Mutations of CDKN2 (MTS1/p16^{INK4A}) and MTS2/p15^{INK4B} genes in human stomach, hepatocellular, and cholangio-carcinomas

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Abbreviations: CDKN2, cyclin dependent kinase inhibitor 2; MTS2, multiple tumor suppressor 2; LOH, loss of heterozygosity; PCR, polymorphism; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR-SSCP, PCR-single strand conformation polymorphism

Abstract

The CDKN2 (MTS1/p16^{INK4A}) and MTS2/p15^{INK4B} genes, encoding cyclin dependent kinase inhibitors, were found to be homozygously deleted at high frequency in cell lines from many different types of cancer and some primary cancers. To determine the frequency of CDKN2 and MTS2 mutations in human stomach, liver, and cholangio- cancers, molecular analyses of CDKN2 and MTS2 were performed on 4 stomach cancer cell lines, 14 primary stomach adenocarcinomas, 11 hepatocellular carcinomas, and 5 cholangiocarcinomas. Two (50%) of the four stomach cancer cell lines (SNU1, SNU5, SNU16 and Kato III) had mutations of the CDKN2 and MTS2 gene: SNU16, a homozygous deletion; SNU5, a nonsense mutation, CGA to TGA (Arg to stop) at codon 72 of the CDKN2 gene. No mutations were observed in the 14 primary stomach cancer tissues. In contrast to the mutations of CDKN2 and MTS2, Northern blot analysis showed that expression of CDKN2 was absent or decreased in all the remaining four stomach cancer cell lines and 11 of the 14 (79%) primary stomach adenocarcinomas. Five of the 11 (45%) hepatocellular carcinomas and one of the 5 (20%) cholangiocarcinomas have possible mutations in CDKN2 exon 2 and MTS2. One of hepatocellular carcinoma was expressed mobility shift on PCR-SSCP analysis and a missense mutation, GAC to GAA (Asp to Glu) at codon 105 of CDKN2 gene. These

results suggest that mutations or inactivation of the CDKN2 gene may be a critical genetic change in the formation of stomach, hepatocellular, and cholangiocarcinomas.

Keyword: CDKN2, MTS2, mutation, PCR-SSCP

Introduction

The development of cancer is affected by environmental factors and genetic factors. Genetically, oncogenes as positive regulators and tumor suppressor genes as negative regulators are involved in carcinogenesis (Levine, 1993). A variety of oncogenes and tumor suppressor genes such as SV40T, E1A/E1B of adenovirus, E6/E7 of HPV-16, p53, Rb, and cyclin dependent kinase inhibitors, have been shown to affect cell growth through the regulation of cell cycle (Hunter and Pines, 1994; Peter and Herskowitz, 1994).

Frequent loss of heterozygosity (LOH) and homozygous deletions of chromosome 9p21 have suggested the presence of tumor suppressor genes in this region. Recently, the CDKN2 and MTS2/p15^{INK4B} genes, encoding inhibitors of cyclin dependent kinases (Serrano *et al.*, 1993; Hannon and Beach, 1994), were shown to be located at chromosome 9p21 and to be candidate tumor suppressor genes, when it was found to be homozygously deleted at high frequency in cell lines from many different types of cancer (Kamb *et al.*, 1994; Nobori *et al.*, 1994). In addition, mutations of CDKN2 have been observed frequently in T-cell leukemias (Hebert, 1994), pancreatic adenomas (Bartsch *et al.*, 1995), 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 the foregoing malignancies, mutations of CDKN2 were identified at low frequency 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 (Zhang *et al.*, 1994), thyroid cancers (Okamoto *et al.*, 1994; Tung *et al.*, 1996) and esophageal cancers (Suzuki *et al.*, 1995;). These varying results have raised the question whether CDKN2 is the target gene involved in all the tumor types showing homozygous deletion at 9p21 or whether another nearby gene could be the tumor suppressor targeted for inactivation (Bonetta, 1994; Cairns *et al.*, 1994; Spruck *et al.*, 1994).

Human stomach, hepatocellular carcinomas and cholangiocarcinomas display multiple abnormalities of particularly chromosomal losses or structural rearrange-

ments at 11p13-15, or 1p (Rodriguez *et al.*, 1990; Ezaki *et al.*, 1996; Cho *et al.*, 1996).

In this study, we show various *CDKN2* and *MTS2/p15^{INK4B}* mutations, including deletions, mutations, and down-regulations in 4 stomach cancer cell lines, 14 stomach cancer tissues, 11 hepatocellular carcinomas, and 5 cholangiocarcinomas.

