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The 9p21 region in bladder cancer cell lines: large homozygous deletions inactivate the *CDKN2*, *CDKN2B* and *MTAP* genes

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Abstract Homozygous and hemizygous deletions of 9p21 are the earliest and most common genetic alteration in bladder cancer. The identification of two cell cycle regulators, *CDKN2* and *CDKN2B*, that map to the common region of deletion has prompted the hypothesis that they are critical tumor suppressor genes in this malignancy. However, controversy as to whether these genes are the only or even the most important target in bladder cancer oncogenesis remains. To more clearly determine the effect of these 9p21 alterations, we mapped the homozygous deletions and performed a detailed mutational and expression analysis for *CDKN2*, *CDKN2B* and a closely linked gene, methylthioadenoside phosphorylase (*MTAP*), in 16 established bladder cancer cell lines. Nine of the 16 lines exhibit large (30 to >2000 kb) homozygous deletions on 9p21. All deletions include at least one exon of *CDKN2*, eight of nine include *CDKN2B*, and six of nine include *MTAP*. *MTAP* function correlates with the genomic deletions. SSCP and sequence analysis does not reveal any inactivating point mutations of *CDKN2* or of *CDKN2B* in any of the cell lines without homozygous deletions, and all express the *CDKN2* and the *CDKN2B* mRNA as well as the encoded p16 protein. The p16 protein levels vary widely and are correlated with absent pRb expression. We conclude that the 9p21 deletions in bladder cancer usually inactivate the *CDKN2*, *CDKN2B*, and *MTAP* genes but that *CDKN2* is the most common target. Other mechanisms for inactivating this gene in bladder cancer appear to be uncommon.

Key words Cell cycle inhibitors · p16 · p15 · MTS · MTS2 · INK4 · INK4b

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Introduction

Loss of heterozygosity (LOH) on chromosome 9 occurs in approximately 70% of primary bladder cancers and appears to be the earliest identifiable genetic event [1, 16, 23, 26]. The majority of samples examined to date exhibit LOH for every chromosome 9 marker tested, confirming earlier cytogenetic observations of monosomy 9 in this disease [21]. In a small minority of samples LOH on 9p occurs with retention of heterozygosity on the long arm [23]. The minimal region of LOH on the 9p arm has been mapped to 9p21 and we and others have shown that homozygous deletions can be demonstrated in this region [1, 3, 26]. Deletions of the 9p21 chromosomal region are not unique to bladder cancer and have in fact been described in a large number of different tumors including lung cancer [19], melanoma [6], mesothelioma [4], gliomas [5], head and neck cancers [28], and oesophageal cancers [27]. This putative tumor suppressor locus is unique in that a large number of homozygous deletions have been described. Initially this finding appeared to be more frequent in cell lines than in primary tumors, suggesting that it was a cell culture artifact [25]. However, we and others have found that homozygous deletions in primary tumors may occur in up to 70% of samples [3, 6, 7, 28].

Two cell cycle inhibitors, *CDKN2* and *CDKN2B*, map to the minimal region of homozygous deletion on 9p21 [9, 10, 18]. In fact, all published homozygous deletions in tumor samples or cancer cell lines include the *CDKN2* locus and most include the *CDKN2B* locus [10, 13]. As such, both of these genes have been proposed as candidate tumor suppressor genes. According to Knudson's hypothesis, if *CDKN2* and/or *CDKN2B* are the operative 9p tumor suppressor gene(s), then the retained allele in those samples with LOH on 9p21 should carry a loss of function mutation in one or both of these genes [11]. However, in the majority of tumor

systems examined, including bladder cancer, very few point mutations in the coding region have been found [2, 25]. It has been argued that this is due to incomplete mutational analysis of these genes or is due to contaminating normal tissue in the primary samples. It has also been suggested that methylation of upstream regulatory factors or other epigenetic phenomena may inactivate gene expression in the absence of homozygous deletion [15].

