate p53-dependent G1 or G2 arrest or apoptosis. p14ARF also has p53-independent actions such as the inhibition of HIF-1 (8), interaction with topoisomerase (9), and the promotion of caspase-9-induced apoptosis (10).

Other members of the INK4 family of cyclin-dependent kinase (CDK) inhibitors such as p27 show haploinsufficiency in mice (11). Mouse models also suggest that partial down-regulation of p16 and/or p14ARF expression alone (12) may yield a phenotypic...
Generally no link between CDKN2A status and clinicopathologic variables has been found in UCC, although associations of LOH/homozygous deletion with larger tumor size and lower recurrence-free survival (15) have been described. As some modes of inactivation can coexist, an incomplete picture of gene status is achieved from studies concentrating on only a single mode of inactivation. We set out to do a comprehensive study of CDKN2A status, investigating gene dosage, allelic status, hypermethylation, mutation, protein expression, and clinicopathologic variables in the same panel of microdissected UCCs.

Materials and Methods

Tissue samples. Tissue was obtained with consent and the approval of the Local Research Ethics Committee from patients undergoing transurethral resection for UCC at St. James’s University Hospital, Leeds, United Kingdom, in 1999 to 2001. A blood sample was collected into EDTA tubes and high molecular weight DNA was extracted using the Nucleon Genomic DNA extraction kit (Tepnel, Manchester, United Kingdom). Fifty tumors were fixed in 10% formalin and paraffin embedded. If possible, an unfixed piece of the same tumor was frozen in liquid nitrogen. Tumors were graded according to the 1973 WHO recommendations and staged according to tumor-node-metastasis classification. Ten-micrometer paraffin-embedded sections were stained with H&E and tumor cells were isolated from contaminating stroma, normal tissue, or immune infiltrate using laser capture microdissection. Despite numerous studies, there are significant differences in reported frequencies and modes of inactivation can coexist, an incomplete picture of gene status is achieved from studies concentrating on only a single mode of inactivation. We set out to do a comprehensive study of CDKN2A status, investigating gene dosage, allelic status, hypermethylation, mutation, protein expression, and clinicopathologic variables in the same panel of microdissected UCCs.

Detection of loss of heterozygosity by fluorescent microsatellite analysis. Monoplex fluorescent PCR was done for microsatellite markers D9S1748 and D9S1749 on 9p and D9S176 and D9S272 on 9q. Primer sequences, sizes of PCR products and locations are available at The Genome Database (http://gdbwww.gdb.org). Forward primers were labeled with tetrachlorofluorescein or 6-carboxyfluorescein. Reactions (25 μL) contained 10 μmol/L of each primer, 1.5 mmol/L MgCl2, 1 unit AmpliTaq Gold (Applied Biosystems, Warrington, United Kingdom), 0.7 to 1.2 = retention of copy number, and >1.2 = overrepresentation. These ratios were used upon the assumption that because the UCC samples had been microdissected, normal tissue contamination of the specimen was <20%.

Fig. 1. CDKN2A locus. Exons are represented as boxes and coding regions are shaded. p16 is encoded by exons 1, 2, and 3. p14^{ARF} is encoded by an alternative first exon, 1′, and the same exons 2 and 3 in an alternative reading frame. Sequences encoding p16 (black) and sequencing encoding p14^{ARF} (gray).
0.2 mmol/L deoxynucleotide triphosphates (Amersham Biosciences International, Berkshire, United Kingdom), 1× PCR buffer, and 5 ng template. Thermocycler conditions were 95°C for 10 minutes followed by 33 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then 72°C for 7 minutes. PCR products were diluted one in four with H2O and 1 μL was added to 10 μL Hi-Di formamide and 0.25 μL Rox 500 standards (Applied Biosystems). Samples were denatured at 95°C for 2 minutes and electrophoresis was done using a 3100 Genetic Analyser (Applied Biosystems), with a 36-cm capillary array and denaturing POP4 polymer (Applied Biosystems). Data was exported into GeneScan software (v 3.1.2). In many cases, a simple visualization of the electropherograms allowed identification of tumors with retention of heterozygosity or LOH (Fig. 2). Where the classification was unclear, allelic balance was calculated using the equation: allelic ratio = (peak height blood allele 1 / peak height blood allele 2) / (peak height tumor allele 1 / peak height tumor allele 2), where allele 1 is the smaller sized allele and allele 2 is the larger one. As samples had been microdissected, normal contamination was minimal. Therefore, if one allele was absent in the tumor (LOH), the peak height produced from contamination was <20% the expected allele height, compared with the allele in the blood. A reduction of >80% of allele height ratio in tumor compared with blood DNA was scored as LOH. Reductions in ratios between 0% and 79% were scored as retention of heterozygosity. A reduction of >80% in height of both alleles relative to the blood was scored as homozygous deletion. PCR reactions that seemed to show homozygous deletion were repeated. To discriminate between a failed PCR and homozygous deletion, the DNA was required to generate product from at least one allele of another marker.

