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DNA Methylation and the Mechanisms of *CDKN2A* Inactivation in Transitional Cell Carcinoma of the Urinary Bladder

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SUMMARY: Alterations of the *CDKN2A* locus on chromosome 9p21 encoding the p16^{INK4A} cell cycle regulator and the p14^{ARF1} p53 activator proteins are frequently found in bladder cancer. Here, we present an analysis of 86 transitional cell carcinomas (TCC) to elucidate the mechanisms responsible for inactivation of this locus. Multiplex quantitative PCR analysis for five microsatellites around the locus showed that 34 tumors (39%) had loss of heterozygosity (LOH) generally encompassing the entire region. Of these, 17 tumors (20%) carried homozygous deletions of at least one *CDKN2A* exon and of flanking microsatellites, as detected by quantitative PCR. Analysis by restriction enzyme PCR and methylation-specific PCR showed that only three specimens, each with LOH across 9p21, had bona fide hypermethylation of the *CDKN2A* exon 1α CpG-island in the remaining allele. Like most other specimens, these three specimens displayed substantial genome-wide hypomethylation of DNA as reflected in the methylation status of LINE L1 sequences. The extent of DNA hypomethylation was significantly more pronounced in TCC with LOH and/or homozygous deletions at 9p21 than in those without (26% and 28%, respectively, on average, versus 11%, $p < 0.0015$). No association of LOH or homozygous deletions at 9p21 with tumor stage or grade was found. The data indicate that DNA hypermethylation may be rare in TCC and that deletions are the most important mechanism for inactivation of the *CDKN2A* locus. The predominance of allelic loss may be explained by its correlation with genome-wide DNA hypomethylation, which is thought to favor chromosomal instability and illegitimate recombination. (*Lab Invest 2000, 80:1513–1522*).

Inactivation of the *CDKN2A* locus at chromosome 9p21 is one of the most frequent genetic changes in human cancers (Kamb et al, 1994). This unusual locus encodes two distinct proteins (Quelle et al, 1995) involved in two pathways crucial for the regulation of cell proliferation and the control of genomic integrity. The p16^{INK4A} protein, encoded by exons E1α, E2, and E3, is a cell cycle regulator that limits the activity of cyclin-dependent kinases and inhibits entry into S-phase when it is accumulated. The p14^{ARF1} protein is encoded by E1β, E2, and E3 in a different reading frame, is induced during cell proliferation and supports activation of p53 by blocking the p53 inhibitor, MDM2. Reported mechanisms of *CDKN2A* inactivation in cancer cells comprise deletion, mutation, and promoter hypermethylation. The frequencies of *CDKN2A* inactivation and the relative importance of each of these inactivation mechanisms differ considerably between different cancers (Ruas and Peters, 1998).

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Loss of chromosome 9 has long been recognized as the most characteristic chromosomal aberration in transitional cell carcinoma (TCC) of the urinary bladder (Knowles, 1999; Orntoft and Wolf, 1998). Many TCC display loss of one entire chromosome 9, others show loss of regions only on the short (9p) or the long arm (9q). Although the genes impaired by changes on 9q have not been identified, overwhelming evidence indicates that *CDKN2A* is an important locus targeted by alterations on chromosome 9p. Homozygous deletions of this locus have been observed in several studies (Balazs et al, 1997; Baud et al, 1999; Orlow et al, 1995, 1999; Packenham et al, 1995; Williamson et al, 1995) and hypermethylation of the CpG island around exon E1α of *CDKN2A* has been observed in cell lines and primary tumors (Akao et al, 1997; Gonzalez-Zulueta et al, 1995; Gonzalgo et al, 1998; Merlo et al, 1995). Additionally, point mutations affecting both reading frames have been found, albeit with low frequency (Baud et al, 1999; Kai et al, 1995), and re-expression of p16^{INK4A} has been shown to suppress the growth of bladder carcinoma cells harboring an inactivated gene (Bender et al, 1998; Gonzalgo et al, 1998; Grim et al, 1997).

Despite ample work in this area, several issues have not been fully resolved. First, some studies in TCC and other carcinomas have observed two distinct regions of loss of heterozygosity (LOH) around *CDKN2A*,

suggesting that more than one tumor suppressor gene may be located in this region (Czerniak et al, 1999). Second, although hypermethylation of the CpG-island encompassing exon 1α is associated with transcriptional repression, the frequency of hypermethylation reported in different studies on TCC (Akao et al, 1997; Gonzalez-Zulueta et al, 1995; Gonzalgo et al, 1998; Merlo et al, 1995) has varied considerably. Third, the relationship between *CDKN2A* alterations and progression of TCC is a matter of debate. On one hand, loss of *CDKN2A* function seems to be required for immortalization of urothelial carcinoma cells in culture (Yeager et al, 1998). Accordingly, several studies have suggested that such alterations are associated with tumor progression (Orlow et al, 1999). On the other hand, loss of chromosome 9, including the region of the *CDKN2A* locus, has been reported in well-differentiated papillary TCC (Balazs et al, 1997; Hartmann et al, 1999; Jung et al, 1999), which rarely progress to invasiveness.

Although not all studies agree, the overall published data on the mechanisms of *CDKN2A* inactivation in TCC suggest that deletions are the most common alteration in this tumor type, whereas point mutations or DNA hypermethylation predominate in other tumors (Ruas and Peters, 1998). Because the various modes of inactivation are probably functionally equivalent, the causes for this difference must be sought in the mechanisms of mutation characteristic for each tumor type. In this regard, it may be relevant that early invasive TCC or their presumptive precursors such as high grade papillary tumors (pTaG3) and carcinoma in situ (CIS) often show a high number of chromosomal alterations (Kallioniemi et al, 1995; Presti et al, 1991; Zhao et al, 1999). This chromosomal instability may be promoted by the loss of p53 function in many TCC that is associated with a high risk of progression (Esrig et al, 1994). However, TCC are also characterized by a high prevalence of genome-wide DNA hypomethylation, which is extensive in some cases (Florl et al, 1999; Jürgens et al, 1996). Although focal DNA hypermethylation contributes to the inactivation of individual genes such as *CDKN2A*, there is evidence that genome-wide DNA hypomethylation in tumors is associated with an increased risk of illegitimate recombination, gene deletion and chromosome loss (Ehrlich, 2000; Schulz, 1998). Moreover, increased frequencies of chromosomal loss and gene deletions have been observed in patients with the inherited immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome in which hypomethylation of certain repetitive sequences occurs (Jeanpierre et al, 1993; Xu et al, 1999), and in mice with decreased levels of methylation caused by targeted deletions of DNA methyltransferase 1 (Chen et al, 1998). Therefore, the high prevalence of DNA hypomethylation might explain the predominance of deletions being the mechanism of *CDKN2A* gene inactivation in TCC.

