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Alterations of *CDKN2* (*MTS1*/*p16*^{INK4A}) gene in paraffin-embedded tumor tissues of human stomach, lung, cervix and liver cancers

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Abbreviations: *CDKN2*, cyclin dependent kinase inhibitor 2; PCR, polymerase chain reaction; Cdk 4, cyclin dependent kinase 4; B-ALL, B-cell acute lymphoblastic leukemia; NSCLC, non small cell lung cancer; SCLC, small cell lung cancer; SSCP, single strand conformation polymorphism; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; FISH, fluorescent in situ hybridization

Abstract

The *CDKN2* (*MTS1*/*p16*^{INK4A}) gene, encoding cyclin dependent kinase inhibitor, was found to be homozygously deleted at a high frequency in cell lines from many different types of cancer and some primary cancers. To determine the frequency of *CDKN2* mutations in most common human cancers in Korea, PCR and PCR-SSCP analyses for the exon 2 of *CDKN2* were performed on each set of 20 formalin-fixed and paraffin-embedded tumor tissues of stomach adenocarcinomas, lung cancers, cervix cancers and hepato-cellular carcinomas. No mutations in exon 2 of *CDKN2* were found in 20 stomach adenocarcinomas. In contrast to rare mutations in stomach adenocarci-nomas, a high frequency of *CDKN2* mutations was identified in other 3 cancers, 11 of 20 (55%) lung cancers (7 of 10 NSCLCs and 4 of 10 SCLCs), 14 of 20 (70%) cervix cancers and 11 of 20 (55%) hepato-cellular carcinomas. These results suggest that mutations of the *CDKN2* gene might be an important genetic change in NSCLCs, cervix cancers and hepatocellular carcinomas.

Keywords: *CDKN2*, cyclin dependent kinase inhibitor, PCR-SSCP

Introduction

Stomach, lung, cervix and hepatocellular cancers are the most common cancers in Korea. Various environmental factors and genetic factors are involved in the formation and development of cancers. Genetically, many human tumors have been reported to have abnormalities in oncogenes as positive regulators or tumor suppressor genes as negative regulators (Levine, 1993). A variety of oncogenes and tumor suppressor genes such as SV40T, E1A/E1B of adenovirus, E6/E7 of HPV-16, p53 and Rb has been identified as affecting cell growth through the regulation of the cell cycle (Hunter *et al*., 1994; Peter and Herskowitz, 1994).

Recently, the *CDKN2* and *MTS2* genes, encoding inhibitors of cyclin dependent kinases (Serrano *et al*., 1993; Hannon and Beach, 1994), were identified to be located at chromosome 9p21 and to be candidate tumor suppressor genes. The protein product of *CDKN2* , p16, prevents pRb phosphorylation by inhibiting Cdk4-cyclin D complex, and thereby regulates the progression of G1 to S phase in the cell cycle. *CDKN2* was found to be homozygously deleted at a high frequency in cell lines from many different types of cancer such as melanoma, leukemia, glioma, breast cancers and astrocytoma (Kamb *et al*., 1994; Nobori *et al*., 1994). In addition, a high frequency of mutations of *CDKN2* have been observed in tissues from pancreatic adenomas (Bartsch *et al*., 1995), T-cell leukemias (Hebert *et al*., 1994), biliary tract cancers (Yoshida *et al*., 1995), esophageal cancers (Mori *et al*., 1994) and non-small cell lung cancers (Washimi *et al*., 1995; Xiao *et al*., 1995). In contrast to the observations in some malignancies, no deletions and rare alterations of *CDKN2* were identified in breast cancers (Xu *et al*., 1994), malignant mesothelioma (Cheng *et al*., 1994), small cell lung cancers (Okamoto *et al*., 1995), head and neck cancers (Zhnag *et al*., 1994), thyroid cancers (Tung *et al*., 1996), ovarian cancers (Campbell *et al*., 1995; Rodabaugh *et al*., 1995) and esophageal cancers (Okamoto *et al*., 1994; Suzuki *et al*., 1995). In some tumors, especially the reported mutation frequency of *CDKN2* was controversial, varying from 0 to 52% in esophageal carcinomas (Mori *et al*., 1994; Okamoto *et al*., 1994; Suzuki *et al*., 1995), 7 to 83% in non-small cell lung cancers (de-Vos *et al*., 1995; Nakagawa *et al*., 1995; Xiao *et al*., 1995), and 4 to 36% in B-ALL (Schroder *et al*., 1995; Guidal-Giroux *et al*., 1996). These varying results might arise in part from the inevitable contamination of nonneoplastic cells in any resected tumor. In order to evaluate more accurately the alterations of *CDKN2* in

tumor cells, we need to examine other specimens rather than surgically resected cancer tissues. Therefore, formalin-fixed and paraffin-embedded tumor tissues may allow us to evaluate more clearly mutations of the *CDKN2* gene in tumor cells.

