Detection of hypermethylation of the $p16^{INK4A}$ gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus


Abstract

**Background/aim**—Inactivation of the $p16^{INK4A}$ ($p16$) tumour suppressor gene by promoter region hypermethylation has been demonstrated not only in many types of tumours, including hepatocellular carcinoma (HCC), but also in early preneoplastic lesions in the lung, colon, oesophagus, and pancreas. The aim of this study was to examine the methylation status of the $p16$ promoter in pre- and/or non-neoplastic liver diseases.

**Patients/Subjects/methods**—The methylation status of $p16$ was evaluated in 22 HCC, 17 cirrhosis, 17 chronic hepatitis, nine primary biliary cirrhosis (PBC), eight autoimmune hepatitis, seven drug induced liver disease, six fatty liver, and three normal liver tissues using methylation specific polymerase chain reaction (MSP), $p16$ protein expression was also examined by immunohistochemical staining.

**Results**—Methylation of the $p16$ promoter was detected in HCC (72.7%, 16/22) and also in cirrhosis (29.4%, 5/17) and chronic hepatitis (23.5%, 4/17), all of which were positive for hepatitis B or C virus infections. Methylation was not detected in any of the other samples. All methylation positive HCC, cirrhosis, and chronic hepatitis samples showed loss of $p16$ expression, and a significant correlation was found between methylation and loss of expression. Analysis of serial samples from individual patients with methylation positive HCC revealed that loss of $p16$ expression with promoter methylation occurred in 18 of 20 patients at the stage of chronic hepatitis without clinically detectable carcinoma.

**Conclusions**—Our results suggest that methylation of the $p16$ promoter and the resulting loss of $p16$ protein expression are early events in a subset of hepatocarcinogenesis and that their detection is useful in the follow up of patients with a high risk of developing HCC, such as those with hepatitis B or C virus infections.

**Keywords:** hypermethylation; $p16$; hepatocarcinogenesis; preneoplastic diseases; hepatitis virus infection; methylation specific PCR

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and one of the leading causes of cancer death in Japan. Hepatitis B virus (HBV) and/or hepatitis C virus (HCV) infections are thought to be involved in hepatocarcinogenesis. Chronic hepatitis and cirrhosis associated with either HBV or HCV precede most HCCs. The process of chronic inflammation and cirrhosis is often accompanied by regeneration of hepatocytes, and the continuous regeneration may predispose hepatocytes to uncontrolled growth and malignant transformation as a result of disruption of cell cycle regulation, senescence, and apoptosis. HBV encodes a gene product that can interact with $p53$ and impair the physiological function of $p53$. The core protein of HCV has been shown to modulate gene transcription, cell proliferation, and cell death. However, the mechanisms underlying virus associated hepatocarcinogenesis are still unclear.

$p16$, a cyclin dependent kinase inhibitor, plays an important role in cell cycle regulation and senescence. $p16$ is one of the most frequently altered tumour suppressor genes in human cancer.

Frequent loss of $p16$ expression has been reported in HCC. However, mutations and homozygous deletions of $p16$ have been shown to be rare in HCC, although conflicting results have been reported. The reported LOH at 9p21, on which $p16$ gene is located, also shows varying frequencies in HCC. The discrepancies in the reported frequencies in $p16$ alterations may be due to differences in risk factors, such as HBV, HCV, and alcohol, and differences in geographic regions. It has been proposed that aberrant methylation of CpG islands, which are CpG dinucleotide rich areas located mainly in the promoter regions of many genes, serves as an alternative mechanism for inactivation of the tumour suppressor gene in cancer. In contrast with the infrequent genetic alterations, frequent methylation of the $p16$ promoter has been observed in HCC, although one study failed to detect methylation in HCC. The discrepancy can be explained by the fact that samples with different ethnic and aetiological backgrounds were analysed and/or that different methods were used. The discrepancy may result from

**Abbreviations used in this paper:**

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; MSP, methylation specific PCR.
Different proportions of specific aetiologies, such as HBV and HCV infections, in the studies.

