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Mutational Analysis of CDKN2 (CDK4I/MTS1) Gene in Tissues and Cell Lines of Human Prostate Cancer

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To study mutation of the CDKN2 gene in prostate cancer, samples from 51 Japanese patients and four human prostate cancer cell lines were examined by single-strand conformation polymorphism analysis and direct sequencing. Only one out of 51 (2%) patients revealed a mutation, which was a 24 bp deletion from the 5′-untranslated region to codon 3, resulting in loss of the initiation site. One of the four cell lines revealed a missense mutation, a GAC→TAC (Asp→Tyr) at codon 84. These results indicate that mutation of the CDKN2 gene is rare in prostate cancer and thus does not contribute significantly to the pathogenesis of human prostate cancer. Prostate cancer cell lines may acquire more frequent abnormality of the CDKN2 gene than tumor tissues.

Key words: CDKN2 gene — MTS1 gene — Prostate cancer — PCR-SSCP

Mutations or deletions of the CDKN2 (CDK4I[4]/p16/MTS1) gene, which is located at chromosome 9p21 and regulates proliferation through the G_1 phase of the cell cycle, occur at high frequency in cell lines derived from many tumors,[1,2] suggesting that the CDKN2 gene is a tumor suppressor gene in human cancers. Germline mutations were frequently detected in familial melanoma kindreds.[3] Somatic mutations of the CDKN2 gene were also detected in esophageal squamous cell carcinoma,[4,5] pancreatic carcinoma[6] and non-small cell lung carcinoma.[7] Therefore, the CDKN2 gene may be related to the tumorigenesis of certain tumor types. Since there have been no studies of the CDKN2 gene in prostate cancer, the present study was undertaken to examine mutations in exons 1 and 2 of this gene in tissue specimens and cell lines of prostate cancer using PCR-SSCP analysis and direct sequencing.

Tumor specimens from 29 cases of surgically removed stage B or C prostate cancer and 22 cases of endocrine therapy-resistant cancers obtained at autopsy were used in the present study; in total, 124 samples were examined, which consisted of 51 from cancerous prostate, 51 from normal tissues and 22 from metastatic cancer foci. Tumor characteristics of these cases are summarized in Table I. All specimens were frozen immediately after the operation or autopsy and stored at −80°C. Resected prostates from surgery were cut in 5 mm serial sections, each section was stained with hematoxylin and eosin, and cancer foci were examined. For autopsy cases, a piece of cancer tissue was removed and examined histologically. Since the number of established human prostate cancer cell lines is small, four representative cell lines (LNCaP,[8] DU145,[9] PC-3[10] and TSU-Prl[11]) were examined. DNAs were prepared by proteinase K digestion and phenol/chloroform extraction. The primers for PCR were prepared according to Kamb *et al.*[1]: 2F, 5′-GAAGAAAGAGGAGGGGCTG-3′ and 1108R, 5′-GCGCTACCTGATTCCAATTC-3′ for exon 1; 42F, 5′-GGAAATTGGAAACTGGAAGC-3′ and 551R, 5′-TCTGAGCTTTGGAAGCTCT-3′ for exon 2. PCR-SSCP analysis was performed according to the reported method.[12] The 5′-terminal of each primer was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara, Kyoto). The PCR mixture was prepared as follows: 50 ng of genomic DNA, 0.125 pmol of each primer, 0.3125 nmol of each deoxynucleotide triphosphate, 10 m*M* Tris-HCl buffer (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 5% dimethyl sulfoxide and 0.0625 U of Taq polymerase (Takara) in a final volume of 5 μl. PCR was performed with 35 cycles of denaturation (94°C, 1 min), annealing (62°C for exon 1 and 55°C for exon 2, 1 min) and extension (72°C, 1 min) by a DNA Thermal Cycler PJ 2000 (Perkin Elmer, Norwalk, CT, USA). The PCR products were diluted with an adequate volume of formamide-dye mixture. One μl of diluted PCR products was heat-denatured (90°C, 3 min), chilled on ice and immediately loaded on 5% polyacrylamide gel containing 45 m*M* Tris-borate buffer (pH 8.3), 4 m*M* EDTA, 0.05% ammonium peroxydisulfate and 5% glycerol, then electrophoresed at 30 W for 2–4 h at 4°C. The gel was vacuum-dried and auto-

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[4] The abbreviations used are: CDK4I, cyclin-dependant kinase 4 inhibitor; MTS1, multiple tumor suppressor 1; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

Table I. Characteristics of 51 Prostate Cancers Examined

Stage	Differentiation			Total
	Well	Moderately	Poorly	
B[a]	5	7	6	18
C[a]	0	6	5[b]	11
D[c]	0	0	22	22
Total	5	13	33	51

a) Removed by total prostatectomy.
b) One case showed a CDKN2 gene mutation.
c) Obtained from cancer-death patients, who initially responded to endocrine therapy, but thereafter relapsed and died.