In this study, we present alterations of the *CDKN2* gene in each 20 formalin-fixed and paraffin-embedded tumor specimens of stomach adenocarcinomas, lung cancers (10 NSCLCs and 10 SCLCs), cervix cancers and hepatocellular carcinomas using PCR and PCR-SSCP analysis on exon 2 of *CDKN2*.

Materials and Methods

Tumor specimens

Formalin-fixed and paraffin-embedded tumor tissues of 20 stomach adenocarcinomas, 20 lung cancers (10 NSCLCs and 10 SCLCs), 20 cervix cancers and 20 hepatocellular carcinomas were obtained at Yeungnam University hospital, Taegu, Korea.

DNA extraction and PCR

The homozygous deletion of *CDKN2* in the fixed and paraffin-embedded tumor tissues was analyzed by PCR. Genomic DNAs from paraffin-embedded tumor tissues were isolated by proteinase K digestion and phenol/ chloroform extraction (McPherson *et al*., 1991). Briefly, one or 2 pieces of 10-μm paraffin section containing > 70% tumor cells were suspended in 200 μl of DNA extraction buffer (10 mM Tris-HCl, pH 8.0, 100 μM EDTA, 0.5% SDS, 20 mg/ml RNase and 0.1 mg/ml proteinase K) and incubated at 37˚C for 4 days. After the mixtures were extracted with an equal volume of phenol, phenol/ chloroform/isoamylalcohol (25:24:1) and chloroform/iso-amylalcohol (24:1), DNAs were precipitated with an equal volume of isopropanol. DNA fragments for exon 2 of the *CDKN2* gene were amplified from 30-50 ng of genomic DNA in 20 μl of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 μM each of dNTPs, 1 μM each of primers, 5 % DMSO and 0.5 U of Taq DNA polymerase (Promega Corp.). Primer sets for the PCR amplification were 35F and 551R for exon 2 of the *CDKN2* gene as described by Kamb *et al*. (1994). The PCR cycles were initial dena-turation of 5 min at 95˚C; 4 cycles of 10 sec at 95˚C, 10 sec at 68˚C and 30 sec at 72˚C; 4 cycles of 10 sec at 95˚C, 10 sec at 66˚C and 30 sec at 72˚C; 4 cycles of 10 sec at 95˚C, 10 sec at 64˚C and 30 sec at 72˚C; 4 cycles of 10 sec at 95˚C, 10 sec at 62˚C and 30 sec at 72˚C; 30 cycles of 10 sec at 95˚C, 10 sec at 60˚C and 30 sec at 72˚C; one cycle of 5 min at 72˚C. *GAPDH* was also amplified as an internal control. Primer sets of *GAPDH* were sense primer, tcacatattctggaggagcc and antisense primer, ggctcaccatgtagcactca. PCR amplification for *GAPDH* was carried in the same mixture used as those for *CDKN2* except 5% DMSO. The PCR cycles for *GAPDH* were initial denaturation of 3 min at 94˚C; 30 cycles of 10 sec at 94˚C, 10 sec at 60˚C and 15 sec at 72˚C; final extension of 3 min at 72˚C. Amplified DNA fragments were separated on 1.5% agarose gels containing ethidium bromide. Deletions of exon 2 of the *CDKN2* gene were estimated by comparing the intensity of amplified *CDKN2* fragments to that of amplified *GAPDH* fragments in the same tumor tissues.

PCR-SSCP analysis

Mutations in exon 2 of the *CDKN2* gene were analyzed by PCR-SSCP (Orita *et al*., 1989). For PCR-SSCP analysis, PCRs were performed as described above with the exception of adding 0.5 mCi of [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol) in a final volume of 5 μl. The PCR products were digested with *Sma* I and mixed with an equal volume of denaturation buffer (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). Samples were heated at 94˚C for 3 min, chilled on ice, and immediately loaded onto a 0.5 × MDE gel (FMC Corp.). The samples were run through the MDE gel at 6-8 watts constant power for 3 h at room temperature. The gels were dried and exposed to x-ray films for 24-72 h at -70˚C.