It has been noted that the homozygous deletions on 9p21 reported to date are significantly larger than the *CDKN2* and *CDKN2B* loci. This suggests that additional genes may be important targets in the oncogenic path. One potential candidate is methylthioadenosine phosphorylase (*MTAP*), a gene critical in the purine nucleotide salvage pathway that is closely linked to *CDKN2* and *CDKN2B* [20].

In order to more clearly determine the extent of the deletions on 9p21, analyze candidate tumor suppressor genes, and analyze the functional effect of these alterations in bladder cancer, we first mapped the exent of the homozygous deletions in 16 established bladder cancer cell lines. We then screened all three exons of *CDKN2* and both exons of *CDKN2B* for inactivating point mutations. Finally, we examined the expression of these two genes and of the closely linked *MTAP* gene at both the RNA and protein level. We elected to analyze cell lines rather than primary tissues in order to obviate the problem with contaminating normal tissue and in order to perform a more complete expression analysis of the target genes. Since similar homozygous deletions have been found in primary tumors [3, 5, 7], we felt that this approach was justified.

Methods

Cell lines

Cell lines were selected to represent various stages and grades of transitional cell bladder cancer. We have previously described the 9p alterations in some of these lines and found that the deletions were similar to those reported in primary tumors [26]. HT1197, HT1376, RT4, T24, UM-UC-3, and 5637 were purchased from ATCC. 253J, 639V, and 647V were a gift from Dr. Richard Williams, University of Iowa. RT112 and ScaBER were a gift from Dr. Giuseppe Barbanti-Brodano, University of Ferrara, Italy. MGH-U3 and MGH-U4 were gifts from Dr Chi-Wei Lin, Massachusetts General Hospital, Boston, Mass. BL-13, BL-17, and BL-28 were gifts from Dr. Derek Raghavan, Roswell Park Memorial Institute, Buffalo, NY. Cell lines were cultured and DNA was isolated as previously described [26]. Total RNA was isolated using the Triazol reagent (Gibco BRL). This reagent was also used to isolate protein.

MTAP expression

MTAP expression was assayed using the principle that in the presence of 5'-methylthioadenosine (MTA), a functional enzyme can rescue cells from azaserine-induced cytotoxicity [22]. Cells were grown in MEM supplemented with 10% horse serum, 1% nonessential amino acids, and 1% sodium pyruvate. Cell growth was assayed using MTT colorimetry [17] in the presence of 10 μM azaserine and 20 μM MTA.

Polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis

Reactions were essentially performed as previously described [10, 12, 18, 29–31] using published primer sequences and protocols. For SSCP analysis of *CDKN2B*, two or three separate PCR reactions for each exon were performed.

For exon 1, primer sequences were AGCCTAAGGGGGTGGGAGAC with CCTGGATTGCTTCTGGGAAAAAG [annealing temperature 58 °C, 10% dimethylsulfoxide (DMSO) added to reaction mixture], CGCTTTTTCCCAGAAGCAATC with TGTTCTCCTCGCCGCATTC (annealing temperature 52 °C, 2% DMSO added to reaction mixture), and GGGAAGAGTGTCGTTAAGTTTACGG with GCACACCTCGCCAACGTAGAC (annealing temperature 59 °C, 10% DMSO added to reaction mixture).

For exon 2 primer sequences were TCTCCCATACCTGCCCCCAC with CCAAGTCCACGGGCAGACG and AGGGCTTCCTGGACACGCTG with GGGTGGGAAATTGGGTAAG (annealing temperature 58 °C, 10% DMSO added for both).

Sequencing

All fragments with abnormal mobility on SSCP were reamplified from genomic DNA and cloned into a PCR II vector using the TA cloning kit (Invitrogen). Plasmids were isolated from at least six colonies using Qiagen tips (Qiagen) and were sequenced in both directions using the Prism cycle sequence kit (ABI) and vector-specific primers as described by the manufacturer. In some cases, additional internal primers were used to confirm sequence alterations. The sequences were analyzed and aligned using AssemblyLign (Kodak).