These issues were addressed in the present study, based on the analysis of 86 TCC samples employing LOH analysis by quantitative multiplex PCR, detection of homozygous deletions by quantitative PCR, DNA hypermethylation analysis by restriction enzyme PCR (RE-PCR) and methylation-specific PCR (MS-PCR), and quantification of genome-wide hypomethylation by Southern blot analysis of LINE L1 sequence methylation.

Results

LOH Analysis

LOH analysis was first performed using five microsatellite markers surrounding the *CDNK2* locus and distributed across 9p21 (Fig. 1). DNA from each tumor sample was compared with normal DNA isolated from leukocytes or with morphologically normal corresponding tissue. LOH analysis was performed in multiplex reactions (Fig. 2) with quantitative evaluation of signal intensities as detailed in the "Materials and

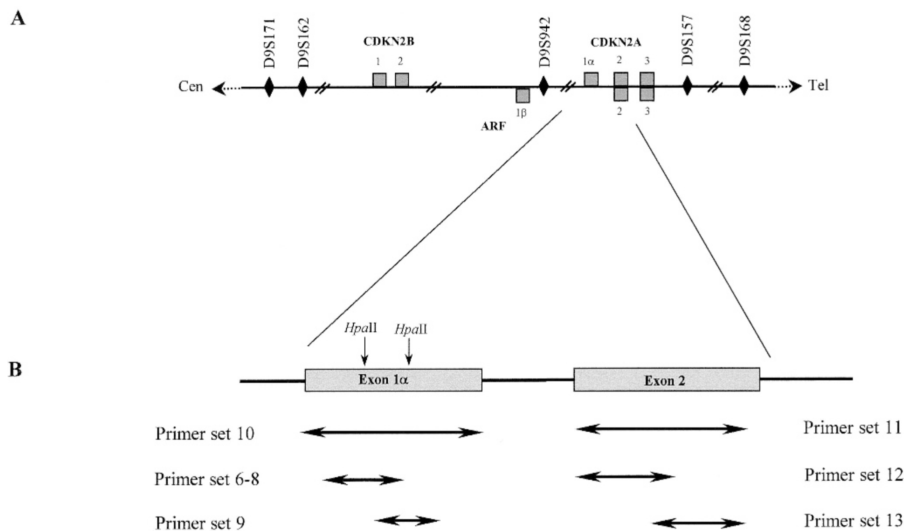

Figure 1.
Partial map of chromosome 9p21. *A*, Locations of the *CDKN2A* and *CDKN2B* loci and the microsatellites analyzed. *B*, DNA fragments amplified by PCR are indicated.

Methods" section. Thirty-four tumors reproducibly showed LOH for at least one marker. In eight specimens, LOH encompassed all informative markers. However, D9S942, D9S157, or both D9S942 and D9S157 showed apparent retention of heterozygosity in 23 tumors, although all flanking markers displayed definitive LOH. Few specimens (eg, specimens 60 and 79) exhibited localized loss (Fig. 3).

In most cases with apparent retention of heterozygosity at D9S942 or D9S157, for both alleles the band intensities were lower than the other microsatellite marker in the same lane, and also lower than the same microsatellite marker for the corresponding normal tissue sample (Fig. 2, Lanes 86 and 94). This situation strongly suggests loss of both alleles, ie, a homozygous deletion encompassing the microsatellites. To verify this assumption, all respective cases were analyzed for deletions of exon 1α and exon 2 of the *CDKN2A* gene by quantitative PCR (see "Materials and Methods"). In most of these cases, a homozygous deletion of exon 2 or both exons was observed (Fig. 3). Likewise, in cases where apparent retention of heterozygosity of DS157 or DS162 was observed alongside that of D9S942 (specimens 50, 91, and 186), homozygous deletions were present in the *CDKN2A* gene. Overall, homozygous deletions of the *CDKN2A* gene were detected in 17 of 34 cases (50%) with LOH.

Hypermethylation Analysis

In tumors with LOH but no homozygous deletion at the *CDKN2A* gene, hypermethylation of the CpG island at E1α was investigated as a possible cause of gene inactivation. Two different methods were used, RE-PCR and MS-PCR. DNA from the bladder carcinoma cell line T24 displaying hypermethylation of this CpG island was used as a positive control in all assays, and DNA from the bladder carcinoma cell line 5637, which lacks Rb and over-expresses p16^{INK4A}, was used as a negative control.

In RE-PCR, DNA from the CpG-island of interest that is not digested by the methylation-sensitive enzyme *Hpa*II is amplified using a primer pair flanking the restriction sites (cf. Fig. 1B). A PCR signal is obtained only if all *Hpa*II recognition sites are methylated. However, a signal can also be obtained from small amounts of remaining undigested DNA after extensive amplification. This amount can be determined by digestion with the methylation-insensitive *Hpa*II isoschizomer *Msp*I. Figure 4 shows that some samples yielded a weak signal after a limited number of PCR cycles even after extensive digestion with the methylation-insensitive restriction enzyme (see "Materials and Methods"). This constitutes a background against which the *Hpa*II signal must be measured to detect and quantify hypermethylation. Dilution of DNA from T24 cells, which contain a strongly hypermethylated E1α, with DNA from 5637 cells, which lack methylation in this region, showed that approximately 5% hypermethylation can be reliably detected over this background (data not shown). Among 16 tumor

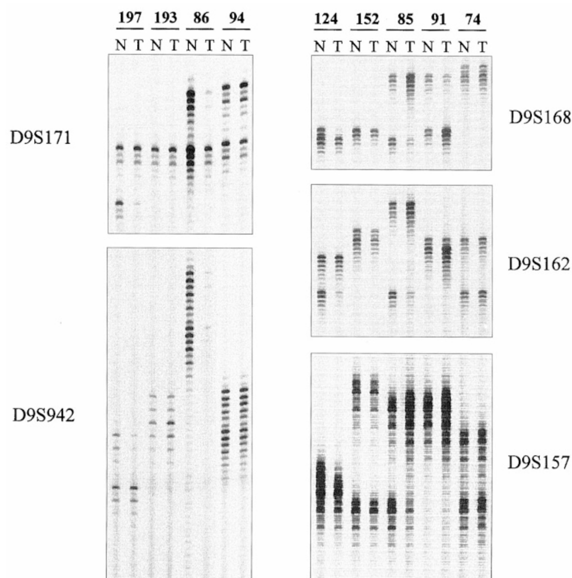

Figure 2.

