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p16 mutations/deletions are not frequent events in prostate cancer

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Summary Cyclin-dependent kinase-4 inhibitor gene (p16INK4a) has recently been mapped to chromosome 9p21. Homozygous deletions of this gene have been found at high frequency in cell lines derived from different types of tumours. These findings suggested therefore, that p16INK4a is a tumour-suppressor gene involved in a wide variety of human cancers. To investigate the frequency of p16INK4a mutations/deletions in prostate cancer, we screened 20 primary prostate tumours and four established cell lines by polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis for exon 1 and exon 2. In contrast to most previous reports, no homozygous deletions were found in prostate cancer cell lines, but one cell line (DU145) has revealed a mutation at codon 76. Only two SSCP shifts were detected in primary tumours: one of them corresponds to a mutation at codon 55 and the other one probably corresponds to a polymorphism. These data suggest that mutation of the p16INK4a gene is not a frequent genetic alteration implicated in prostate cancer development.

Keywords: prostate cancer; p16; mutation

Cyclin-dependent kinases (CDKs) are key enzymes in driving cells through the cell cycle into mitosis. Their activity is tightly controlled by phosphorylation and dephosphorylation of the CDK itself and with association with regulatory subunits. Binding of a specific cyclin is a prerequisite for kinase activity. On the other hand, CDK inhibitory subunits have been recently described. p21 and p27 share a region of homology, and form with CDKs and cyclins ternary complexes that can inhibit a variety of CDKs (Polyak et al., 1994; Nasmith and Hunt, 1993). p16INK4a was the first identified member (Serrano et al., 1992) of a growing family of proteins that specifically inhibit CDK4 and CDK6 (Kamb et al., 1994; Nobori et al., 1994; Hannon and Beach, 1994). These two CDKs act together with cyclin D to control Rb phosphorylation and passage through the starting point of the cell cycle. This is a crucial step in controlling cell growth and perturbation of this pathway is thought to be implicated in carcinogenesis (Kamb et al., 1994). Moreover, the gene encoding p16 protein, (p16INK4a/CDKN1A/MTS1) has been recently located in the chromosomal region 9p21, a critical area of allelic loss in a wide spectrum of human tumours (Serrano et al., 1993; Kamb et al., 1994; Nobori et al., 1994). Interestingly, a high frequency of homozygous deletions in the p16INK4a gene in cell lines derived from a variety of human tumours including bladder and kidney have been reported (Kamb et al., 1994). The mutation rate found in cell lines could even rival that of the p53 tumour-suppressor gene. However, in primary tumours the rate of p16INK4a mutations seems to be rather low and varies in a tumour type-dependent manner (Mori et al., 1994; Cairns et al., 1994; Spruck et al., 1994).

Here we have investigated the alterations of p16INK4a in cell lines and primary tumours of the prostate using the polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis.

Materials and methods

Patient samples, cell lines and DNA isolation

Four established prostatic cell lines (PC3, DU145, TSUPrl, LNCaP) were grown in RPMI medium containing 10% of calf serum until they reach confluency. DNA was extracted according to Miller et al. (1988). Twenty prostate cancer samples of various Gleason grades (Gg2–Gg5) were obtained by transurethral resection of the prostate, and then frozen in liquid nitrogen. Areas containing at least 70% of tumour cells as judged by step sectioning, were subjected to DNA extraction (Miller et al., 1988).

Polymerase chain reaction (PCR)

We amplified exon 1 and exon 2 for the analysis of p16INK4a gene alterations in prostate cancer. Primer sequences used for amplification were: 5'-GAAGAAGAGGGGCTG-3' and 5'-GCCGTACCTGATTCATTTGC-3' for exon 1. Two overlapping primer sets were taken to cover the entire 500 bp long exon 2: 5'-GCACCCACCGGTGTC-3' and 5'-GGAATTTGAAAACGTGAAG-3' for exon 2. PCR amplification were: 5'-GAAGAAGAGGGGCTG-3' and 5'-GCCGTACCTGATTCATTTGC-3' for exon 1. Two overlapping primer sets were taken to cover the entire 500 bp long exon 2: 5'-GCACCCACCGGTGTC-3' and 5'-GGAATTTGAAAACGTGAAG-3' for exon 2. PCR products displaying a shift on SSCP analysis were sequenced both directly, as described before (Jacoby et al., 1994) and after being cloned into TA cloning vector (pCR II; Invitrogen).