Materials and Methods

Cell lines and tumors

Four human stomach cancer cell lines (SNU1, SNU5, SNU16 and Kato III) and one hepatocellular cancer cell line (HepG2) were purchased from Korea Cell Line Bank (KCLB) (Seoul, Korea), and grown in RPMI1640 medium containing iron-supplemented 10% bovine serum (Hyclone, Logan, UT) and 50 µg/ml of gentamycin. Tumor tissues from patients of stomach (14 cases), hepatocellular carcinomas (11 cases) or cholangiocarcinoma (5 cases) were directly obtained from surgery and frozen at -70°C prior to use for DNA or RNA extraction.

DNA extraction and PCR

The homozygous deletion of *CDKN2* and *MTS2/p15^{INK4B}* in cell lines and tumor tissues was analyzed by PCR. Genomic DNAs were isolated by proteinase K digestion and phenol/chloroform extraction as described by Sambrook *et al.* (1989). DNA fragments for exons 2 of *CDKN2* and *MTS2* were amplified from 100 ng of genomic DNA in 40 µl of PCR mixture containing 10 mM Tris/Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 100 µM each of dNTPs, 1 µM each of primers, 5% dimethyl sulfoxide and 1 U of AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ). Primer sets for the PCR amplification were 35F, 42F and 551R for exon 2 of *CDKN2*, and 89F and 50R for exon 2 of *MTS2* as described by Kamb *et al.* (1994). The PCR cycles were initial denaturation of 5 min at 95°C; 4 cycles of 10 s at 95°C, 10 s at 68°C and 30 s at 72°C; 4 cycles of 10 s at 95°C, 10 s at 66°C and 30 s at 72°C; 4 cycles of 10 s at 95°C, 10 s at 64°C and 30 s at 72°C; 4 cycles of 10 s at 95°C, 10 s at 62°C and 30 s at 72°C; 30 cycles of 10 s at 95°C, 10 s at 60°C and 30 s at 72°C; one cycle of 5 min at 72°C. Glyceraldehyde 3-phosphate dehydrogenase (*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 out in the same mixture used for *CDKN2*, except for emission of the 5% dimethyl sulfoxide. The PCR cycles for *GAPDH* were initial denaturation of 3 min at 94°C; 30 cycles of 10 s at 94°C, 10 s at 60°C and 15 s at 72°C; final extension of 3 min at 72°C. Amplified DNA fragments were separated on 1.5% agarose gels containing ethidium bromide.

PCR-single strand conformation polymorphism (PCR-SSCP) analysis and sequencing

Mutations in exon 2 of the *CDKN2* gene were analyzed by PCR-SSCP (Orita *et al.*, 1989) and direct sequencing of the PCR products. For PCR-SSCP analysis, PCRs were performed as described above with the exception of adding 0.5 µCi of [α -³²P]dCTP (specific activity, 3000 Ci/mmol) in a final volume of 5 ml. 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 to 94°C for 3 min, chilled on ice, and immediately loaded onto a 0.5×MDE gel (FMC, Rockland, ME). 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 4 to 72 h at -70°C. To confirm the presence of a mutation, direct sequencing of PCR-generated exon 2 fragments of the *CDKN2* gene was performed using a Sequenase PCR sequencing kit (Amersham, Buckinghamshire, UK) with a 42F primer, according to the instructions supplied by the manufacturer.

Southern blot analysis

Ten micrograms of DNA extracted from cell lines and tumor tissues was digested with *Eco*RI, separated in 0.7% agarose gels, and transferred to Nylon membranes (Schleicher and Schuell, Dassel, Germany). The DNA fragment of exon 2 of *CDKN2* was cloned in a pGEM-T vector (Promega, Madison, WI) and used as a probe for detecting *CDKN2* and *MTS2* DNA in cell lines and tumor tissues. After baking the DNA blotted membranes at 80°C for 2 h, and the membranes were hybridized with a ³²P-labeled *CDKN2* probe at 42°C for 12 to 18 h. The blots were washed three times in 1×SSC, 0.1% SDS at 60°C for 15 min, then three times in 0.1×SSC, 0.1% SDS at 60°C for 15 min, and exposed to X-ray films at -70°C with intensifying screens. Equal loading and transfer of DNA were estimated with a *GAPDH* probe.

Northern blot analysis

Total RNAs from stomach cancer tissues and cell lines were extracted by using Tri-reagent (Molecular Research Center, Cincinnati, OH). Thirty micrograms of total RNA was separated in a 1% formaldehyde agarose gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The DNA fragment of exon 2 of *CDKN2* was used as a probe for detecting *CDKN2* RNA in cell lines and tumor tissues. Equal loading and transfer of RNA were estimated with a *GAPDH* probe. The down-regulation of *CDKN2* in the tumor tissues and cancer cell lines was compared with expression of *CDKN2* to that of *GAPDH* in the each specimen.