Loss of heterozygosity (LOH) at 9p21 in bladder cancers. Typical results of LOH analysis. PCR products from paired normal (*N*) and tumor (*T*) specimens were loaded next to each other. *Left*, Microsatellite markers D9S171 and D9S942: Cases 197 and 94 show LOH for both markers, Case 193 shows LOH for D9S942 and D9S171 is noninformative. Sample 86 shows LOH for D9S171 and retention of both alleles of marker D9S942. *Right*, Microsatellite markers D9S168, D9S162, and D9S157: Cases 124 and 85 show LOH for all three markers; Sample 91 shows LOH only at D9S168 and D9S162. Cases 152 and 74 are noninformative or retain heterozygosity at the three markers.

Bladder tumor	D9S171	D9S162	D9S942	D9S157	D9S168	HD *CDKN2*
139	LOH	not informative	LOH	not informative	LOH	homozygous deletion
85	LOH	LOH	LOH	LOH	LOH	
27	LOH	LOH	LOH	LOH	not informative	
159	LOH	LOH	LOH	LOH	LOH	
118	not informative	not informative	LOH	LOH	LOH	
193	not informative	not informative	LOH	LOH	not informative	
13	LOH	LOH	not informative	not informative	LOH	
102	LOH	LOH	LOH	not informative	not informative	
114	not informative	LOH	retention	LOH	LOH	
98	LOH	LOH	retention	LOH	LOH	
93	LOH	LOH	retention	LOH	LOH	
86	LOH	LOH	retention	LOH	LOH	homozygous deletion
195	LOH	LOH	retention	LOH	LOH	homozygous deletion
122	LOH	LOH	retention	LOH	LOH	homozygous deletion
138	LOH	LOH	retention	LOH	LOH	homozygous deletion
57	LOH	LOH	retention	LOH	LOH	homozygous deletion
92	LOH	LOH	retention	LOH	LOH	homozygous deletion
124	LOH	LOH	retention	LOH	LOH	homozygous deletion
135	LOH	LOH	retention	not informative	LOH	homozygous deletion
81	LOH	not informative	retention	LOH	LOH	homozygous deletion
15	LOH	LOH	retention	LOH	not informative	homozygous deletion
176	LOH	LOH	retention	LOH	LOH	homozygous deletion
186	LOH	LOH	retention	retention	LOH	homozygous deletion
50	LOH	retention	retention	not informative	not informative	homozygous deletion
91	LOH	LOH	not informative	retention	LOH	homozygous deletion
89	LOH	LOH	not informative	not informative	LOH	homozygous deletion
157	LOH	LOH	not informative	LOH	not informative	homozygous deletion
100	LOH	LOH	not informative	retention	LOH	
182	not informative	LOH	LOH	retention	LOH	
169	LOH	LOH	LOH	retention	LOH	
60	not informative	retention	LOH	retention	retention	
197	LOH	not informative	LOH	retention	microsatellite instability	
79	retention	retention	retention	LOH	not informative	
39	retention	LOH	LOH	LOH	LOH	

Figure 3.

Summary of LOH analysis data. □, retention of heterozygosity; ■, LOH; ▨, not informative; ▦, microsatellite instability; ■, homozygous deletion.

samples showing LOH but no evidence for homozygous deletions at the *CDKN2A* locus, 3 samples (specimens 102, 182, and 193) were clearly and reproducibly positive in this assay. In contrast, in 17 samples without LOH, no samples had hypermethylation of the E1α CpG island above background. Furthermore, in several specimens with homozygous deletions, no samples showed evidence of hypermethylation.

In MS-PCR, DNA treated with bisulfite, which converts cytosine to uracil but does not react with methylcytosine, is amplified using two distinct primer pairs (Table 1), which selectively recognize either the converted or non-converted sequence. By diluting T24 DNA with 5637 DNA, the sensitivity of the method was determined to be less than 1:10. Using this method, the same three tumor samples with LOH in the 9p21 region (specimens 102, 182, and 193) showed hypermethylation, which was not found in any of the other samples from this group (Fig. 5). Among 21 carcinomas without evidence of LOH at 9p21, no samples were positive. Likewise, as expected, hypermethylation was detected in none of the eight specimens with homozygous deletions.

Genome-Wide Hypomethylation

Genome-wide hypomethylation was investigated in 84 TCC specimens with sufficient available DNA by Southern blot analysis of the methylation status of LINE L1 sequences, which comprise between 10% and 15% of the human genome (Kazazian and Moran, 1998). These sequences are almost completely methylated in normal somatic tissues. Their hypomethylation in TCC can be used as a measure of overall decreased methylation (Florl et al, 1999). In line with this previous report, L1 hypomethylation was detected in almost all of the tumor specimens (Fig. 6). As is evident in Figure 6, many of the samples without alterations at chromosome 9p21 had low degrees of hypomethylation, with an average level of hypomethylation of 11 $\pm$ 13% (mean $\pm$ SD). By comparison, the average level of hypomethylation in the samples with LOH at 9p21 but no homozygous deletion was 26 $\pm$ 18% and the average level of the samples with homozygous deletions was 28 $\pm$ 18%. The difference between either of the latter groups and the first group was statistically significant (Wilcox rank sum test: $p = 0.0015$ and 0.0001, respectively). All three specimens with hypermethylation at *CDKN2A* also displayed pronounced genome-wide hypomethylation.

Relation to Clinical Parameters

The relationship between alterations on chromosome 9p21 and clinical parameters is compiled in Table 2. Neither the presence of LOH at 9p21 nor the presence of homozygous deletions showed any significant relationship to tumor stage or grade. Specifically, the percentage of LOH at 9p21 or homozygous deletions at *CDKN2A* was almost the same between muscle-invasive tumors and earlier stages and between tumors with or without metastases. The relationship to tumor grade was also not statistically significant, although none of three well-differentiated tumors, but all G4 carcinomas, exhibited alterations. However, the frequencies of LOH and homozygous deletion were almost the same between moderately and poorly differentiated tumors.