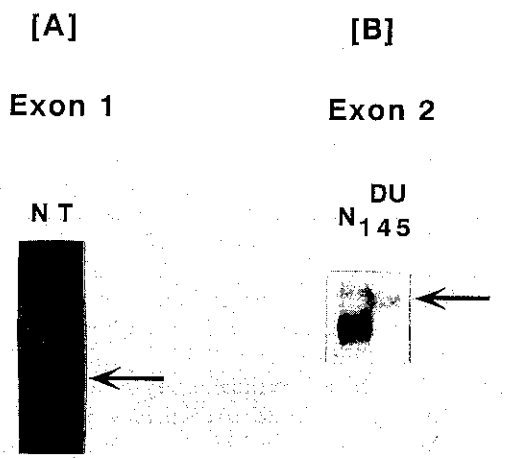

Fig. 1. SSCP analysis of CDKN2 gene. N: normal tissue, T: primary cancer tissue. DU145: a prostate cancer cell line. (A) Exon 1 of case No. 8 (stage C cancer patient). (B) Exon 2 of DU145. Arrows indicate abnormally shifted bands.

radiographed at −80°C. Duplicate examinations were performed to confirm conformation abnormality. Abnormal bands detected by SSCP analyses were eluted from gels and amplified by PCR according to the manufacturer's instruction (Gene Amp PCR Reagent kit, Perkin Elmer). Amplified DNA fragments were electrophoresed in low melting point agarose gels (Sea Plaque GTG, FMC Bio Products, Rockland, ME, USA). After electrophoresis, the fragments were separated from gels, and purified by phenol/chloroform extraction, followed by ethanol precipitation. The purified PCR products were sequenced by means of the cycle sequencing method described previously.[13] The sequencing primer was the same as that used for the preceding PCR.

Variant SSCP patterns were detected in exon 1 of one stage C case (No. 8) and exon 2 of DU145 cell line (Fig. 1). The stage C case contained a normal allele fragment

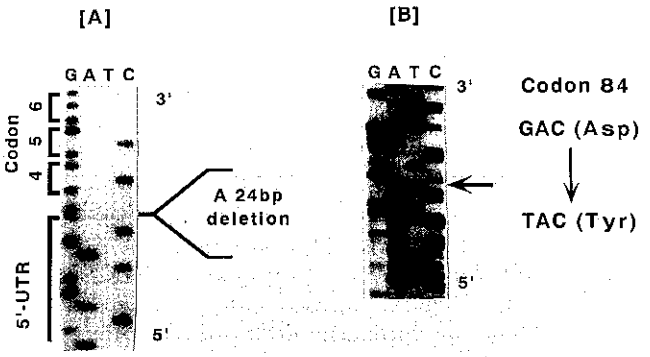

Fig. 2. Sequencing analysis of CDKN2 gene. Codon numbers are used according to Okamoto *et al.*[14] (A) A 24-base-pair deletion was detected from the 5′-untranslated region (5′-UTR) to the second position of codon 3 in case No. 8. (B) G to T transversion at codon 84, resulting in a substitution of Try (TAC) for Asp (GAC) in the DU145 cell line.

in addition to an abnormal band. Since the sample was confirmed histologically, the normal fragment was at least partly derived from tumor cells. DU145 cell line was found to have a loss of the normal allele in SSCP analysis. Direct DNA sequencing confirmed a 24-base-pair deletion from the 5′-untranslated region to the first position of codon 3,[14] resulting in loss of the initiation site in the stage C case (No. 8, Fig. 2A). DU145 cell line revealed a point mutation, G to T transversion, at the first position of codon 84, resulting in a substitution of Tyr for Asp (Fig. 2B). No abnormality was found in the other specimens and cell lines.

The status of CDKN2 gene has been examined in many tumors. Frequent mutations were found in certain types of tumors, but recent studies performed on other primary tumors such as bladder,[15,16] lung, head and neck, brain, kidney,[17,18] breast[19] and sporadic melanoma[20,21] found rather low ratios of CDKN2 gene mutations. In the present study, only 1 of 51 prostate cancer cases (2%) carried a mutated CDKN2 gene. On the other hand, 1 of 4 prostate cancer cell lines revealed a mutated CDKN2 gene, suggesting that CDKN2 gene alterations may be more frequent in cell lines than in primary tumors. This might occur as a result of adaptation to cell culture, as suggested by others,[15–19] who found relatively low frequencies of CDKN2 gene alterations in primary tumors when compared to cell lines.

Concerning primary tumors, homozygous deletion of the CDKN2 gene was observed frequently in T-cell lineage acute lymphoblastic leukemia (83%),[22] pancreatic adenocarcinoma (37%),[6] and glioblastoma multiforme (40–80%).[1,23–26] Hemizygous deletion at this gene locus was also found frequently in pancreatic carcinoma (48%),[6] glioblastoma multiforme (25–28%)[21,24] and

esophageal carcinoma (25%),[5] which carried a mutation in the other copy of this allele as seen in the DU145 cell line in the present study. These cases clearly sustain two-hit mutation inactivation of CDKN2 gene.[16] However, loss of CDKN2 function may be incomplete in other tumors,[17] which rarely revealed mutations of the CDKN2 gene in spite of frequent loss of heterozygosity at this gene locus. The stage C case (No. 8) had a mutated allele and a normal allele of the CDKN2 gene. In this case, CDKN2 function may remain to some extent. This seems consistent with the dosage effect of the CDKN2 gene on cell growth.[16] Taking into account the infrequent loss of chromosome 9p in prostate cancer,[27] we conclude that mutation of CDKN2 gene is not a critical genetic change in the pathogenesis of prostate cancer.

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