SSCP analysis

The reaction product (3 µl) was then mixed with 10 µl of loading buffer containing 96% formamide. Samples were denatured at 94°C for 3 min, chilled on ice for at least 5 min and 2 µl was loaded onto a 6% non-denaturing polyacrylamide gel with or without 10% glycerol. Gels were electrophoresed at 5 W (with glycerol) and 3 W (without glycerol) for 16 h at room temperature, using 0.5 x Tris-borate- EDTA buffer. Gels were dried and exposed to (RPN 120-122) Amersham film at —80°C for 3 days. The reaction product (3 µl) was then mixed with 10 µl of loading buffer containing 96% formamide. Samples were denatured at 94°C for 3 min, chilled on ice for at least 5 min and 2 µl was loaded onto a 6% non-denaturing polyacrylamide gel with or without 10% glycerol. Gels were electrophoresed at 5 W (with glycerol) and 3 W (without glycerol) for 16 h at room temperature, using 0.5 x Tris-borate- EDTA buffer. Gels were dried and exposed to (RPN 120-122) Amersham film at —80°C for 3 days. The reaction product (3 µl) was then mixed with 10 µl of loading buffer containing 96% formamide. Samples were denatured at 94°C for 3 min, chilled on ice for at least 5 min and 2 µl was loaded onto a 6% non-denaturing polyacrylamide gel with or without 10% glycerol. Gels were electrophoresed at 5 W (with glycerol) and 3 W (without glycerol) for 16 h at room temperature, using 0.5 x Tris-borate- EDTA buffer. Gels were dried and exposed to (RPN 120-122) Amersham film at —80°C for 3 days.

Sequence analysis

PCR products displaying a shift on SSCP analysis were sequenced both directly, as described before (Jacoby et al., 1994) and after being cloned into TA cloning vector (pCR II; Invitrogen).
Results and discussion

We have studied the mutation frequency in the MTS1/p16\textsuperscript{INK4} gene, in prostate cancer. Exon 1 and exon 2 represent the major part of the coding sequence (98% of the p16 protein), whereas exon 3 makes up only 11 coding nucleotides (2% of the total coding sequence). Amplification of tumour genomic DNA using primers for exon 1 and exon 2 of the p16\textsuperscript{INK4} gene resulted in bands of the predicted size 350, 240 and 340 bp respectively when separated on 2% agarose gel. A PCR product of the expected size was obtained in each of the four prostatic cell lines, indicating that none of them has a homozygous deletion of the p16 gene.

We subsequently investigated the presence of point mutations in exon 1 and exon 2 fragments of the p16\textsuperscript{INK4} gene by SSCP analysis, a technique that is sensitive enough to detect more than 80% of mutations (Sheffield et al., 1993). The screening of almost the entire coding sequence (98%) of p16\textsuperscript{INK4} revealed band migration shifts, for exon 2, in one cell line (DU145) and two primary tumours (see example in Figure 1, sample 21).

The corresponding PCR products were sequenced. This revealed a missense mutation in DU145 at codon 76 (GAC→TAC) resulting in a change from aspartic acid to tyrosine (see example in Figure 2). The shift found in one of the primary tumours (case 166) was a mutation at codon 55 (CTG→CCG) leading to a change from leucine to proline. It remains to be determined whether the substitution of the Asp with Tyr and Leu with Pro at codons 76 and 55 respectively plays a part in prostate cancer development. The shift in the other primary tumour (case 154) corresponded to a migration shift displayed by one primary tumour (case 154) in the establishment of DU145 cell lines and two primary tumours, we were not able to find more than two mutations: one in the DU145 cell line, and one in primary tumours. These results indicate that mutation of the p16\textsuperscript{INK4} gene is not a frequent genetic change in the formation of primary prostate cancer. Moreover, in contrast to several reports in which homozygous deletion in cultured cell lines can reach 85% (Kamb et al., 1994), homozygous deletions in established lines of prostate cancer were not found in the four analysed cases. Parallel to our observations on primary prostate tumours, analysis of tumours from several organs such as astrocytomas (Ueki et al., 1994), breast (Xu et al., 1994), bladder (Cairnes et al., 1994; Spruck et al., 1994), lung, brain and kidney tumours (Cairns et al., 1994) revealed p16\textsuperscript{INK4} mutations in only a small fraction of primary tumours. Alternatively, higher frequencies have been found in some tumour types: 16% in head and neck squamous cell carcinoma and up to 51% in oesophageal squamous cell carcinomas have been detected recently (Mori et al., 1994). Therefore, the p16\textsuperscript{INK4} gene has certainly a key role in certain cases, but not in all tumour types.

Figure 1 A representative example of SSCP analysis of p16\textsuperscript{INK4} gene (exon 2) in primary tumour and established cell lines of the prostate. A migration shift displayed by one primary tumour (sample 21).

![Figure 1](image1.png)

Figure 2 A representation of DNA sequencing of the SSCP shift observed in DU145 prostate cancer cell line. A base substitution from cytidine to adenine in the non-coding strand is indicated by an arrow. This base substitution leads to a change from guanine to thymine at codon 76 and results in an amino acid change from aspartic acid to tyrosine.

![Figure 2](image2.png)
References


