Detection of Aberrant $p16^{INK4A}$ Methylation in Sera of Patients with Liver Cirrhosis and Hepatocellular Carcinoma

Hepatocellular carcinomas (HCCs) show genomic alterations, including DNA rearrangements associated with HBV DNA integration, loss of heterozygosity, and chromosomal amplification. The genes most frequently involved are those encoding tumor suppressors. The $p16^{INK4A}$ tumor suppressor gene frequently displays genetic alteration in HCC tissues. The present study was performed to examine the incidence of methylated $p16^{INK4A}$ in the sera of liver cirrhosis (LC) and HCC patients, and to evaluate its role as a tumor marker of HCC. The sera of 23 LC patients and 46 HCC patients were examined in this study. The methylation status of $p16^{INK4A}$ was evaluated by methylation-specific PCR of serum samples. Methylated $p16^{INK4A}$ was detected in 17.4% (4/23) of LC patients and in 47.8% (22/46) of HCC patients. No association was demonstrated between $p16^{INK4A}$ methylation and serum AFP level. As the status of $p16^{INK4A}$ methylation was not associated with serum AFP level, it may have a role as a tumor marker of HCC.

Key Words : *$p16^{INK4A}$ Methylation; PCR, Methylation-Specific; Carcinoma, Hepatocellular; Liver Cirrhosis*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cause of mortality in cancer patients in Korea, with a death rate of approximately 21 per 100,000 (1). No treatment modality has been successful in increasing survival time in patients with advanced HCC, whereas effective treatments, such as liver transplantation, surgical resection, and local ablation therapies, are available for patients with small HCCs (2-6).

Although alpha-fetoprotein (AFP) measurement and ultrasonography are useful surveillance tests for detecting HCCs at a stage at which they may be treated, both tests have limitations: AFP shows low sensitivity and specificity, while the results of ultrasonography are dependent on the skill of those performing the examination and on the condition of the patient.

Inactivation of tumor suppressor genes is important in the development of cancers, and leads to abnormal proliferation, transformation, invasion, and metastasis (7). Inactivation of tumor suppressor genes can occur due to silencing as a result of methylation of tumor suppressor gene promoters, as well as genetic mutation, loss of heterozygosity (LOH), or deletion of homozygosity (8-10).

The tumor suppressor gene $p16^{INK4A}$ is located on chromosome 9p21 and encodes the p16 protein, which binds selectively to CDK4 to inhibit activation of the CDK4/cyclin D complex in G1 phase (11). Recent studies have indicated the occurrence of structural changes in $p16^{INK4A}$ in HCC (12, 13).

Inactivation of this gene, which normally inhibits progression to the G1 phase of the cell cycle, is involved in the initiation of tumors. The methylation of $p16^{INK4A}$ is known to silence transcription of the gene (14).

The degree of $p16^{INK4A}$ methylation shows a wide range of variation (from 0 to 94%) in tumor tissues of HCC patients (8, 15-22), and this change has been detected in the sera of such patients (20).

This study was performed to evaluate the incidence of methylated $p16^{INK4A}$ in the sera of liver cirrhosis (LC) and HCC patients, and to examine its role as a tumor marker of HCC.

MATERIALS AND METHODS

Subjects

This study included 23 patients with cirrhosis and 46 with HCC. Cirrhosis patients were selected on the basis of clinical, biochemical, and radiological findings, and follow-up was performed for at least 6 months to exclude undetected cancers. HCC patients (23) were selected consecutively from those diagnosed according to the criteria of the European association for the study of the liver (EASL) (Table 1).

Methods

Methylation-specific PCR (MSP) (24) was used to detect

abnormal methylation of the *p16^INK4A* gene. Serum AFP was measured by radioimmunoassay (BioSource Europe S.A., Nivelles, Belgium).

Isolation and Quantification of DNA

To obtain serum, blood specimens from each patient were centrifuged at 3,000 RPM for 20 min. Sera were stored at -70°C until DNA extraction. A total volume of 2 mL of serum was treated with an equal volume of 1% sodium dodecyl sulfate, 0.5 mg/mL proteinase K (1 × SDS/PK) for over 16 hr at 58°C. The solution was extracted twice with an equal volume of PC-8 [250 mL of Aquaphenol, supplemented with pH 8.0 buffer (Qbiogene Inc., Carlsbad, CA, U.S.A.), 40 mL of distilled water, 2.5 mL of 0.5 M EDTA, and 200 mL of chloroform], and 7.5 M ammonium acetate was added to the supernatant. After addition of glycogen, the DNA was precipitated with ethanol. The DNA pellet was washed twice with 70% ethanol, dried, and dissolved in LoTE (30 mM Tris-HCl, 0.3 mM EDTA).