Mutation-specific PCR. One microgram of tumor DNA plus 10 μg salmon sperm DNA (Sigma, St. Louis, MO) in a total volume of 100 μL H2O was treated with the CpGenome DNA modification kit (Intergen, Oxford, United Kingdom) and eluted into 60 μL Tris-EDTA. Methylation-specific PCR was done for p16 and p14 ARF promoter regions on tumors without homozygous deletion, as described previously (23). PCR products were separated in 2% agarose gels and visualized under ultraviolet light. PCR and homozygous deletion, the DNA was required to generate fragment A-AGCTTCCTTTCCGTCATGC/ GCAGCAC-CACCAGGTGT, fragment B-AGCCCAACATGCCCG/GAC-AGGT-CACGGGCGAG, and fragment C-TGGACGTTTGGCAGTGC/GAAACGTCTGCAGGTCACCAATTC. PCR products were denatured at 95°C for 5 minutes and then cooled to 65°C. Denaturation high-performance liquid chromatography was carried out at the temperatures determined by the “DHPLC Melt” program (24). A Transgenomic WAVE Nucleic Acid Fragment Analysis system and DNAsep column (Transgenomic, Cheshire, United Kingdom) were used. Data analysis was by visual inspection of chromatograms by two independent observers. Cases with possible polymorphisms or mutations were sequenced.

Immunohistochemistry. For detection of p16, 5-μm deparaffinized and rehydrated sections were treated with 3% hydrogen peroxide (Sigma), Avidin Biotin blocking kit (Vector Laboratories, Peterborough, United Kingdom), and then the catalyzed signal amplification system (DakoCytomation, Buckinghamshire, United Kingdom). Primary antibody was mouse anti-p16 (Ab-7; Labvision, Suffolk, United Kingdom) at 1:1,600 for 1 hour. Slides were counterstained with hematoxylin, dehydrated, and mounted. Positive control for p16 expression was cervical carcinoma. p16 was scored as negative, heterogeneous, or strong expression in >50% of tumor cells (25) by three independent observers without knowledge of tumor identity or genotype.

Statistical analyses. Statistical analyses of homozygous deletion of CDKN2A exon 2 (p16) or 1b (p14ARF) with tumor presentation, stage, grade, or recurrence were done using a two-tailed Fisher’s exact test and SAS (version 8.0) computer software. The stages and grades of the 45 tumors, for which gene dosage and clinicopathologic information was available were pTa G1 (six cases), pTa G2 (19 cases), pT1 G2 (five cases), pT1 G3 (eight cases), pT2 G2 (two cases), and pT2 G3 (five cases). pT1 and pT2 tumors were grouped together as invasive tumors. Statistical analyses were also done in relation to LOH on 9p or 9q or on both 9p and 9q. P ≤ 0.05 was taken to indicate a statistically significant relationship. P values were not adjusted for multiple comparisons.