Results

Mutations of *CDKN2* and *MTS2/p15^{INK4B}* in stomach cancer cell lines and stomach cancer tissues

Mutations of *CDKN2* and *MTS2* were found in two (50%) of the four stomach cancer cell lines; SNU16 had homozygous deletions in the *CDKN2* and *MTS2* genes and SNU5 had a nonsense mutation, CGA to TGA (Arg to stop) at codon 72 of *CDKN2* (Figure 1). No deletions of exon 2 of *CDKN2* and *MTS2* were observed in 14 stomach cancer tissues from PCR and Southern blot analysis (Figure 2). The PCR-SSCP analysis and direct sequencing from the foregoing amplified DNA fragments of exon 2 of *CDKN2* showed no sequence alterations in these all cancer samples (data not shown). In contrast to rare mutations of *CDKN2* in the stomach adenocarcinomas, it is of interest that the down-regulation of *CDKN2* in these tissues was identified at high frequency by Northern blot analysis. The mRNA expression of *CDKN2* gene was absent or

decreased in all the remaining stomach cancer cell lines and 11 of the 14 (79%) stomach cancer tissues (Figure 3). The mRNA expression of *CDKN2* gene of hepatocellular carcinoma cell line (HepG2) was also decreased. These results suggest that although the mutations of *CDKN2* and *MTS2* were observed at lower frequency in stomach cancer tissues than in cell lines, the down-regulation of the *CDKN2* gene was identified at high frequency both in the stomach cancer cell lines and tissues.

Mutations of *CDKN2* and *MTS2/p15^{INK4B}* in hepatocellular carcinomas and cholangiocarcinomas

All the 11 hepatocellular carcinomas and the 5 cholangiocarcinomas showed the amplified DNA fragments of *CDKN2* and *MTS2*. However, 4 of the 11 hepatocellular carcinomas (H6, H9, H10, and H11) and one of the 5 cholangiocarcinomas (C5) have possible homozygous deletions of the *CDKN2* and *MTS2* genes in Southern blot analysis (Figure 4). The amplified fragments of *CDKN2*

Figure 1. Mutations of *CDKN2* and *MTS2/p15^{INK4B}* in stomach cancer cell lines. **A**, PCR of exon 2 of *CDKN2* and *MTS2/p15^{INK4B}* in stomach cancer cell lines. The amplified DNAs were resolved on a 1.5% agarose gel containing ethidium bromide. SNU16 had homozygous deletions in exons 2 of the *CDKN2* and *MTS2* genes. **B**, Direct sequencing of the amplified DNAs showed that SNU5 had a nonsense mutation, CGA to TGA (Arg to stop) at codon 72 of the *CDKN2* gene.

Figure 2. PCR and Southern blot analyses of the *CDKN2* and *MTS2/p15^{INK4B}* genes in human stomach adenocarcinomas. **A**, PCR analysis of exons 2 of *CDKN2* and *MTS2* in human stomach adenocarcinomas (13 cases: S1-S13). **B**, Southern blot analysis in 14 cases of human adenocarcinoma and cancer cell lines (SNU1, SNU16 and KatohII). Ten microgram of DNA extracted from cancer tissues and cancer cell lines was digested with *EcoRI*, separated on a 0.7% agarose gel and Southern blotted. The membrane was hybridized with a ³²P-labeled *CDKN2* exon 2 probe. The equal loading and transfer of DNA were measured with a *GAPDH* probe.

Figure 3. The mRNA expressions of the *CDKN2* gene by Northern blot analysis in the tissues of human stomach adenocarcinomas (S1-S14) and cancer cell lines. Thirty microgram of RNA extracted from the tissues and cell lines were separated on a 1% formaldehyde agarose and blotted to a nitrocellulose membrane. The membrane was hybridized with a 32 P-labeled *CDKN2* exon 2 probe. The equal loading and transfer of RNA were measured with a *GAPDH* probe. Note absence of *CDKN2* expression in SNU16 having a homozygous deletion of *CDKN2*, down-regulation in SNU1, SNU5, KatoIII, HepG2 and 11 of the 14 stomach adenocarcinomas.

and *MTS2* in the 11 hepatocellular carcinomas and the 5 cholangiocarcinomas might, however, be due to DNAs derived from normal tissues. PCR-SSCP analysis and direct sequencing of all cases of the amplified DNA fragments of exon 2 of *CDKN2* showed that one hepatocellular cancer tissue (H7) had mobility shifts, and a point mutation, GAC to GAA (Asp to Glu) at codon 105 of *CDKN2* (Figure 5). Although cell lines derived from hepatocellular carcinomas and cholangiocarcinomas were not examined and the numbers of tumor samples were small, the frequency of mutations of *CDKN2* and *MTS2* in hepatocellular carcinomas and cholangiocarcinomas may be high (45% in hepatocellular carcinomas and 20% in cholangiocarcinomas). Therefore, mutations of the *CDKN2* and *MTS2* genes may be an important genetic change in hepatocellular carcinomas and cholangiocarcinomas.