Discussion

In accord with cytogenetic data identifying loss of chromosome 9 as the most frequent chromosomal alteration in TCC, molecular analyses have consistently shown a high rate of LOH at 9p21, often in the range of 30% to 40%, as was found in the present study. The reported frequencies of homozygous deletions at the *CDKN2A* locus have been more variable (Balazs et al, 1997; Baud et al, 1999; Orlow et al, 1995, 1999; Packenham et al, 1995; Williamson et al, 1995), the frequency in the present series (17 of 86, 20%) being in the higher range. These differences may reflect patient selection and geographical variation to some extent, but may be compounded by differences in methods. The high frequency of homozygous deletions detected in this study may be because of the use of a combination of multiplex PCR LOH analysis and a quantitative assay for gene copy number, as suggested by others (Cairns et al, 1998). Of note, a remarkably sensitive indicator of homozygous deletion in our study was an apparent retention of heterozygosity at microsatellite markers close to the *CDKN2A*

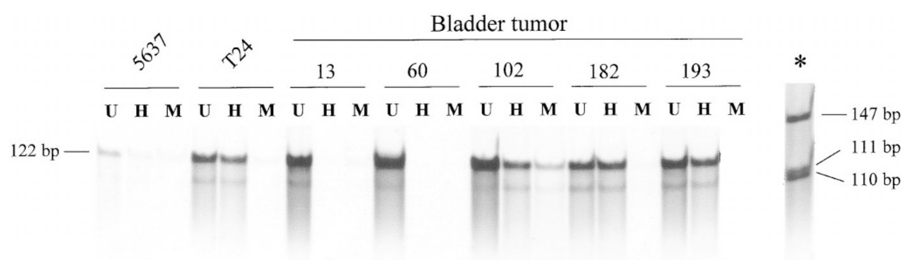

Figure 4.

Methylation analysis by restriction enzyme PCR (RE-PCR). Analysis of *CDKN2A* exon 1α in two human bladder cell lines and five bladder tumor samples. DNA was digested with *Hpa*II (**H**), or *Msp*I (**M**), or left undigested (**U**) before PCR amplification with primers described in Table 1. Uncut DNA (**U**) was amplified in all cases. After digestion with *Msp*I, either no signals or weak signals were obtained, indicating completeness of the restriction enzyme digestion. Cases 102, 182, and 193 and the T24 cell line show significant methylation as evidenced by a strong signal in the H lane. * Molecular weight marker pUC-*Msp*I digest.

Table 1. Oligonucleotides Used

Primer set	Designation	Target	Product size	Primer sequence	Accession number	Use
Set 1	AFM158×f12a AFM158×f12m	D9S168	227–247 bp	5′-*ggtttgtggtctttgtaagg-3′ 5′-tggtttgtttgtataactatcattg-3′	GDB188111	LOH
Set 2	AFM067×d3a AFM067×d3m	D9S157	133–149 bp	5′-*agcaaggcaagccacatttc-3′ 5′-tggggatgcccagataactatatc-3′	GDB187914	LOH
Set 3	CT.29.F CT.29.R	D9S942	100–130 bp	5′-*gcaagattccaaacagta-3′ 5′-ctcatcctgcggaaaccatt-3′	GDB370738	LOH
Set 4	AFM115yb4a AFM115yb4m	D9S162	172–196 bp	5′-*gcaatgaccagttaaggttc-3′ 5′-aattcccacaacaaatctcc-3′	GDB188003	LOH
Set 5	AFM186×c3a AFM186×c3m	D9S171	159–177 bp	5′-*agctaagtgaacctcatctctgtct-3′ 5′-accctagcactgatggtatagtct-3′	GDB188218	LOH
Set 6	p16-W sense p16-W antisense	p16 exon 1α	140 bp	5′-cagagggtggggcggaccgc-3′ 5′-cgggccgcggccgtgg-3′	X94154	MS-PCR
Set 7	p16-M sense p16-M antisense	p16 exon 1α	150 bp	5′-ttattagagggtggggcggatcgc-3′ 5′-gaccccgaaccgcgaccgtaa-3′	X94154	MS-PCR
Set 8	p16-U sense p16-U antisense	p16 exon 1α	151 bp	5′-ttattagtgggtggggtggattgt-3′ 5′-caaccccaaaccacaaccataa-3′	X94154	MS-PCR
Set 9	exon 1α sense exon 1α antisense	p16 exon 1α	122 bp	5′-agccttcggctgactggctgg-3′ 5′-ctggatcggcctccgaaccgta-3′	U12818	RE-PCR
Set 10	HD exon 1α/F HD exon 1α/R	p16 exon 1α	340 bp	5′-gaagaaagaggaggggctg-3′ 5′-gcgctacctgattccaattc-3′	U12818	HD
Set 11	HD exon 2/F HD exon 2/R	p16 exon 2	476 bp	5′-ggaaattggaaactggaagc-3′ 5′-tcagatcatcagtcctcacc-3′	U12819	HD
Set 12	HD exon 2/F SSCP exon 2/R	p16 exon 2	285 bp	5′-ggaaattggaaactggaagc-3′ 5′-caccagcgtgtccaggaagc-3′	U12819	SSCP
Set 13	SSCP exon 2/F HD exon 2/R	p16 exon 2	260 bp	5′-gccgaccccgccactctcac-3′ 5′-tcagatcatcagtcctcacc-3′	U12819	SSCP
Set 14	GAPDHE×G/F GAPDHE×G/R	GAPDH	160 bp	5′-aacgtgtcagtggtggacctg-3′ 5′-agtgggtgtcgctgttgaagt-3′	J04038	HD

LOH, loss of heterozygosity; MS-PCR, methylation-specific PCR; RE-PCR, restriction-enzyme PCR; HD, homozygous deletion; SSCP, single-strand conformation polymorphism.

* IRD 800-labeled.