Results

CDKN2A status. Gene dosage at CDKN2A exon 2 and exon 1b was successfully measured in 46 of 50 tumors. Three samples were excluded as DNA yield or quality was not sufficient and one was excluded because it was found to be a biopsy of a previous resection site. Homozygous deletion of exons 2 and 1b was detected in 16 of 46 (35%) and 10 of 46 (22%) of tumors, respectively. Specific deletion of p14ARF exon 1b was not detected. Exon 2–specific deletions were identified in six cases. In five of these, homozygous deletion was accompanied by underrepresentation of p14ARF exon 1b and in one case, with retention of exon 1b gene dosage (Table 1; Fig. 3). A reduction in gene dosage was the most common event at CDKN2A. Exon 2 was underrepresented in 20 of 46 (43%) of tumors and exon 1b in 21 of 46 (46%). We were interested to determine whether frequent reduction in CDKN2A gene dosage corresponded to LOH in the 9p21 region. Microsatellite marker-based LOH...
analysis was successfully done in 41 of 46 tumors (Figs. 2 and 3). Five cases were either noninformative for the markers investigated, constitutive DNA was not available, or the PCR reaction repeatedly failed. LOH of at least one marker on 9p was detected in 15 of 46 tumors (33%). Underrepresentation of exon 2 was accompanied by 9p LOH in 6 of 18 (30%) and of exon 16 in 11 of 20 assessable cases (55%). Allelic balance at a 9q marker (D9S972 or D9S176) was measured to obtain information on the relative status of 9q to 9p in the same tumors. LOH on 9q was detected in 24 of 44 tumors (55%). Predicted loss of an entire parental homologue (i.e., LOH of at least one marker on both q and p arms) was detected in 12 of 40 tumors (30%). Overall, CDKN2A was altered by a reduction in gene dosage and/or LOH in 36 of 46 tumors (78%) indicating that alteration of this locus is involved in the genesis of the vast majority of UCC.

**Mutation and methylation analyses.** No mutations were detected in CDKN2A exons 1a, 1b, 2, or 3. Previously reported polymorphisms (26, 27) were detected in exon 3 in tumors 355, 463, and 467 and in exon 2 in tumor 393. For tumors without homozygous deletion, PCR was done with primers specific for either the nonmethylated or methylated p16 or p14ARF promoter sequence. As these tumors were not microdissected, all should have produced a product with at least one primer set. DNA from UCC 364 failed to produce a PCR product for either methylated or unmethylated p16 and therefore was not included in the analysis. In contrast to the 56% previously described (18), hypermethylation of p14ARF promoter was not seen in any tumor but was detected in positive control DNA (Fig. 4). Hypermethylation of the p16 promoter was detected in 1 of 18 tumors examined (UCC 372) and was always detected in the positive control DNA (data not shown). All other tumors produced a product for the nonmethylated p16 sequence. No product was produced from negative or no template controls with either primer pair.

**Immunohistochemistry.** Immunohistochemistry was done on paraffin sections of 43 of 46 tumors and was concordant with CDKN2A status in that homozygous deletion of exon 2 correlated with absence of p16 expression in tumor cells (Fig. 5A). Twelve of 15 cases with homozygous deletion showed a complete lack of p16 staining in tumor cells yet positive internal control cells in the stroma. Only three tumors had staining patterns potentially discrepant with gene dosage. One case (387) was negative in its major papillary component but did contain a small invasive component with positive staining. UCCs 446 and 360 (gene dosages indicating homozygous deletion) had rare positive cells. It is possible that these areas were not captured during microdissection and consequently the tumors were scored as having homozygous

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**Table 1. Patterns of CDKN2A gene dosage as determined by quantitative real-time PCR**

<table>
<thead>
<tr>
<th>Exon 1β</th>
<th>Exon 2</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD</td>
<td>HD</td>
<td>10/46 (22)</td>
</tr>
<tr>
<td>Underrepresentation</td>
<td>HD</td>
<td>5/46 (11)</td>
</tr>
<tr>
<td>Retention</td>
<td>HD</td>
<td>1/46 (2)</td>
</tr>
<tr>
<td>Underrepresentation</td>
<td>Underrepresentation</td>
<td>16/46 (35)</td>
</tr>
<tr>
<td>Retention</td>
<td>Underrepresentation</td>
<td>4/46 (9)</td>
</tr>
<tr>
<td>Retention</td>
<td>Retention</td>
<td>10/46 (22)</td>
</tr>
</tbody>
</table>

Abbreviation: HD, homozygous deletion.

