Featured Article

p16<sup>INK4A</sup> Hypermethylation Is Associated with Hepatitis Virus Infection, Age, and Gender in Hepatocellular Carcinoma

Xin Li, Ai-Min Hui, Lin Sun, Kiyoshi Hasegawa, Guido Torzilli, Masami Minagawa, Tadatoshi Takayama, and Masatoshi Makuuchi

Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan

ABSTRACT

Purpose: The tumor suppressor gene p16<sup>INK4A</sup> is mainly inactivated by an epigenetic change involving promoter hypermethylation in hepatocarcinogenesis. The possible clinical impact of p16<sup>INK4A</sup> methylation and the potential risk factors for this epigenetic alteration have not been thoroughly investigated.

Experimental Design: We studied the methylation status and mRNA and protein expression of p16<sup>INK4A</sup> in 50 hepatocellular carcinomas and corresponding nonneoplastic liver lesions using methylation-specific PCR, reverse transcription-PCR, and immunohistochemical techniques.

Results: p16<sup>INK4A</sup> hypermethylation was observed in 58% (29 of 50) of the hepatocellular carcinomas and 16% (6 of 38) of the corresponding chronic hepatitis and cirrhosis tissue samples. p16<sup>INK4A</sup> methylation was significantly associated with mRNA and protein expression (P < 0.001 and P = 0.003, respectively). All of the p16<sup>INK4A</sup>-methylated tumors were positive for hepatitis B virus or hepatitis C virus markers, but none of the virus-negative tumors exhibited p16<sup>INK4A</sup> methylation (P = 0.006). The frequency of p16<sup>INK4A</sup> hypermethylation tended to be higher in hepatitis C virus-related tumors (23 of 32, 72%) than in hepatitis B virus-related tumors (6 of 13, 46%; P = 0.1). Aberrant methylation of p16<sup>INK4A</sup> was also related significantly to increasing age, female gender, and normal levels of serum PIVKA-II (P = 0.02, 0.04, and 0.04, respectively). No statistically significant difference in survival was observed between patients with p16<sup>INK4A</sup> hypermethylation and those without.

Conclusions: Our observations suggest that p16<sup>INK4A</sup> hypermethylation may contribute to hepatocarcinogenesis from an early stage and that multiple risk factors, such as viral infections, age, and gender, may be associated with p16<sup>INK4A</sup> hypermethylation in hepatocarcinogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, with the highest incidence in Asia and Africa and an increasing incidence in occidental countries (1). Most HCCs arise in the liver, with underlying diseases induced by persistent hepatitis virus infection, including chronic hepatitis and cirrhosis. In the complex multistage process of hepatocarcinogenesis, the accumulation of genetic and epigenetic alterations involving the inactivation of tumor suppressor genes and the activation of oncogenes is required for the emergence of a fully malignant tumor. The p16<sup>INK4A</sup> gene, located on chromosome 9p21, encodes a critical negative regulator of cell cycle progression and is one of the most frequently inactivated tumor suppressor genes detected in various tumor types (2–4). The p16<sup>INK4A</sup> protein binds to cyclin-dependent kinases 4 and 6 and inhibits the ability of cyclin-dependent kinase 4/6 to phosphorylate the retinoblastoma protein, resulting in G1-phase arrest. The p16<sup>INK4A</sup> gene was initially thought to be inactivated through endogenous DNA damage events involving homozygous deletions and mutations. However, recent studies have suggested that the p16<sup>INK4A</sup> gene can also be silenced epigenetically in human neoplasias through the hypermethylation of the 5’ cytosine phospho guanine island within the promoter region (4). Accumulating evidence suggests that aberrant promoter methylation is generally correlated with the transcriptional inactivation of defined tumor suppressor genes, which has emerged as a common molecular defect in neoplastic cells. The cause of aberrant DNA methylation in cancer cells remains largely unknown.

We and others shown previously that p16<sup>INK4A</sup> is frequently down-regulated in HCC but that mutations or homozygous deletions in this gene are rare (5–7). Recently, several studies have identified aberrant promoter methylation as the main mechanism underlying the inactivation of p16<sup>INK4A</sup> in HCC (8–17). To better understand the functional effect and possible clinical significance of p16<sup>INK4A</sup> hypermethylation and to document the factors that might influence the p16<sup>INK4A</sup> methylation status in HCC, we examined the methylation status of the p16<sup>INK4A</sup> gene promoter and the expression levels of p16<sup>INK4A</sup> mRNA and protein in a series of surgically resected HCC specimens and corresponding non-neoplastic liver lesions after reviewing the available clinical follow-up information on the patients.