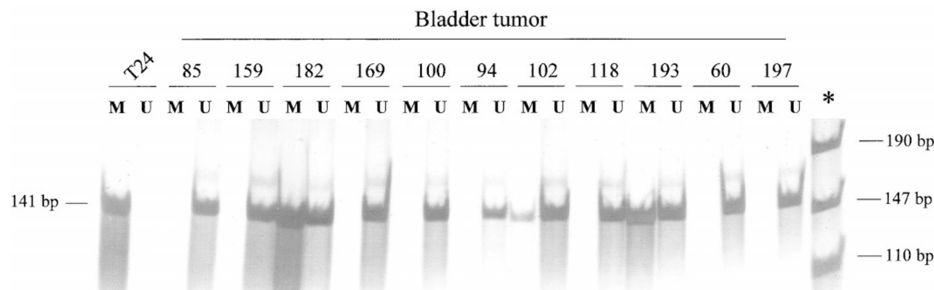

Figure 5.

Methylation-specific polymerase chain reaction (MS-PCR) analysis of *CDKN2A* exon 1α. Amplification of bisulfite-treated DNA from bladder cancer cell line T24 and 11 bladder tumor samples. Primer sets for unmethylated (*U*) and methylated (*M*) DNA were used as described in Table 1. The same samples as in Figure 4 react positively. * Molecular weight marker pUC-*Msp*I digest.

locus (Fig. 2). This apparent retention, probably caused by contamination with stromal cells, may have inspired reports of distinct regions of LOH at 9p in TCC and other cancers. Indeed, few studies have been performed on microdissected material, presumably because most TCC contain a high proportion of tumor cells anyhow, the amount of DNA obtained is limited, and the concentration and quality of DNA are difficult to ascertain for subsequent analyses. However, if the amounts of DNA used and the intensity of the PCR signal in multiplex reactions are closely monitored, this experimental artifact can obviously be used to identify cases with homozygous deletions.

Because all previous studies agree that point mutations in the coding region of the *CDKN2A* gene are rare in bladder cancer (Kai et al, 1995; Packenham et

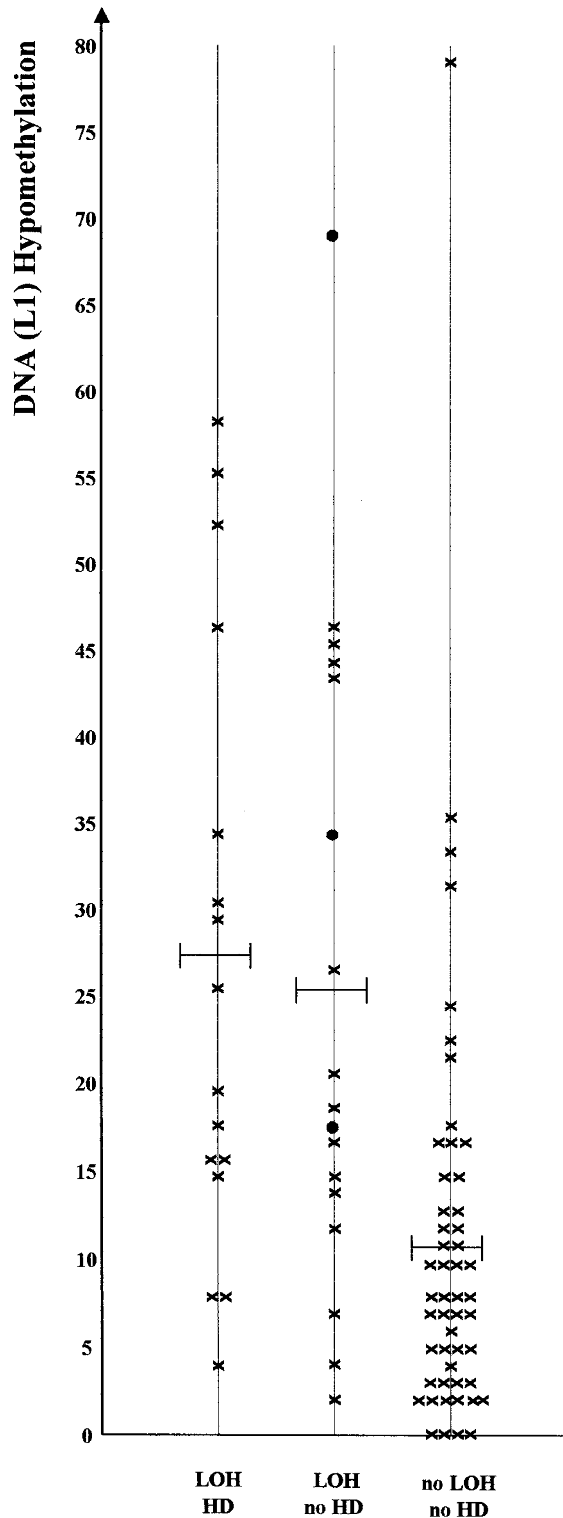

Figure 6.
Genome-wide hypomethylation and deletions at *CDKN2A* in bladder cancers. The extent of DNA hypomethylation, as reflected by L1 LINE. Hypomethylation is indicated for each transitional cell carcinoma (TCC) specimen. The three cases showing hypermethylation at *CDKN2A* in RE-PCR and MS-PCR (see Figs. 4 and 5) are indicated by a filled circle. *Left column*, Homozygous deletion at *CDKN2A* with LOH at flanking markers; *center column*, LOH at 9p21; *right column*, no alterations detected at 9p21. Average values for each group are indicated by horizontal bars. (HD, homozygous deletion)

al, 1995), we have not performed a systematic search for mutations in the present series of tumors. A preliminary screen by PCR, single-strand conformation polymorphism (SSCP), and sequencing has identified four cases with potential mutations yielding a figure in the expected range. However, even when the number of homozygous deletions and of point mutations affecting *CDKN2A* was somewhat underestimated, a substantial number (up to 13 of 34 cases) of carcinoma specimens with LOH without either of these alterations would remain. In three of these cases, DNA hypermethylation was detected by two independent methods (Figs. 4 and 5). Because hypermethylation was not detected in any specimen without LOH, the overall frequency of hypermethylation was lower than that in other studies (Akao et al, 1997; Gonzalez-Zulueta et al, 1995; Gonzalgo et al, 1998; Merlo et al, 1995). Technical reasons may account for this discrepancy. Specifically, RE-PCR tends to yield false positives because of incomplete digestion of DNA and over-amplification, particularly when used on archival tissue. False positives in MS-PCR can result from incomplete conversion of cytosines in the bisulfite reaction (Rein et al, 1998). However, because the highest frequency of hypermethylation has been reported in Japanese patients (Akao et al, 1997), geographical factors cannot be excluded as a cause of the differences. Nevertheless, the present data suggest that in initial reports the frequency of *CDKN2A* inactivation by hypermethylation in TCC may have been overestimated. Moreover, our data raise the possibilities that either a further unidentified mechanism exists for inactivation of the *CDKN2A* locus or that a sizable number of cases with LOH are associated with hemizygosity rather than complete inactivation of this gene.