Recently, aberrant methylation of the p16 promoter has also been reported in early preneoplastic lesions in the lung, colon, oesophagus, and pancreas. These findings suggest that loss of p16 function, often due to promoter methylation, may be an early event in the pathogenesis of various types of tumours. In this regard, it is of interest that weak amplification of methylated DNA has been seen in a small number of non-tumour liver tissues in which strong signals of methylated DNA were detected in the corresponding tumour tissues. Although these findings suggest that de novo methylation of the p16 promoter could occur in premalignant or early subcellular malignant changes in hepatocarcinogenesis, the functional significance of these observations has not been elucidated. In this study, we analysed 5′ CpG island methylation of p16 and correlated the results with immunohistochemical expression of p16 protein in 89 specimens from HCCs, pre- and/or non-neoplastic liver diseases, with or without hepatitis virus infections, and normal livers.

Materials and methods

Tissue specimens

Twenty-two HCC (mean age 59.2 (SD 9.3) years; 19 males and three females; nine HBV positive and 13 HCV positive), 17 cirrhosis (58.2 (9.7) years; 15 males and two females; seven HBV positive and 10 HCV positive), 17 chronic hepatitis (44.0 (13.7) years; nine males and eight females; seven HBV positive and 10 HCV positive), nine primary biliary cirrhosis (PBC) (55.9 (11.4) years; one male and eight females), eight autoimmune hepatitis (AIH) (61.9 (4.5) years; one male and seven females), seven drug induced liver disorder (45.1 (16.2) years; two males and five females), six fatty liver (46.7 (15.3) years; two males and four females), and three normal liver (29.0 (14.0) years; two males and one female) tissue specimens were obtained surgically by needle biopsy or at autopsy. HBV and HCV infections were diagnosed by HBs antigen (LPIA-200; Diatron laboratories, Tokyo, Japan) and anti-HCV antibody (Immunocheck-HCV Ab; International Reagent, Kobe, Japan), respectively. All specimens were diagnosed pathologically and frozen at −80°C.

Bisulphite modification

The principles of the method have been described previously. In the chemical modification of cytosine to uracil by bisulphite treatment, all cytosines are converted to uracil, but those that are methylated are resistant to this modification and remain as cytosine. One can design polymerase chain reaction (PCR) primers to distinguish methylated from unmethylated DNA in bisulphite modified DNA, taking advantage of the sequence differences resulting from bisulphite modification.

Genomic DNA (2 µg) extracted from frozen tissues by the standard phenol/chloroform procedure was denatured in 0.2 M NaOH for 15 minutes. Sodium bisulphite (Sigma Chemical, St Louis, Missouri, USA) was added to a final concentration of 3.1 M, and hydroquinone (Sigma Chemical) was added to a final concentration of 0.5 mM. The reaction was performed at 50°C for 16 hours. Modified DNA was then purified using Wizard DNA purification resin (Promega, Madison, Wisconsin, USA) according to the manufacturer’s protocol and eluted with 50 µl of water. DNA was treated with NaOH (final concentration 0.3 M) for five minutes at room temperature, followed by ethanol precipitation. Modified DNA was resuspended in water and used immediately or stored at −20°C.
Table 1  Summary of methylation and expression of p16 in hepatocellular carcinoma (HCC) tissues

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+, positive; −, negative; ±, partial; ND, not determined.

FC, fibrous capsule around HCC; Fc-inf, cancer cell infiltration through fibrous capsule; Sf, septal fibrous band in HCC; Vp, tumour thrombus in portal vein; Im, intrahepatic metastasis.

Virus B, HBsAg positive; C, HCV antibody positive; LC, liver cirrhosis; CH, chronic hepatitis.

Grade I, well differentiated HCC; grade II, moderately differentiated HCC; grade III, poorly differentiated HCC.

METHYLATION SPECIFIC PCR (MSP)

Bisulphite modified DNA was subjected to MSP using primers specific for unmethylated p16 (5'-TTATAGGGGTGGGGTTGGAT TGT-3') or methylated 5'-CAACCCCAAACACCAAAC CATAA-3') or methylated p16 (5'-TTAT TAGGGGTGGGGCGGATCGC-3' and 5'-GACCCCGAACCGCGACCGTAA-3'). PCR reactions were performed in a volume of 50 µl containing 1×PCR buffer, 0.25 mM dNTP, 1 mM of each primer, and 2.5 U of Taq polymerase. PCR conditions were as follows: one cycle of 95°C for five minutes; 35 cycles of 95°C for four seconds, 60°C (unmethylated) or 65°C (methylated) for 30 seconds, and 72°C for 30 seconds; and one cycle of 72°C for four minutes. PCR samples were then electrophoresed on a 3% agarose gel.