Results

No deletions of exon 2 of the *CDKN2* gene and no mobility shifts of the amplified DNA fragments were identified in the 20 paraffin-embedded specimens of stomach adenocarcinomas (Figure 1).

Nine of the 20 (45%) lung cancers had deletions in exon 2 of the *CDKN2* gene. In details, 7 of the 10 (70%) NSCLCs and 2 of the 10 (20%) SCLCs were identified to have deletions in this gene (Figure 2). PCR-SSCP analysis showed that 2 (L13 and L17) of the 8 SCLCs without deletions of the *CDKN2* gene had mobility shifts of the amplified DNA fragments (Figure 5). Thus, 4 of the 10 (40%) SCLCs might have had mutations in the *CDKN2* gene.

Thirteen of the 20 (65%) cervix cancers had deletions in exon 2 of the *CDKN2* gene (Figure 3). One (C19) of the 7 cervix cancers without deletions showed mobility shifts of the amplified DNA fragments on PCR-SSCP analysis (Figure 5). Therefore, 14 (70%) cervix cancers might have had mutations in this gene.

Ten of the 20 hepatocellular carcinomas had deletions in exon 2 of the *CDKN2* gene (Figure 4). One (H18) of the 10 hepatocellular carcinomas without deletions showed mobility shifts on PCR-SSCP analysis (Figure 5). Eleven (55%) hepatocellular carcinomas might have had muta-tions in this gene.

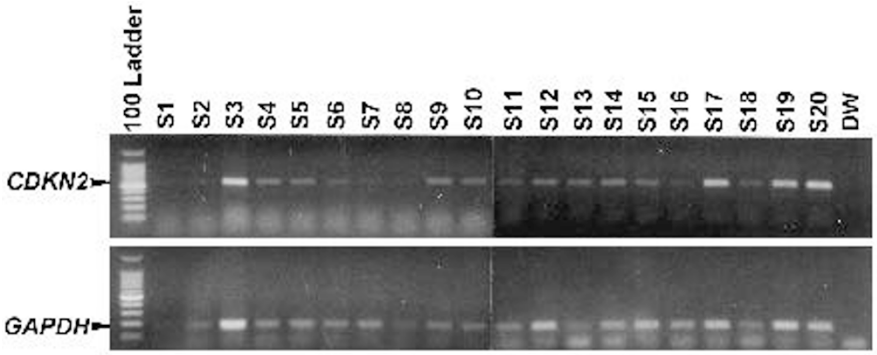

Figure 1. PCR of *CDKN2* in the 20 paraffin-embedded specimens of stomach adenocarcinoma. The amplified DNAs were resolved on a 1.5% agarose gel containing ethidium bromide. No deletions were found in these samples.

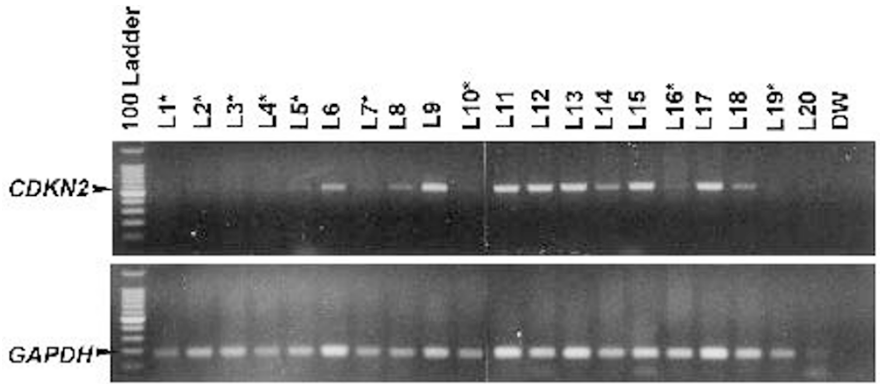

Figure 2. PCR of *CDKN2* in the 20 paraffin-embedded specimens of lung cancer. L1 to L10 specimens are NSCLCs and L11 to L20, SCLCs. *These specimens had deletions in exon 2 of *CDKN2*.