Northern and Southern blotting

Northern and Southern blotting was performed as previously described [32]. For the *CDKN2* Southern analysis the entire cDNA (kindly provided by Dr. David Beach) was used as a probe. This probe recognizes all three *CDKN2* exons as well as exon 2 of *CDKN2B*. For the Southern analysis of the first exon of *CDKN2B* and for the Northern analysis of *CDKN2B*, a 0.45-kb *BAM*H1 fragment from the cDNA (kindly provided by Dr. David Beach), which recognizes only the first exon, was used as a probe.

Western blotting

Proteins were separated on a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to nitrocellulose and detected with anti-p16 (Pharmingen) using ECL detection system (Amersham), or anti-pRb (Pharmingen) using nitroblue tetrazolium (NBT) colorimetry (Promega).

Results

To assess whether the cell lines exhibit 9p21 loss of heterozygosity, we examined the IFNA and D9S171 dinucleotide repeat markers that flank the minimal

region of deletion on 9p21. Although no normal counterpart exists for the established cell lines, only 2 of 16 lines were heterozygous at the IFNA locus and 3 of 16 were heterozygous at the D9S171 locus (data not shown). This is significantly lower than would be expected from the known heterozygosity indices for these markers ($P < 10^{-6}$). In addition, two cell lines, MGH-U3 and MGH-U4, demonstrated homozygous deletion of the IFNA dinucleotide marker.

To extend our previous observations of homozygous deletions in bladder cancer cell lines in this region [28], all the cell lines were subjected to a combination of PCR and Southern blot analysis and the data are summarized in Fig. 1. All deletions were confirmed by repeating the PCR at least 2 times and by Southern blotting. Nine of the 16 established cell lines (56%) had homozygous deletions. The deletions ranged in size from approximately 100 kb (ScaBER) to greater than 2 Mb (RT4) and all included at least one exon of *CDKN2*. Eight of the deletions also included the *CDKN2B* locus and six included the *MTAP* gene. To confirm the homozygous deletions of *MTAP*, selected cell lines were subjected to a cell culture assay which has been shown to reliably detect the inactivation of this gene (see "Methods"). Two cell lines with large deletions (RT4 and UM-UC-3) and one with a smaller deletion (253J) all died in the selective media, whereas two cell lines without deletions (HT1197 and T24) continued to proliferate.

To assess whether any of the cell lines without homozygous deletions had mutations of the *CDKN2* or *CDKN2B* candidate tumor suppressor genes, SSCP analysis of the entire coding region as well as a majority of the 3′ noncoding region was carried out for both genes. All abnormally migrating bands were sequenced. We identified an intronic C to A change 135 bp upstream from the start of the second exon of *CDKN2B* in HT1197, but there was no apparent effect on the mRNA expression (see below). We also identified a G to T change (leucine to leucine) in codon 105 of *CDKN2* in 639V.

To screen for mutations in upstream noncoding regions, six of the seven cell lines without homozygous deletions were assessed for *CDKN2* and *CDKN2B*