Because monosomy of chromosome 9 is a frequent change in superficial TCC, it is not surprising that, in most investigations, LOH at 9p21 was not related to tumor stage or grade, as was found in the present study. However, because homozygous deletion of *CDKN2A* provides an alternative pathway to simultaneous loss of p53 and Rb function in vitro (Markl and Jones, 1998; Yeager et al, 1998), the finding in the present study that the frequency of homozygous deletions did not increase with tumor stage and grade (Table 2) is somewhat surprising. Notably, the specimens in this study did not include well-differentiated papillary tumors (pTaG1), but had all progressed to require major surgical intervention. The cumulative findings are best explained by the hypothesis that inactivation of *CDKN2A* is permissive for the progression of TCC but is not the actual stimulus.

Taken together, the data corroborate the hypothesis that chromosomal loss and deletions are the most important causes underlying *CDKN2A* inactivation in TCC. Because DNA hypomethylation has been experimentally linked to chromosome instability, we previously speculated that the high rate of chromosomal alterations found in TCC are related to the high prevalence of hypomethylation (Schulz, 1998). According to this hypothesis, the extent of DNA hypomethylation

Table 2. Relationship of Alterations at 9p21 to Stage and Grade in TCC*

	pTa			pT1			pT2			pT3			pT4		
	Σ	LOH	HD	Σ	LOH	HD	Σ	LOH	HD	Σ	LOH	HD	Σ	LOH	HD
G1	2	—	—	—	—	—	1	—	—	—	—	—	—	—	—
G2	3	1	2	4	3	2	8	4	2	7	2	3	1	—	—
		33%	66%		75%	50%		50%	25%		29%	43%			
G3	1	—	—	6	3	3	13	3	2	23	10	8	12	6	2
					50%	50%		23%	15%		44%	35%		50%	17%
G4	—	—	—	—	—	—	—	—	—	2	1	1	2	1	—
											50%	50%		50%	

TCC, transitional cell carcinoma; HD, homozygous deletion.
* One case of carcinoma in situ is not included.

should be related to the frequency of deletions overall and at any particular locus. The present study demonstrates this relationship for the *CDKN2A* locus (Fig. 6). All carcinomas with LOH or homozygous deletions displayed significant DNA hypomethylation and their average extent of hypomethylation was significantly higher. Significant hypomethylation was also observed in the three tumors exhibiting hypermethylation of the *CDKN2A* exon 1α underlining our previous demonstration that hypermethylation of the calcitonin gene and genome-wide hypomethylation coexist in individual bladder carcinoma cells (Jürgens et al, 1996). Because different types of tumors differ considerably in the frequency of genome-wide DNA hypomethylation (Ehrlich, 2000; Schulz, 1998), the present data provide an explanation why deletions are the most prevalent mechanism of *CDKN2A* inactivation in TCC, whereas point mutations predominate in certain other tumors.

Material and Methods

Cell Lines

The human bladder carcinoma cell lines T24, 5637, and SD were cultured in DMEM with Glutamax I, sodium pyruvate, 4.5 g/l glucose, supplemented with 15% fetal bovine serum (Biochrom, Berlin, Germany), and 100 μg/ml of penicillin/streptomycin (Life Technologies, Inc., Eggertshausen, Germany) at 37° C in a humidified atmosphere with 5% CO_2.

Patients and Tissue Samples

TCC samples were obtained by cystectomy, nephroureterectomy, or transurethral resection from 86 patients between 1994 and 1999. Tumors and corresponding normal tissue were dissected after surgery, frozen in liquid nitrogen, and stored at −80° C. Blood samples from tumor patients were routinely collected for DNA extraction. Grading and staging were performed according to the 1997 Union Internationale Contre le Cancer (UICC) classification for bladder carcinoma. Among the 86 patients, 64 were male and 22 female. Ages ranged from 41 to 97 years with a mean age of 58 years. In all, 6 tumors were staged as pTa, 10 carcinomas as pT1, 22 as pT2, 32 as pT3, 15 as pT4, and 1 as pCIS. Three tumors were graded as G1, 23 as G2, 55 as G3, and 4 as G4, respectively. One case of carcinoma in situ was not graded.

DNA Extraction

High molecular weight genomic DNA from cell lines, tissue, and whole blood was isolated using the blood and cell culture DNA kit (Qiagen, Hilden, Germany). Frozen tissues were crushed to a fine powder with a mortar and pestle before extraction.

LOH Analysis

Matched pairs of normal and tumor DNA samples were screened for LOH at 9p21 at the five microsatellite loci: D9S168, D9S157, D9S942, D9S162, and D9S171. Primer sequences (Table 1) were obtained from the Genome DataBase. Testing of markers was performed in two multiplex PCR analyses. Template DNA (100 ng) was amplified in a total volume of 50 μl containing 150 μM of each dNTP, 1.5 mM $MgCl_2$, 15 pmol of each primer (forward primers modified at the 5'end with IRD800), and 2 U of DyNAzymeII *Taq* polymerase (Biozym, Göttingen, Germany). After initial denaturation at 95° C for 5 minutes, 26 cycles of 30 seconds at 94° C, 30 seconds at 55° C, and 1 minute at 72° C each were performed for primer sets 3 and 5, and 26 cycles of 94° C for 45 seconds, 55° C for 45 seconds, and 72° C for 45 seconds each for primer sets 1, 2, and 4. All reactions included a final elongation step at 72° C for 10 minutes.