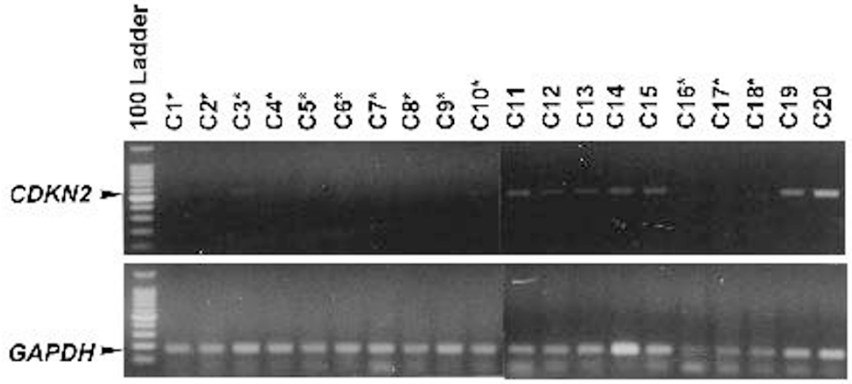

Figure 3. PCR of *CDKN2* in the 20 paraffin-embedded specimens of cervical cancer. *These specimens had deletions in exon 2 of *CDKN2*.

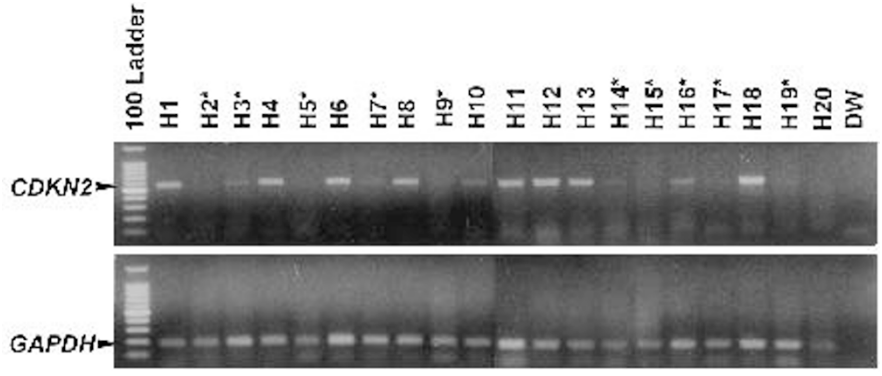

Figure 4. PCR of *CDKN2* in the 20 paraffin-embedded specimens of hepatocellular carcinoma. *These specimens had deletions in exon 2 of *CDKN2*.

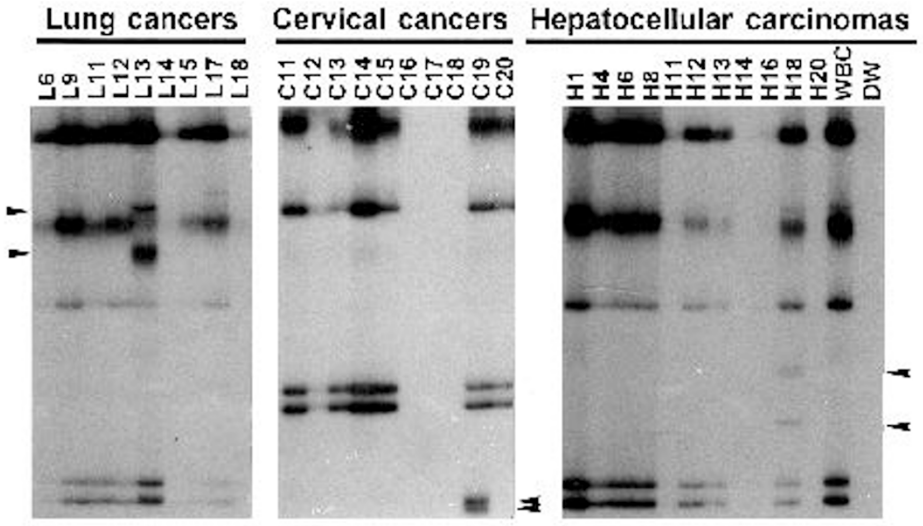

Figure 5. PCR-SSCP analysis in the paraffin-embedded tumor specimens without deletions in *CDKN2*. Arrowheads indicate mobility shifts in L13, L17, C19 and H18.