Discussion

To investigate the importance of *CDKN2* and *MTS2/p15^{INK4B}* mutations in development of stomach, hepatocellular and cholangio cancers, we examined mutations of the *CDKN2* and *MTS2* genes in 4 stomach cancer cell lines, 14 stomach adenocarcinomas, 11 hepatocellular carcinomas, and 5 cholangiocarcinomas using PCR for exon 2 of *CDKN2* and *MTS2*, PCR-SSCP and direct sequencing of the amplified DNA fragments of exon 2 of *CDKN2*, by both Southern and Northern blot analysis.

Figure 4. PCR of exons 2 of the *CDKN2* and *MTS2* genes **A** and Southern blot analysis **B** in human hepatocellular carcinomas (cases, H1-H11) and cholangiocarcinomas (cases, C1-C5). Southern blot analysis showed that some samples (H6, H9, H11, and C5) might have homozygous deletions in both of the *CDKN2* and *MTS2* genes.

Figure 5. PCR-SSCP analysis and direct sequencing of the amplified DNA fragments in the human hepatocellular carcinomas and cholangiocarcinomas. **A**, PCR-SSCP analysis. Arrow-heads indicate mobility shifts in H7. **B**, Direct sequencing of the amplified DNA fragments showed that H7 had a missense mutation, GAC to GAA (Asp to Glu) at codon 105 of the *CDKN2* gene.

Mutations of the *CDKN2* and *MTS2* genes were detected in 2 stomach cancer cell lines but were not found in any of the 14 stomach cancers. Our data from stomach cancer cell lines and tumor tissues indicate that the frequency of *CDKN2* alterations in the cell lines is much higher than in the tumor tissues. A similar observation has been reported in stomach cancers by Igaki *et al.* (1995). They also showed that no mutations of the *CDKN2* gene were observed in 19 surgical specimens of gastric adenocarcinomas. One possible explanation for the difference in the frequency of the mutations in *CDKN2* and *MTS2* between cell lines and primary tumors from patients could

be that DNA fragments of *CDKN2* can be amplified from DNAs derived from normal tissues, because cancer tissues inevitably contain residual normal tissues. A second possibility is that cancer cells with mutations of the *CDKN2* gene take advantage of acquiring immortality. Again, after immortalization, cell lines may become more prone to deletion of one of these loci. The absence of mutations in the *CDKN2* and *MTS2* genes in stomach cancer tissues suggests that mutations of the *CDKN2* and/or *MTS2* genes may not be a critical genetic change in the formation of stomach cancers. However, mRNA expressions of *CDKN2* gene by Northern blot analysis were decreased or absent in 11 of the 14 (79%) stomach cancer tissues and in all the stomach cancer cell lines without *CDKN2* mutations. These results indicate that the inactivation of the *CDKN2* gene may be involved in the tumorigenesis of these cancers. Some investigators have shown that inactivation of the *CDKN2* gene is frequently associated with de novo methylation of a 5' CpG island of the gene in human cancers such as breast, prostate, renal and colon cancers, which have been reported to show low frequency of *CDKN2* mutations, potentially representing a common pathway of tumor suppressor gene inactivation in human cancers (Gonzalez-Zulueta *et al.*, 1995; Herman *et al.*, 1995; Merlo *et al.*, 1995). Therefore, further studies on the aberrant methylation of the *CDKN2* gene in stomach cancers may be needed.

On the other hand, we could observe relatively frequent mutations of the *CDKN2* and *MTS2* genes in the hepatocellular carcinomas (5 of the 11, 45%) and cholangiocarcinomas (1 of the 5, 20%). There have been no reports on the mutation frequency of *CDKN2* in primary hepatocellular carcinomas. In a study of primary biliary tract cancers and cell lines, Yoshida *et al.* (1995) showed that 4 of the 4 (100 %) biliary tract cancer cell lines had homozygous deletions in *CDKN2* and/or *MTS2* genes, and 16 of the 25 (67 %) primary tumors had point mutations in the *CDKN2* genes but no mutations in the *MTS2* gene were found in these cancers. However, we could identify homozygous deletion in both of the *CDKN2* and *MTS2* genes in one of the 5 cholangiocarcinomas. Presently, the role of *CDKN2* in carcinogenesis and development of human cancer is controversial. However, our data in stomach, liver and cholangio-carcinomas suggest that inactivation of the *CDKN2* gene due to mutations or hypermethylation may play an important role in the development of these cancers.

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