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**Fig. 3.** Gene dosage and allelic balance at CDKN2A and 9q. Boxes represent gene dosage relative to PFKL, determined by quantitative real-time PCR: retention of copy number (white), underrepresentation (gray), homozygous deletion (HD, black). Allelic balance determined by microsatellite marker analysis: retention (R), LOH, and noninformative/no data (−). UCC 372 had hypermethylation of p16 promoter region.
deletion by genetic analysis. p16 expression was detected in all tumors without homozygous deletion of exon 2, but there was no clear correlation between gene dosage and level of protein expression. The pattern of p16 expression ranged from homogeneous to heterogeneous (Fig. 5B). Tumor 447 (underrepresentation of exon 2 with LOH) contained distinct areas of negative and positive staining that seemed to be two tumor clones (Fig. 5C).

**Correlation of CDKN2A status with clinicopathologic variables.** Clinicopathologic information was obtained in retrospect. Eleven tumors were disease recurrences and the remainder were first presentations. Clinicopathologic data was not available for UCC 500. Thirty-two of 45 patients (71%) experienced recurrent disease during the follow-up period. Homozygous deletion of exon 2 or LOH on 9p, 9q, or both 9p and 9q were associated with invasion (Table 2). Homozygous deletion of exon 2 or exon 1β was not predictive of disease recurrence but was more common in recurrences rather than initial presentations. Similarly, LOH on 9q was also more common in recurrences but when present in the initial tumor was not predictive of disease recurrence.

**Discussion**

We have confirmed that chromosome 9 is a major target for alteration in UCC. Thirty-four of 40 (85%) assessable cases (those with gene dosage information for CDKN2A plus allelic balance information for at least one marker on 9p and 9q) had some alteration of the chromosome. CDKN2A is likely to be the key target of these chromosome 9 alterations as only 10 of 46 UCC (22%) retained gene dosage of both p16 and p14ARF. One of these tumors (UCC 372) potentially had silencing of the p16 gene by promoter hypermethylation. As
The frequency of homozygous deletion detected here was similar to that previously detected by us using the same method on a separate panel of nonmicrodissected UCC (22). However, a clear advantage of the use of microdissected material and consequently a higher gene dosage ratio threshold for retention of copy number was the ability to detect a higher frequency of underrepresentation than previously reported.

Of the six tumors with p16 exon 2–specific deletions, four were accompanied by a reduction in gene dosage of p14ARF and LOH at flanking markers. This may represent LOH at 9p21, with a targeted p16-specific homozygous deletion, consistent with the classic two-hit hypothesis of tumor suppressor gene inactivation. A reduction in gene dosage of p14ARF exon 1β accompanied by homozygous deletion of p16 exon 2 is likely to be of phenotypic significance. Mice null for p16 and homozygous for p19ARF are tumor prone but those null for p16 only are not (33). Similarly, Carnero et al. (13) showed that incomplete inhibition of p19ARF expression in mouse embryonic fibroblasts was sufficient to cause a phenotypic effect in vitro. These studies indicate that a reduction in p19ARF gene dosage may have an independent role in mouse tumorigenesis. Whether this is also the case in human cancer is not yet clear. A reduction in gene dosage (underrepresentation) was the most frequent event at CDKN2A, indicating that the locus may be haploinsufficient. Indeed, a statistically significant association of LOH in the CDKN2A region with invasive tumors and high grade was observed and we were unable to find evidence for inactivation of the retained allele by any other mechanism. LOH was seen at flanking markers and so is the likely cause of underrepresentation in approximately one third of cases. LOH of one or more markers on 9p21 was seen in 33% of tumors, but it is likely that we have underestimated the frequency of 9p LOH due to the frequent homozygous deletion in our tumor panel. Certainly, in samples 498, 387, 446, and 351, a large homozygous deletion spanning the 9p21 region encompassed all the markers we investigated. Up to 40% of stage T1 UCC and virtually all higher stage tumors are aneuploid not diploid (36). Therefore, aneuploidy or small hemizygous deletions could explain the 11 cases where exon 2 and the 12 cases where exon 1β were underrepresented in the absence of detectable LOH. These tumors had retained at least one copy of both alleles in the CDKN2A region but had fewer copies of 9p than of PFKL. It is also possible that apparent underrepresentation is a reflection of a heterogeneous tumor containing some cells with homozygous deletion and some with retention of copy number. Indeed, “heterogeneous” and “clonal” patterns of p16 expression were visible in some tumors (Fig. 5B and C). Alternatively, tumors showing a reduction in gene dosage but no LOH may contain small hemizygous deletions targeting the exons but not flanking markers.