Although rare mutations in the *CDKN2* gene were found in stomach adenocarcinomas, a high frequency of its mutations were identified in NSCLCs (70%), cervix cancers (70%), and hepatocellular carcinomas (55%).

Discussion

To investigate the importance of *CDKN2* mutations in tumors which occur at a high incidence in Korea, we examined the mutations on exon 2 of the *CDKN2* gene in each set of 20 formalin-fixed and paraffin-embedded tumors of stomach adenocarcinomas, lung cancers, cervix cancers and hepatocellular carcinomas using PCR and PCR-SSCP analysis.

Mutations of the *CDKN2* gene were not found in the 20 paraffin-embedded specimens of stomach adenocarcinoma. We could identify rare mutations of *CDKN2* in 14 surgically resected stomach cancer tissues. A similar observation has been reported in stomach cancers by Igaki et al. (1995). They also showed that no mutations of *CDKN2* were observed in 19 surgical specimens of gastric adenocarcinomas. The rare mutations of *CDKN2* in paraffin-embedded or surgical specimens of stomach adenocarcinomas suggests that the mutations of *CDKN2* may not be a critical genetic change in the formation and progression of stomach cancers. However, we could observe that the expression of the *CDKN2* gene was decreased or absent at a high frequency in stomach cancer tissues and stomach cancer cell lines by Northern blot analysis. These results indicate that the inactivation of the *CDKN2* gene due to hypermethylation rather than mutations may be involved in the tumorigenesis and progression of these cancers (Gonzalez-Zulueta *et al*., 1995; Merlo *et al*., 1995; Herman *et al*., 1995). Therefore, further studies on the aberrant methylation of the *CDKN2* gene in paraffin-embedded specimens of stomach cancers may be needed.

On the other hand, we could identify highly frequent mutations of the *CDKN2* gene in paraffin-embedded specimens of 3 other cancers; 55% in lung cancers (70% in NSCLCs and 40% in SCLCs), 70% in cervix cancers and 55% in hepatocellular carcinomas.

In lung cancers, the frequency of *CDKN2* alterations has shown a clear specificity in terms of the affected histological subtype, which is much higher in NSCLCs than in SCLCs, and varied from 7 to 83% in NSCLCs (de-Vos *et al*., 1995; Nakagawa *et al*., 1995; Xiao *et al*., 1995). Xiao *et al*.(1995) reported that codeletion of *p15* and *p16* was found in 15 of 18 (83%) primary NSCLCs by FISH technique. Our results on the mutation frequency (70%) in the paraffin-embedded tissues of NSCLCs might be nearly the same as those reported by Xiao *et al* (1995). Washimi *et al*. (1995) reported that the deletions of *CDKN2* were absent in 20 SCLC cell lines derived from patients. However, Geradts *et al*. (1995) showed that the inactivation of *CDKN2* was found in some SCLC cell lines and 8 of 17 (47%) lung cancers by Western blot analysis or immunochemical staining of p16 for paraffin-embedded specimens.

Our data from cervix cancers suggest that the alterations of *CDKN2* are frequent genetic alterations in these cancers. Kelley *et al*. (1995) showed that no alterations of *CDKN2* were observed in 8 HPV-positive and 2 HPV-negative cervix cell lines. However, there have been no reports on the mutation frequency of *CDKN2* in tumor tissues of cervix cancers.

Previously, we found that the mutational frequency of *CDKN2* in surgical specimens of hepatocellular carcinomas was as high as 45% (5/11). In the present study in paraffin-embedded tumor specimens, the frequency (55%) of mutations was observed to be nearly the same as that of surgical specimens.

Presently, the role of *CDKN2* in carcinogenesis and the progression of human cancers is controversial. Geradts *et al*. (1995) pointed out that the invariable presence of nonneoplastic elements in any resected tumor is res-ponsible for an unknown but possibly large number of inaccurate results in the published studies, virtually all of which are based on homogenates of primary or metastatic tumors and showed that frequent abnormalities in *CDKN2* were detected in paraffin-embedded specimens of breast, lung, bladder and colon cancers by immunohistochemical staining of p16.

In the present study, we found that the mutation frequency of *CDKN2* was higher in paraffin-embedded specimens than in surgical specimens of lung, cervix and hepatocellular cancers. The alterations of *CDKN2* may be a critical genetic change in these cancers and molecular analyses in formalin-fixed and paraffin-embedded specimens of human cancers may help to define more clearly *CDKN2* alterations.

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