PCR products were analyzed with an automated infrared DNA electrophoresis system (LI-COR DNA 4000/4200, MWG-Biotech, Ebersberg, Germany). After addition of 4 μl of formamide loading buffer to the 1:6-diluted PCR product, the sample was heated to 95° C for 5 minutes and snap-cooled. From each mixture, 1.5 μl per lane was loaded onto a 6% denaturing polyacrylamide gel (SequaGel, National Diagnostics, Atlanta, Georgia). Before loading the samples, the gel was pre-run for 30 minutes at 1000 V. Electrophoresis was carried out at a constant voltage of 1000V at 50° C. The data are presented as an radioautograph-like image that is stored in TIF format using the image manipulation subprogram of the LI-COR Base ImagIR software package. Band analysis

was performed using OneDScan 1.2 software (MWG-Biotech). An allele was regarded as lost when the intensity of remaining signal was less than 60% of the signal from the same allele in the matching control DNA of the same patient, as previously described (An et al, 1996).

MS-PCR

MS-PCR was performed as described by Herman et al (1996). Briefly, 0.5 to 1 μg of DNA from each sample was treated with bisulfite using the CpGenome DNA modification kit (Oncor, Heidelberg, Germany). Aliquots from the reaction mixture were used in two separate PCR reactions with primer pairs for *CDKN2A* exon 1α specific for converted (non-methylated) or unconverted (methylated) DNA (Herman et al, 1996). PCR products were separated on 6% polyacrylamide gels (AccuGel 29:1, National Diagnostics, Atlanta, Georgia) and visualized by silver staining. DNA from the bladder carcinoma cell line T24 was used as a positive control with each batch of samples.

RE-PCR Methylation Analysis

One microgram of DNA was digested with 10 U of methylation-sensitive *Hpa*II or methylation-insensitive *Msp*I restriction enzymes overnight. Digestion was then continued for 2 hours with 5 U of freshly added enzyme to ensure complete digestion. After 10 minutes of incubation at 65° C to inactivate the enzymes, 100 ng of each DNA sample was amplified in a total volume of 50 μl containing 150 μM each of dNTP, 5% DMSO, 5 mM $MgCl_2$, 40 pmol of primer set 9, and 1.2 U of *Taq* polymerase. Cycling consisted of an initial denaturation at 95° C for 5 minutes, 30 cycles each of 95° C for 30 seconds, 67° C for 30 seconds, and 72° C for 60 seconds. The final elongation step was at 72° C for 10 minutes. PCR products were separated on 6% polyacrylamide gels and visualized by silver staining.

Homozygous Deletion Testing

Testing for homozygous deletion was performed using primer set 11 for *CDKN2A* exon 2, primer set 10 for exon 1α, and primer set 14 for *GAPDH* as an internal standard. One hundred nanograms of template DNA was amplified in a total volume of 50 μl containing 150μM dNTP, 1.5 mM $MgCl_2$, 5% DMSO, 20 pmol of each primer, and 2 U of *Taq* polymerase. Forward primers were modified at the 5'-end with IRD 800. For exon 1α, the PCR conditions were initial denaturation at 95° C for 5 minutes, and 30 cycles each of 95° C for 1 minute, 60° C for 1 minute, and 72° C for 1 minute. The final elongation step was at 72° C for 10 minutes. For p16 exon 2 and *GAPDH*, cycling consisted of an initial denaturation at 95° C for 5 minutes, and 30 cycles each of 95° C for 45 seconds, 56° C for 45 seconds, and 72° C for 1 minute. The final elongation step was at 72° C for 10 minutes. For separation of the PCR products, an 8% denaturing polyacrylamide gel was used on the automated sequencer. Control PCR was performed using different ratios of DNA from normal lymphocytes and from the bladder carcinoma cell line SD with a homozygous deletion of *CDKN2A*.

Mutation analysis

For mutation analysis by PCR-SSCP, exon 2 of *CDKN2A* was amplified as two overlapping fragments of 285 bp and 260 bp, using primer sets 12 and 13, respectively. Thirty-five PCR cycles were carried out consisting of 45 seconds each at 95° C, 55° C or 66° C, and 72° C, preceded by initial denaturation and final elongation as above. The 50 μl reaction mix contained 150 μM NTPS, 6 mM or 1.5 mM $MgCl_2$, 5% DMSO, 20 pmol of primer, and 2 U of *Taq* polymerase. Sample aliquots were diluted 1:5 in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, heated at 90° C for 3 minutes, electrophoresed on an 8% partially denaturing polyacrylamide gel at 300V and 4°C for 26 hours. DNA was visualized by silver staining.

LINE-1 Methylation Analysis

Genome-wide hypomethylation was analyzed on Southern blots using a LINE L1 probe as described (Florl et al, 1999; Jürgens et al, 1996). In normal somatic tissues, L1 sequences are almost completely methylated. Hypomethylation results in the appearance of lower size bands (0.5 to 4 kb) after digestion with *Hpa*II. The data are expressed as percent hypomethylation, which is the intensity of these bands after *Hpa*II digestion compared with *Msp*I digestion. The value in normal bladder tissue is 0.5% to 1% (Florl et al, 1999).

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References

Akao T, Kakehi Y, Itoh N, Özdemir E, Shimizu T, Tachibana A, Sasaki MS, and Yoshida O (1997). A high prevalence of functional inactivation by methylation modification of p16^{INK4A}/CDKN2/MTS1 gene in primary urothelial cancers. Jpn J Cancer Res 88:1078-1086.

An H-X, Niederacher D, Picard F, van Roeyen C, Bender HG, and Beckmann MW (1996). Frequent allele loss on 9p21-22 defines a smallest common region in the vicinity of the CDKN2 gene in sporadic breast cancer. Genes Chromosomes Cancer 17:14-20.

Balazs M, Carroll P, Kerschmann R, Sauter G, and Waldman FM (1997). Frequent homozygous deletion of cyclin-dependent kinase inhibitor 2 (MTS1, p16) in superficial bladder cancer detected by fluorescence in situ hybridization. Genes Chromosomes Cancer 19:84-89.

Baud E, Catilinia P, and Bignon YJ (1999). p16 involvement in primary bladder tumors: Analysis of deletions and mutations. Int J Oncol 14:441-445.

Bender CM, Pao MM, and Jones PA (1998). Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 58:95-101.

Cairns JP, Chiang PW, Ramamoorthy S, Kurnit DM, and Sidransky D (1998). A comparison between microsatellite and quantitative PCR analyses to detect frequent p16 copy number changes in primary bladder tumors. Clin Cancer Res 4:441-444.

Chen RZ, Petterson U, Beard C, Jackson-Grusby L, and Jaenisch R (1998). DNA hypomethylation leads to elevated mutation rates. Nature 395:89-93.