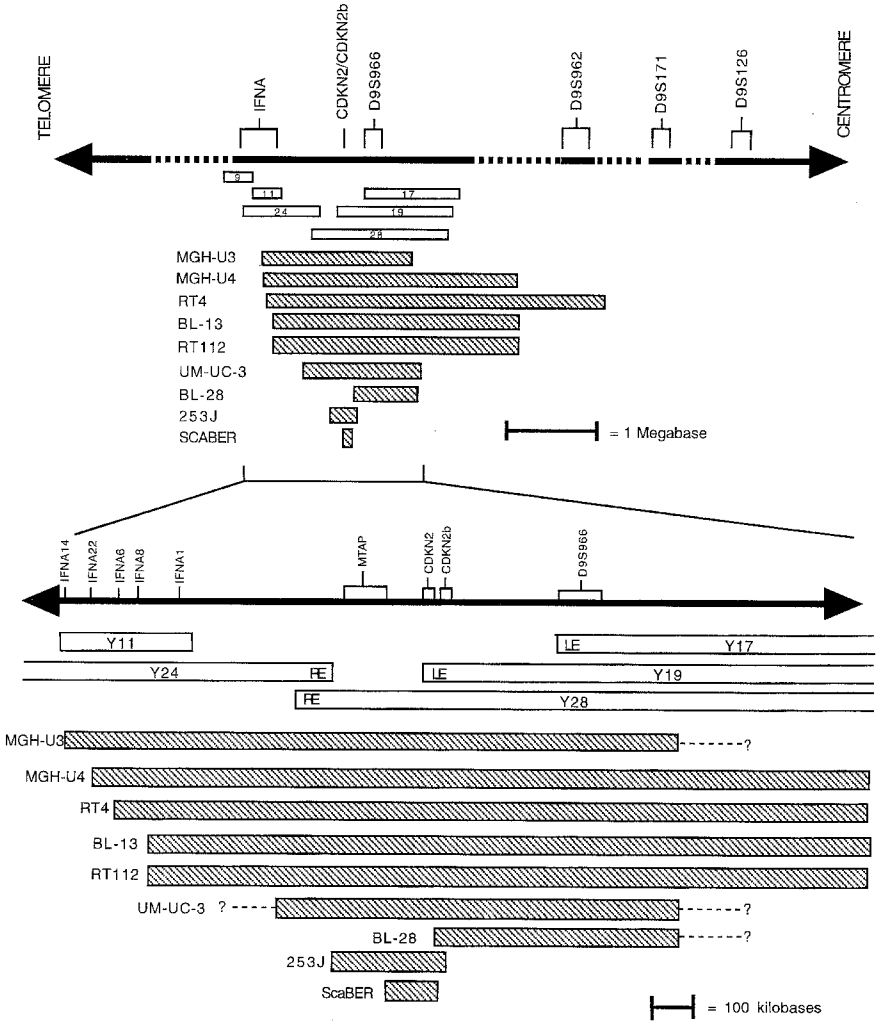

Fig. 1 Homozygous deletions in bladder cancer cell lines. Deletions are represented by *shaded bars* and were mapped as described in text. *Open bars* represent individual YACs from a contig mapped to the region [21]. Approximate scale is indicated. The relative order of the YAC contig, D9S962, D9S171, and D9S126 is indicated but these have not yet been physically linked. The distance from IFNA to D9S171 is approximately 4 cm