The frequency of p16 hypermethylation (6%) detected in our study was lower than previously reported (18) and methylation of the p14ARF promoter was not seen. The reason for the discrepancy with the study of Dominguez et al. is unclear but may be because of the small size of our tumor panel. The only other study that has examined p14ARF and p16 promoter methylation in bladder tumors, detected hypermethylation of p14ARF in 5% of cases and of p16 in 9% (37).

Other studies have concluded that mutations at CDKN2A are rare in UCC. Accordingly, we found no evidence of point

### Table 2. Statistically significant ($P \geq 0.05$) associations between CDKN2A status and clinicopathologic variables

<table>
<thead>
<tr>
<th>Status</th>
<th>n</th>
<th>Association</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD of p16 exon 2</td>
<td>45</td>
<td>Invasion (pT1 and above)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>Tumor was not a first presentation</td>
<td>0.03</td>
</tr>
<tr>
<td>HD of p14ARF exon 1β</td>
<td>45</td>
<td>Invasion (pT1 and above)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>Tumor was not a first presentation</td>
<td>0.02</td>
</tr>
<tr>
<td>LOH 9p</td>
<td>44</td>
<td>Tumor was not a first presentation</td>
<td>0.006</td>
</tr>
<tr>
<td>LOH on 9p and 9q</td>
<td>41</td>
<td>Invasion (pT1 and above)</td>
<td>0.008</td>
</tr>
</tbody>
</table>

NOTE: Two-tailed Fisher’s exact test.

Abbreviations: HD, homozygous deletion; LOH, less of heterozygosity.
mutations. Three common single nucleotide polymorphisms were identified. As these occurred in the 3' untranslated region, they do not cause an amino acid change but may affect expression of p16 or p14ARF (38). Neither single nucleotide polymorphism is reported to affect bladder cancer risk, but both have been associated with shorter survival of bladder cancer patients and one with disease progression (27).

A key clinical problem in UCC is disease recurrence after initial resection and currently all patients are followed by regular cystoscopies to treat recurrences early and to prevent progression. However, up to 30% of patients remain tumor free and therefore undergo unnecessary investigations. Current clinical and pathologic variables cannot reliably assess recurrence risk or identify the smaller group of patients with tumors that are destined to progress to muscle invasion and who would benefit from more aggressive initial therapy. Many studies have considered the relationship between the status of p16 and/or p14ARF genes and have found no relationship with recurrence (39) or stage and grade (34). However, LOH of 9p21 has been linked with a more aggressive phenotype (40) and an association of LOH/homozygous deletion with reduced recurrence-free survival and larger tumor size has been reported (15). Here we have found statistically significant associations between CDKN2A status with invasion beyond the basement membrane and the presence of recurrent disease. As the tumor panel was small, we feel that this highlights potential relationships of interest that merit further investigation rather than providing robust correlations. However, high accuracy of detection of homozygous deletion may render such associations clear even in a small population.