IMMUNOHISTOCHEMISTRY

Tissue sections (4–5 µm) were deparaffinised and incubated in target retrieval solution (Dako, Carpinteria, California, USA) at the recommended dilution and heated by steam for 15 minutes. Slides were incubated overnight at 4°C with a mouse monoclonal anti-p16 antibody (Neomarkers, Union City, California, USA) diluted to a final concentration of 4 µg/ml. Negative control slides were treated with normal rabbit immunoglobulin under similar conditions. Slides were then rinsed with phosphate buffered saline and incubated with the secondary antibody using the Dako LSAB 2 System (Dako), developed with diaminobenzidine (Kanto Chemical, Tokyo) for five minutes, and counterstained with haematoxylin for one minute.

STATISTICAL ANALYSIS

Methylation of the p16 promoter was assessed for associations with clinicopathological characteristics using the χ² or Fisher’s exact test. A p value <0.05 was considered statistically significant.

Results

METHYLATION OF THE p16 PROMOTER IN VARIOUS LIVER DISEASES

Methylation of the p16 promoter was detected in 72.7% (16/22) of HCC (fig 1A), 29.4% (5/17) of cirrhosis (fig 1B), and 23.5% (4/17) of chronic hepatitis tissue samples (fig 1B, fig 2, table 1). In contrast, none of the nine PBC, eight autoimmune hepatitis, seven drug induced liver disease, six fatty liver, or three non-viral liver diseases showed p16 promoter methylation (fig 1C, fig 2). All methylation positive tissue samples were positive for either HBV or HCV (fig 2, table 1). Positivity for p16 promoter methylation was not different between HBV and HCV positive patients in HCC (7/10 v 9/13), cirrhosis (2/7 v 3/10), or chronic hepatitis (1/7 v 3/10). Unmethylated bands were also detected in all methylation positive cases (fig 1A, 1B). There was no significant correlation between methylation of the p16 promoter and age, type of viral infection, tumour cell differentiation, tumour size, or histological findings in HCC (table 1).

p16 EXPRESSION

All normal liver tissues (fig 3A) and those with non-viral liver diseases (fig 3B–E) showed positive nuclear staining for p16. In contrast, none of the nine PBC, eight autoimmune hepatitis, seven drug induced liver disease, six fatty liver, or three non-viral liver diseases showed p16 expression (fig 3F, 3G). Partial loss of p16 expression (fig 3F, 3G) was observed in four of the five methylation positive chronic hepatitis tissues but in only one of the 13 methylation negative chronic hepatitis tissues (p=0.002) (fig 1B). One complete
and four partial losses of p16 expression were observed in the five methylation positive cirrhosis tissues while only three of 12 methylation negative cirrhosis tissues showed partial loss of p16 expression (fig 1B; p=0.009). Complete (fig 3I) or partial loss of p16 nuclear staining was observed in 81.8% (18/22; 8/9 HBV positive and 10/13 HCV positive) and 9.1% (2/22; both HCV positive) of HCCs, respectively. All of the 16 methylation positive cases showed complete loss of p16 expression (fig 1A, table 1), and a significant correlation was found between methylation and complete loss of expression (p=0.002).

Analysis of serial samples from individual patients with methylation positive HCC revealed that loss of p16 expression with promoter methylation occurred in 18 of 20 patients at the stage of chronic hepatitis without clinically detectable carcinoma (data not shown).

Discussion
In this study, we analysed the methylation status of the 5' CpG island of the p16 gene promoter in a series of HCC and pre- and/or non-neoplastic liver diseases with or without hepatitis virus infections. Methylation of the p16 promoter was detected in 72.7% of HCCs, 29.4% of cirrhosis, and 23.5% of chronic hepatitis tissues, all of which were positive for HBV or HCV infections. In contrast, methylation was not detected in any of the tissue samples from non-viral liver diseases, including PBC, autoimmune hepatitis, drug induced liver disease, and fatty liver, suggesting that this epigenetic change may be related to hepatitis virus infections.