Czerniak B, Chaturvedi V, Li L, Hodges S, Johnston D, Ro JY, Luthra R, Logothetis C, von Eschenbach AC, Grossman HB, Benedict WF, and Batsakis JG (1999). Superimposed histologic and genetic mapping of chromosome 9 in progression of human urinary bladder neoplasia: Implication for a genetic model of multistep urothelial carcinogenesis and early detection of urinary bladder cancer. Oncogene 18:1185-1196.

Ehrlich M (2000). DNA hypomethylation and cancer. In: Ehrlich M, editor. DNA alterations in cancer. Natick: Eaton Publishing, 273-291.

Esrig D, Elmajian D, Groshen S, Freeman JA, Stein JP, Chen S-C, Nichols PW, Skinner DG, Jones PA, and Cote RJ (1994). Accumulation of nuclear p53 and tumor progression in bladder cancer. N Engl J Med 331:1259-1264.

Florl AR, Loewer R, Schmitz-Dräger BJ, and Schulz WA (1999). DNA methylation and expression of L1 LINE and HERV-K provirus sequences in urothelial and renal cell carcinoma. Br J Cancer 80:1312-1321.

Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, van Turnout JM, and Jones PA (1995). 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.

Gonzalgo ML, Hayshida T, Bender CM, Pao MM, Tsai YC, Gonzales FA, Nguyen HD, Nguyen TT, and Jones PA (1998). The role of DNA methylation in expression of the p19/p16 locus in human bladder cancer cell lines. Cancer Res 58: 1245-1252.

Grim J, d'Amico A, Frizelle S, Zhou J, Kratzke RA, and Curiel DT (1997). Adenovirus-mediated delivery of p16 to p16-deficient human bladder cancer cells confers chemoresistance to cisplatin and paclitaxel. Clin Cancer Res 3:2415-2423.

Hartmann A, Moser K, Kriegmair M, Hofstetter A, Hofstaedter F, and Knuechel R (1999). Frequent genetic alterations in simple urothelial hyperplasias of the bladder in patients with papillary urothelial carcinoma. Am J Pathol 154:721-727.

Herman JG, Graff JR, Myohänen S, Nelkin BD, and Baylin SB (1996). Methylation-specific PCR: A novel assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826.

Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, Fischer A, and Viegas-Pequignot E (1993). An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. Hum Mol Genet 2:731–735.

Jung I, Reeder JE, Cox C, Siddiqui JF, O'Connell MJ, Collins L, Yang Z, Messing EM, and Wheeless LL (1999). Chromosome 9 monosomy by fluorescence in situ hybridization of bladder irrigation specimens is predictive of tumor recurrence. J Urol 162:1900-1903.

Jürgens B, Schmitz-Dräger BJ, and Schulz WA (1996). Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. Cancer Res 56:5698-5703.

Kai M, Arakawa H, Sugimoto Y, Murata Y, Ogawa M, and Nakamura Y (1995). Infrequent somatic mutation of the MTS1 gene in primary bladder carcinomas. Jpn J Cancer Res 86:249-251.

Kallioniemi A, Kallioniemi O-P, Citro G, Sauter G, DeVries S, Kerschmann R, Caroll P, and Waldman F (1995). Identification of gains and losses of DNA sequences in primary bladder cancer by comparative genomic hybridization. Genes Chromosomes Cancer 12:213-219.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, and Skolnick MH (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436-440.

Kazazian HH and Moran JV (1998). The impact of L1 retrotransposons on the human genome. Nat Genet 19:19-24.

Knowles MA (1999). The genetics of transitional cell carcinoma: Progress and potential clinical application. BJU Int 84:412-427.

Markl ID and Jones PA (1998). Presence and location of TP53 mutation determines pattern of CDKN2A/ARF pathway inactivation in bladder cancer. Cancer Res 58:5348-5353.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, and Sidransky D (1995). 5'CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1:686-692.

Orlow I, Lacombe L, Hannon GJ, Serrano M, Pellicer I, Dalbagni G, Reuter VE, Zhang ZF, Beach D, and Cordon-Cardo C (1995). Deletion of the p16 and p15 genes in human bladder tumors. J Natl Cancer Inst 87:1524-1529.

Orlow I, LaRue H, Osman I, Lacombe L, Moore L, Rabbani F, Meyer F, Fradet Y, and Cordon-Cardo C (1999). Deletions of the INK4A gene in superficial bladder tumors. Am J Pathol 155:105-113.

Orntoft TF and Wolf H (1998). Molecular alterations in bladder cancer. Urol Res 26:223-233.

Packenham JP, Taylor JA, Anna CH, White CM, and Devereux TR (1995). Homozygous deletions but no sequence mutations in coding regions of p15 or p16 in human primary bladder tumors. Mol Carcinog 14:147-151.

Presti JC, Reuter VE, Galan T, Fair WR, and Cordon-Cardo C (1991). Molecular genetic alterations in superficial and locally advanced human bladder cancer. Cancer Res 51:5405-5409.

Quelle DE, Zindy F, Ashmun RA, and Sherr CJ (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 83:993-1000.

Rein T, DePamphilis ML, and Zorbas H (1998). Identifying 5-methylcytosine and related modifications in DNA genomes. Nucleic Acids Res 26:2255-2264.

Ruas M and Peters G (1998). The p16INK4A/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378: F115-F177.

Schulz WA (1998). DNA methylation in urological malignancies. Int J Oncol 13:151-167.

Williamson MP, Elder PA, Shaw ME, Devlin J, and Knowles MA (1995). p16 (CDKN2) is a major deletion target at 9p21 in bladder cancer. Hum Mol Genet 4:1569-1577.

Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, and Viegas-Pequinot E (1999). Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature 402:187-191.

Yeager TR, DeVries S, Jarrard DF, Kao C, Nakada S, Moon TD, Bruskewitz R, Stadler WM, Meisner LF, Gilchrist KW, Newton MA, Waldman FM, and Reznikoff CA (1998). Overcoming cellular senescence in human cancer pathogenesis. Genes Dev 12:163-174.

Zhao J, Richter J, Wagner U, Roth B, Schraml P, Zellweger T, Ackermann D, Schmid U, Moch H, Mihatsch MJ, Gasser TC, and Sauter G (1999). Chromosomal imbalances in noninvasive papillary bladder neoplasms (pTa). Cancer Res 59:4658-4661.