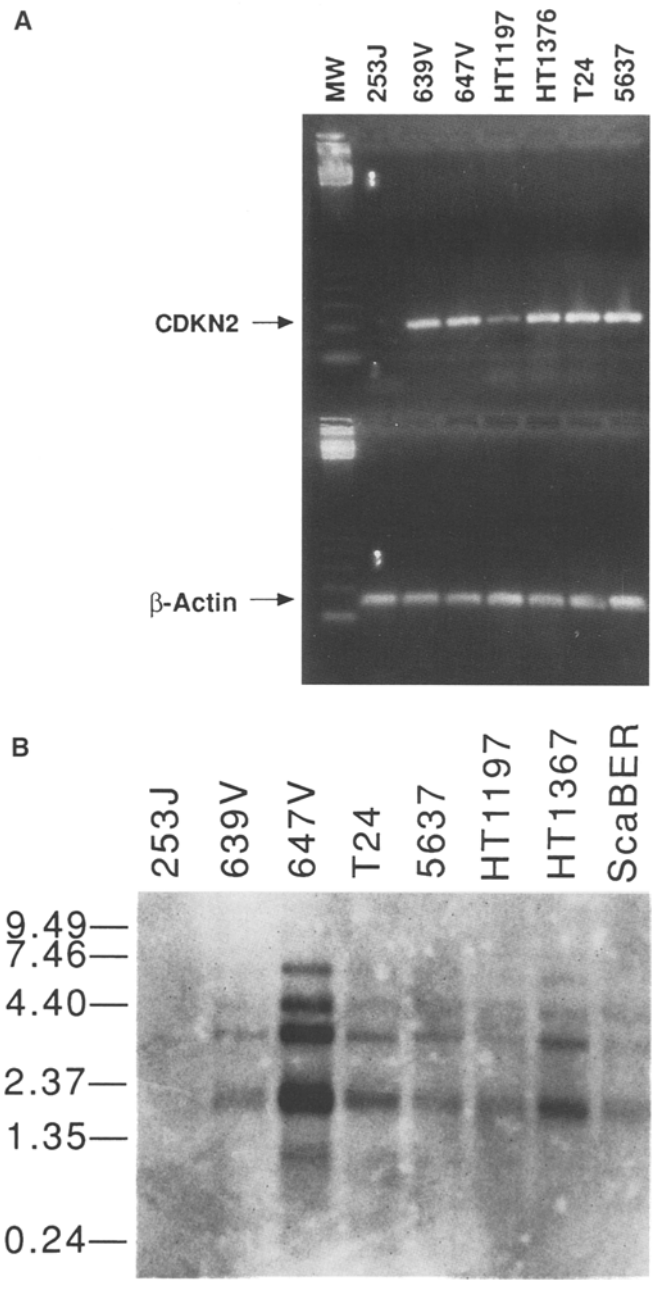

Fig. 2A, B mRNA expression of *CDKN2* and *CDKN2B* in Bladder cancer cell lines without 9p21 homozygous deletions. **A** *CDKN2* expression as assessed by nonquantitative RT-PCR, 253J is a cell line with homozygous deletion of *CDKN2* (see Fig. 1). **B** *CDKN2B* expression as assessed by Northern blot. The reported cDNA sequence is 900 bp [8]. It is presumed that the higher molecular weight bands are alternatively spliced variants since they are codeleted in 253J, but they were not further analyzed. Equal loading was demonstrated by probing with B-actin

mRNA expression. Due to low expression of *CDKN2*, nonquantitative RT-PCR was used to assess mRNA expression of this gene. Figure 2 shows that both genes are expressed in all of the established cell lines without homozygous deletions.

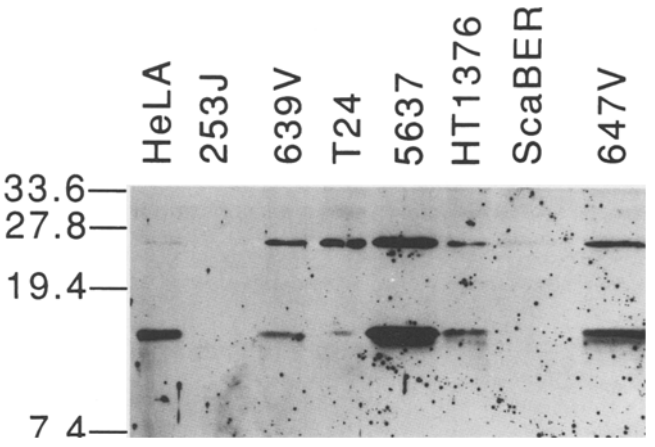

Fig. 3 p16 Western blot. HeLa cells were used as a positive control. The identity of the higher molecular weight band has not been characterized. Equal loading was demonstrated by staining the membrane with fast green

Finally, the hallmark of a mutated gene is a dysfunctional or absent protein product. As an initial screen we assayed the levels of p16 via Western blotting in those cell lines without deletions and found that p16 was expressed in five of five established cell lines (Fig. 3). As previously reported [30], p16 protein levels appeared to inversely correlate with normal pRb expression. The only exception to this observation was in the HT1376 cell line, in which no pRb was detected but p16 levels were still relatively low. It is noteworthy that we have also been unable to detect p16 in normal human bladder epithelium using Western blotting (data not shown).

In sum, in the bladder cancer cell lines studied here, we found homozygous genomic deletions in the 9p21 chromosomal region that always included at least one exon of *CDKN2*. We have, however, been unable to detect any additional subtle alterations of either *CDKN2* or *CDKN2B* that would unequivocally lead to an altered protein product. More importantly, all cell lines without any genomic alterations expressed the p16 protein.