All of the HCC, cirrhosis, and chronic hepatitis samples with promoter methylation showed loss of expression, and a significant correlation between methylation and loss of
expression was observed. Therefore, methyla-
tion of the p16 promoter detected in this study
appears to have a functional significance. Fur-
thermore, it is notable that the majority of
patients with methylation positive HCC had
loss of p16 expression with promoter methyla-
tion at the stage of chronic hepatitis without
clinically detectable carcinoma. Our results
suggest that methylation of the p16 promoter
is one of the earliest events in a subset of hepati-
tis virus associated hepatocarcinogenesis. Our
results are consistent with the findings of
several recent studies indicating that inactiva-
tion at the stage of chronic hepatitis without
clinical detectable carcinoma. Our results
confirm recently reported observations of
frequent methylation of the p16 promoter in HCC and extend the observations to
colon,22 oesophagus,23 and pancreas. Thus
our results confirm previous reports.

In all of the methylation positive cases,
unmethylated bands were also detected. One
possible explanation is that these unmethylated
bands were due to contaminated normal
tissues in the tumour, such as inflammatory
cells and normal liver cells. Considering the
loss of expression, both alleles appear to be
inactivated by biallelic methylation or methyla-
tion in conjunction with genetic alterations,
such as mutation, LOH, and homozygous
deletion, as shown in a previous study.5 Several
HCC, cirrhosis, and chronic hepatitis samples
without methylation also showed loss of p16
expression, suggesting that the above men-
tioned genetic alterations play a role in
inactivation of the p16 gene. In contrast with
our results, several previous studies have failed
to detect methylation of the p16 promoter in
chronic non-tumour liver diseases, including
those of viral origin.10 11 These conflicting
observations may be due to differences in the
methods for detection of methylation and/or in
the ethnic and aetiological backgrounds of
individuals. However, these factors in our study
were similar to those in the study of Matsuda
and colleagues.5 Nevertheless, our observation
is unlikely to be simply overestimation of
methylation as we found a good correlation
between methylation and loss of expression.

All methylation positive HCC showed com-
plete loss of p16 expression while the majority
of methylation positive cirrhosis and chronic
hepatitis samples showed partial loss of expres-
sion. It has been suggested that accumulation
of methylated cytosine in the 5’ CpG island of
p16 may silence this gene in a dose dependent
manner.25 It has been reported that partial loss
of p16 expression was associated with lower
levels of methylation than complete loss,
suggesting that methylation, and loss of expres-
sion, of p16 occurs in a progressive manner.25
27 Gradual loss of p16 expression associated with
progressive p16 promoter methylation has also
been reported in the escape of mammmary
epithelial cells from senescence.29 Therefore,
partial loss of p16 expression in cirrhosis and
chronic hepatitis may be due to the heterogen-
eity of methylation in hepatocytes, and the
population of cells with methylated alleles may
increase during progression to HCC. It seems
intriguing to speculate that cells with loss of
p16 expression are more likely to progress to
tumorous cells than are those with normal p16
function.

The precise mechanisms leading to aberrant
methylation of CpG islands in neoplastic cells
are not known. Moreover, little is known of the
link between viral infections and methylation
machinery. Recently, human immuno-
deficiency virus has been shown to induce
methylation of interferon γ through increased
dNA methyltransferase activity.20 Interestingly,
Sun et al have reported increased levels of DNA
methyltransferase mRNA in liver tissues from
chronic hepatitis and cirrhosis compared with
those in normal liver tissues.21 However, several
twisting lines of experimental evidence suggest that
there is no correlation between DNA methyl-
transferase activity and aberrant methylation
of endogenous genes.22 Therefore, aberrant methy-
lation of the p16 promoter may not be caused
directly by elevated DNA methyltransferase
activity in hepatocarcinogenesis. Other mecha-
nisms such as loss of protection against de novo
methylation may underlie aberrant methylation
of p16 during hepatocarcinogenesis.17 18 Our
results warrant further investigation.

Although further study is necessary to clarify
the link between hepatitis virus infections and
methylation of p16 in hepatocarcinogenesis,
detection of p16 promoter methylation could
be a useful molecular marker to follow up
patients with a high risk of developing HCC,
such as those with HBV or HCV infections. It
would be of interest to prospectively examine
whether patients with p16 methylation positive
cirrhosis or chronic hepatitis have a higher risk
of developing HCC compared with those with-
out p16 methylation.

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scriptional activity, and association with transcription
protein cooperate with rats and transform primary rat
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Detection of hypermethylation of the $p16^{INK4A}$ gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus


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