In agreement with previous studies, homozygous deletion of exon 2 or exon 1β was not predictive of tumor recurrence. However, a higher frequency of homozygous deletion was detected in tumors that were recurrent disease compared with initial presentations. This suggests that although homozygous deletion of CDKN2A is not predictive of recurrence, it may be an event involved in the development of the recurrent phenotype. If so, this raises the question of whether some primary UCC are already "preprogrammed" to recur or whether this phenotype is acquired later. If homozygous deletion of p16 is necessary for recurrence, post-resection gene therapy to restore p16 expression in residual tumor cells or the induction of p16 expression by pharmacologic means may be a way to overcome this key clinical problem. CDKN2A status in primary and recurrent tumors from the same patient has not yet been examined and this will be an interesting area of investigation.

An association of LOH on 9q but not on 9p with recurrent tumors was also observed suggesting that loss of function of a 9q gene is independently involved in recurrence. Indeed, TSC1 and DBC1, tumor suppressor genes implicated in UCC, reside on 9q (41, 42). Our present results therefore support the notion that genes on both arms of this chromosome play a key role in UCC development. LOH on both 9p and 9q simultaneously was seen in approximately one third of tumors. LOH spanning both p and q arms has been associated with an increased risk of recurrence (43), which has been attributed to loss of the TSC1 gene at 9q34 rather than the 9p21 region. Here, this association was not observed but LOH of both chromosome arms was associated with invasion indicating a contribution to tumor phenotype. An association of p16 loss with invasion in UCC and other tumors has been suggested (44, 45). However, we report the first significant association of homozygous deletion of exon 2 with invasion. p16 has cell cycle–independent functions in promoting anoikis (4), matrix-dependent cell spreading (46), and directional motility (7), all of which could contribute to invasion. An association of LOH on 9p21 with invasive tumors (pT1 and above) was also identified and was more significant than that with homozygous deletion. Overall, LOH of 9p21 was the most frequent event at CDKN2A and was always accompanied by either a reduction in gene dosage or homozygous deletion of exon 2 or both exon 2 and exon 1β. As LOH was not always accompanied by homozygous deletion, this raises the possibility that underrepresentation of CDKN2A rather than complete deletion may be sufficient to contribute to the invasive phenotype and that subsequent homozygous deletion is not of any further benefit to invasion but may contribute to other characteristics. LOH on 9q was not associated with invasion. Thus, LOH of CDKN2A seems of greatest significance to the invasive phenotype.

The status of p14ARF exon 1β was not associated with stage or grade. However, this does not rule out a functional role for inactivation of this gene in UCC. The role of p14ARF as a tumor suppressor in human cancer remains unclear. Its loss can cause inappropriate cell cycling. However, cell cycle–independent functions have also been described, including an interaction with topoisomerase I (9), which is involved in DNA supercoiling during transcription and replication. Interestingly, unlike the p53-dependent functions, this function required exon 2–but not exon 1β–encoded sequence (30). p14ARF is also reported to inhibit the transcriptional ability of HIF-1 (8), and p19ARF has effects on angiogenesis in mouse models (47, 48), a role which may be important in papillary UCC, which are highly vascular. Thus, p53-independent functions of p14ARF have the potential to be tumor suppressive and might be associated with clinicopathologic variables such as microvessel density, which were not investigated in this study.

In conclusion, the use of laser capture microdissection and quantitative real-time PCR has allowed us to make accurate measurements of CDKN2A gene dosage and to uncover potential phenotype-genotype relationships that now require confirmation in a larger tumor panel. Complementation of gene dosage data with LOH, methylation, and mutations analyses has provided the most comprehensive study of the CDKN2A in UCC to date. A key question is whether underrepresentation of CDKN2A is sufficient for a phenotypic effect and whether homozygous deletion of either or both genes confers some additional advantage. The frequent but not compulsory coinactivation of the two genes suggests that both have important and discrete tumor suppressor activities, as expected from their different physiologic roles. With this knowledge, we are better placed to investigate genotype-phenotype relationships and clinical significance of inactivation of these genes in UCC. As a model for the role of deletion of CDKN2A in UCC is developed, it will be important to consider that the two pleiotropic proteins it encodes can potentially interact with factors involved in cell cycle control, angiogenesis, apoptosis, and cell migration.

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