Modification Reaction

Bisulfite conversion of genomic DNA was performed using the reagents provided with a CpGenome™ DNA modification kit, according to the manufacturer's protocols (Intergen, Edinburgh, U.K.). The modified DNA was eluted into 20 μL of TE (10 mM Tris-HCl, 1 mM EDTA), and was either used immediately as a template for MSP or stored at -20°C.

Methylation-specific Polymerase Chain Reaction (MSP)

Bisulfite-modified DNA was amplified using the primers

provided with a CpG WIZ™ *p16^INK4A* amplification kit (Intergen, Edinburgh, U.K.). The PCR mixture contained 1 × universal PCR buffer, dNTPs (each at 0.25 mM), methylated or unmethylated primer, 1 unit of AmpliTaq Gold polymerase (Perkin Elmer, Wellesley, MA), and modified DNA (100 ng) in a final volume of 12.5 μL. Water was used as a negative control, while positive control DNA was supplied with the kit. Amplification was carried out in a thermal cycler (MWG-Biotech AG, Ebersberg, Germany) as follows: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min; with a final extension of 10 min at 72°C. PCR products (12.5 μL) were separated on non-denaturing 10% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Statistical Analysis

Statistical analysis was performed using the SPSS™, version 11.0 for Windows software (SPSS Inc., Chicago, IL, U.S.A.). The significance of variations between groups was examined by chi-square test, Fisher's exact test, or Student's t-test.

RESULTS

Clinical Features of Patients

The average age of patients was 57 ± 11 yr (mean standard deviation), and ranged from 30 to 86. The patient population consisted of 45 men and 24 women. Forty patients (58.0%)

Table 1. Diagnostic criteria for hepatocellular carcinoma

Cytohistological criteria

Non-invasive criteria (restricted to cirrhotic patients)
 1. Radiological criteria: two coincident imaging techniques*
　　Focal lesion >2 cm with arterial hypervascularization
 2. Combined criteria: one imaging technique associated with AFP
　　Focal lesion >2 cm with arterial hypervascularization
　　AFP levels >400 ng/mL

*Four techniques considered. US, spiral CT, MRI, and angiography.

Table 2. Clinical and laboratory features of cirrhosis and HCC patients

	Liver cirrhosis	HCC	*p* value
Number of patients	23	46	
Sex (M/F)	13/10	32/14	0.290*
Age (yr)	58±14	56±9	0.624[†]
Etiology (%)			0.010*
Chronic hepatitis B	43.5 (10/23)	65.2 (30/46)	
Chronic hepatitis C	13.0 (3/23)	21.7 (10/46)	
Alcohol	34.8 (8/23)	4.3 (2/46)	
Unknown	8.7 (2/23)	10.9 (5/46)	
Alpha-fetoprotein (ng/mL)	82.9±197.7	11,327.9±23,485.9	0.003[†]

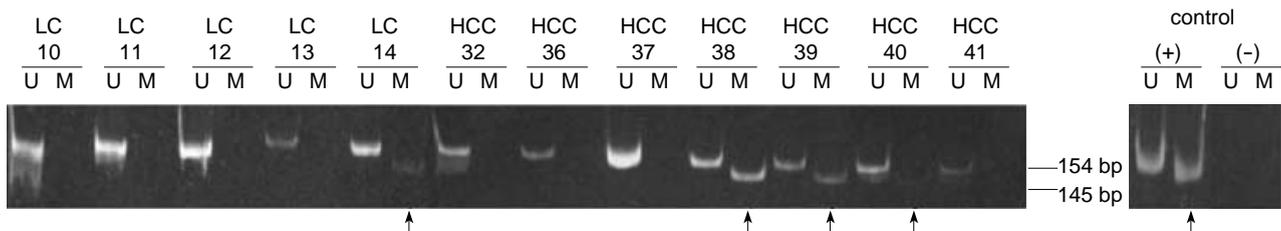*Fisher's exact test. [†]Student's t-test.

Fig. 1. Detection of aberrant *p16^INK4A* methylation (sample numbers 14, 38, 39 and 40, as indicated with arrows) in the sera of patients with liver cirrhosis (LC) and hepatocellular carcinoma (HCC). U, unmethylated; M, methylated; bp, base pairs.

Table 3. Association between p16^{INK4A} methylation and serum AFP levels in patients with HCC*

| AFP (µg/L) | Methylation (%) | | p value |
	Positive	Negative	
≥ 20 (n=28)	67.0	61.0	0.690†
< 20 (n=16)	33.0	39.0	

*Two patients were excluded due to long interval between the AFP test date and serum sampling for methylation. †chi-square test.