Discussion

Although chromosome 9 losses were first described in bladder cancer a number of years ago [22], the critical gene or genes are still controversial. It has been hypothesized that these losses distinguish a locus in which a tumor suppressor gene resides and that this gene can be identified by detecting point mutations in the remaining allele [12]. This paradigm has been followed in characterizing known tumor suppressor genes such as *RB*, *NF1*, and *BRCA1*. In contrast to all other

known and putative tumor suppressor loci, however, there is a high incidence of homozygous deletions on 9p21 in both cell lines and primary samples. Using a large number of cell lines with nested deletions, Kamb et al. were able to identify two cell cycle inhibitor genes, *CDKN2* and *CDKN2B*, that map to the common region of deletion [10]. In order to examine the 9p21 region and potential mechanisms for inactivating genes at that locus, we mapped the homozygous deletions and undertook a mutation and expression analysis of *CDKN2*, *CDKN2B*, and *MTAP* in a panel of 16 bladder cancer cell lines.

Our results show that homozygous deletion is the most common alteration seen in this region and six of eight homozygous deletions include *MTAP*, seven of eight include *CDKN2B*, and all include at least one exon of *CDKN2*. Despite analyzing the DNA, RNA, and protein, we were unable to demonstrate other mechanisms for inactivating p16, the *CDKN2* product and the most promising candidate tumor suppressor gene. Merlo et al. have suggested methylation of upstream sequences as a mechanism of inactivating this locus [15]. These investigators reported that in those cell lines or primary tumors in which the upstream CpG island was methylated, no RNA expression was detected. Although we did not directly assess the methylation status of the *CDKN2* locus in our cell lines, the RNA and protein data suggest that this mechanism is not operative. We should also note that an alternatively spliced *CDKN2* RNA species has been described and that our RT-PCR experiment for detecting *CDKN2* expression will detect both transcripts [14]. It does not appear as if a protein is translated from the alternative transcript and it is not clear what role this trascript plays. Nevertheless, the expression of p16 protein in all bladder cancer cell lines without 9p21 homozygous deletions leads us to conclude that alternative mechanisms of inactivating the *CDKN2* gene are rare.

Our results differ from previous publications where a small intragenic deletion of *CDKN2* was detected in ScaBER and a mutation introducing a stop codon in the second exon of *CDKN2* was detected in 639V [13]. It is possible that the ScaBER line that we have worked with has accumulated the additional deletions noted here during propagation in culture. Since normal-sized p16 protein was readily detected in 639V, a stop codon cannot be present in the *CDKN2* gene from our cell line. Finally, and perhaps most importantly, we find that p16 protein levels vary widely and are correlated most closely with pRb expression. In fact, there is no detectable protein expression in normal bladder and, as such, lack of protein expression in a tumor sample may not be an adequate assay to detect p16 alterations.

It is not clear why there is such a high incidence of homozygous deletions in this region. One potential explanation is that there are chromosomal structural factors that predispose this region to deletions. Alternatively, another closely linked target gene (or genes) could be commonly codeleted. Two genes which are frequently codeleted, *CDKN2B* and *MTAP*, have already been identified. The functional importance, in terms of oncogenic progression, of these codeletions remains to be determined. The frequent codeletion of *MTAP* does, however, provide a rather unique opportunity to explore this phenomenon in new therapeutic approaches for bladder cancer. Chemotherapeutic approaches to take advantage of the selective killing of cells deficient in *MTAP* have already been proposed and may potentially be useful in the therapy of metastatic bladder cancer.

In summary, it appears that large homozygous deletions of 9p21 occur frequently in bladder cancer cell lines, and that the *CDKN2* gene is the most common target. Although subtle alterations in *CDKN2* expression patterns may still be important in those bladder cancers without 9p21 homozygous deletions, point mutations of this gene are uncommon. Finally, there is no adequate explanation for the high incidence of large homozygous deletions in this chromosomal region and we thus continue to assess the functional importance of other genes mapped to the region.

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