Table 4. Association between p16^{INK4A} methylation and tumor size

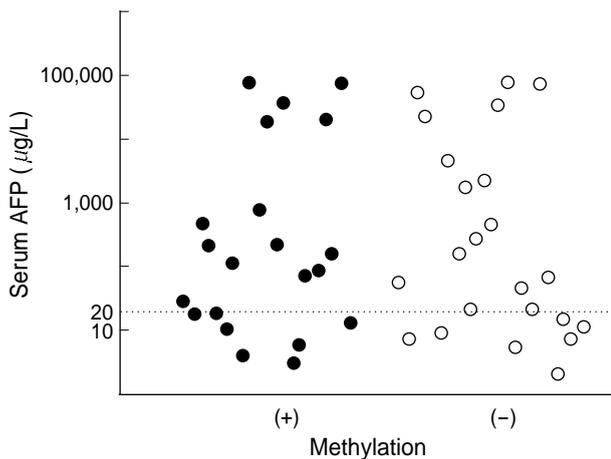| Size (cm) | Methylation (%) | | p value |
	Positive	Negative	
>3 (n=25)	63.6	45.8	0.226†
≤3 (n=21)	36.4	54.2	

*Chi-square test.

Fig. 2. Serum AFP levels of 44 HCC patients according to status of p16^{INK4A} methylation.

were positive for HBsAg and 13 (18.8%) were positive for anti-HCV antibody. The clinical and laboratory features of each group are shown in Table 2.

Detection of Aberrant p16^{INK4A} Methylation in Serum Using MSP

Methylated p16^{INK4A} was detected in 47.8% (22/46) of HCC patients, a significantly higher rate than the 17.4% (4/23) seen in LC patients (p=0.014) (Fig. 1).

Sensitivity, Specificity, and Positive Predictive Value of the Detection of p16^{INK4A} Methylation in the Diagnosis of Hepatocellular Carcinoma

Aberrant serum p16^{INK4A} methylation showed 47.8% (22/46) sensitivity, 82.6% (19/23) specificity, and 84.6% (22/26) positive predictive value in the diagnosis of HCC.

Association of p16^{INK4A} Methylation Status and Serum AFP Level or Tumor Size in Patients with Hepatocellular Carcinoma

There were no associations between the status of p16^{INK4A} methylation and serum AFP level in HCC patients (Table 3, Fig. 2) or tumor size (Table 4).

DISCUSSION

The tumor suppressor gene p16^{INK4A} exhibits variations, including methylation, that are involved in the process of carcinogenesis. A causal relationship between genetic variation and methylation has yet to be determined. Kondo et al. (8) studied LOH, microsatellite instability, and DNA methylation in HCC and in non-cancerous surrounding tissue by microdissection. Their results suggested that methylation often occurs in both tissue types, that it precedes LOH, and that methylation is involved in the early genesis of HCC as the cause of LOH.

The reported rate of incidence of p16^{INK4A} methylation is quite variable, ranging from 0% to 94% in HCC (8, 15, 22), and from 29.4% to 83% in cirrhosis (8, 21, 22). This may be due to the lack of a standardized method of detection and to the diversity in the clinical courses of patient groups. There have been few serum studies of p16^{INK4A} methylation in HCC patients. Wong et al. (20) reported abnormal p16^{INK4A} methylation in 60% of sera and in 73% of tissue in HCC patients. In the present study, abnormal p16^{INK4A} methylation was detected in 48% of the sera of HCC patients. Further studies on plasma or sera are required; large variations in incidence may be expected due to differences in sample materials, detection methods, and/or subjects selected.

Wong et al. (20) reported that p16^{INK4A} methylation was not detected in the plasma of patients with either liver cirrhosis or hepatitis, whereas in the present study it was detected in the sera of 17.4% of cirrhosis patients. We infer that abnormal p16^{INK4A} methylation can also be detected in the sera of patients with cirrhosis because it is frequently found in non-tumorous tissues (8). AFP and albumin mRNA were also detected in the sera of hepatitis and cirrhosis patients, as well as in those of HCC patients (25). More meticulous methods may be required to detect p16^{INK4A} methylation in cirrhosis patients, as the amount of circulating DNA is lower in the sera of cirrhosis patients than in that of HCC patients. The incidence of methylated p16^{INK4A} DNA did not differ between small (≤3 cm) and large HCCs, and was not correlated with AFP levels in the sera of HCC patients. Wong et al. (26) reported a significant correlation between the methylation of circulating DNA and serum AFP levels in HCC patients. There is a danger in generalizing from this result, however, as that study included only six cases of HCC without methylation.

Our study suggested that methylated p16^{INK4A} DNA may

play an important role as a tumor marker in detection of HCC. The state of serum $p16^{INK4A}$ methylation discriminated HCC from cirrhosis with a sensitivity of 47.8% and a specificity of 82.6%. As there is no correlation between serum methylation and AFP, it may be a useful complementary tool to the AFP test.

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