MATERIALS AND METHODS

Patients and Specimens. Tumor and corresponding noncancerous liver tissue specimens were obtained from 50 patients with HCC whose frozen tumor samples had been stored in the...
surgical pathology files of our department between August 1996 and December 1998. The frozen tissue samples were flash-frozen in liquid nitrogen immediately after surgical resection and stored at −80°C until analysis. The patients consisted of 38 men and 12 women, ranging in age from 29 to 82 (median, 62.5) years. After Tumor-Node-Metastasis staging (18), the 50 primary HCCs were divided into 4 stage I lesions, 24 stage II lesions, 19 stage III lesions, and 3 stage IV lesions. Pathologic diagnoses were made based on the classification of the Liver Cancer Study Group of Japan (19). Of the 50 patients, one exhibited a normal liver, 8 had fibrosis, 12 had chronic hepatitis, and 29 had cirrhosis. Serologic examinations indicated that 13 patients were hepatitis B surface antigen-positive, 32 were antitheatpeptit C virus antibody (anti-HCV)-positive, and 5 were negative for both hepatitis viral markers. Written informed consent was obtained from each patient, and the protocol of the study was approved by the local ethics committee.

Methylation-Specific PCR Analysis. We extracted genomic DNA from the 50 tumors and the 46 corresponding noncancerous tissues for which frozen samples were available using a DNA isolation kit (GenomicPrep DNA Isolation kit, Amersham Pharmacia Biotech, Piscataway, NJ). After being modified by treatment with sodium bisulfite, we screened the methylation status of the p16INK4A promoter by PCR using a commercial primer kit (cytosine phospho guanine WIZ p16 Amplification kit, Intergen Company, Purchase, NY): an M primer set, for detecting the methylated DNA sequence; a U primer set, for detecting the unmethylated DNA sequence; and a W primer set, recognizing any unmodified DNA and serving as a control monitoring the efficiency of the bisulfite reaction. Water blanks were used as negative controls. We separated the amplified products electrophoretically on 3% agarose gels and visualized them using ethidium bromide staining.

Reverse Transcription-PCR Analysis. Forty-eight frozen tumors and 36 corresponding noncancerous liver tissue samples from which RNA could be extracted after DNA preparation were used to isolate the total RNA using a RNA isolation method described previously (5). We reverse-transcribed the total RNA using a random hexadeoxynucleotide primer (Takara method described previously (5). We reverse-transcribed the samples from which RNA could be extracted after DNA prep-

RESULTS

p16INK4A Methylation. Methylation-specific PCR analysis showed aberrant p16INK4A methylation in 29 (58%) of the 50 HCCs, which generate a methylated PCR product (Fig. 1A). Of the 46 corresponding noncancerous tissues, a methylation band was not found in the one normal liver tissue specimen or in the seven fibrosis tissue specimens; weak methylation bands were observed in 2 (17%) of the 12 chronic hepatitis tissue specimens and 4 (15%) of the 26 cirrhotic liver tissue specimens. All of the tumor tissues corresponding to these six methylated noncancerous lesions exhibited strong methylation bands. Consistent with previous reports (9, 12, 13), a band of unmethylated products was observed in all samples. No product was observed in negative controls (water blanks). The W primer set did not produce any or only a very slight amount of product, indicating that the bisulfate reactions are efficient.

p16INK4A Expression. Of the 48 tumors examined, p16INK4A mRNA amplification products were not observed in 31 (65%) tumors; the other 17 (35%) expressed detectable levels of p16INK4A mRNA (Fig. 1B). Twenty-seven of the 36 corresponding noncancerous tissues exhibited detectable p16INK4A mRNA products. To confirm whether a loss of p16INK4A mRNA expression was actually present, all analyses were repeated at least twice. Close level of the β-actin expression was detected in all samples (Fig. 1C).

p16INK4A immunoreactivity appeared as a dark brown nuclear stain. Twenty-one (42%) of the 50 tumors were positive for p16INK4A protein expression, with the percentage of immu-

![Fig. 1 Methylation-specific PCR and reverse transcription-PCR analyses of p16INK4A in HCC. Representative methylation-specific PCR (A) and reverse transcription-PCR (B) results for seven HCC samples are shown. Bands in the M Lanes represent the methylated PCR products of p16INK4A, whereas bands in the U Lanes represent unmethylated p16INK4A. Cases 3 and 23 exhibit unmethylated p16INK4A genes and positive mRNA expression; cases 4, 21, 31, 42, and 47 exhibit methylated p16INK4A genes and undetectable mRNA expression. C, control β-actin mRNA is expressed in all cases, including the five cases in which p16INK4A mRNA expression was not detected.](https://clincancerres.aacrjournals.org/content/7/19/7485/F1.large.jpg)
nereactive cells ranging from 1 to 60% (Fig. 2). p16\textsuperscript{INK4A} protein expression was significantly related to mRNA expression ($P < 0.001, \chi^2$ test).

Association of p16\textsuperscript{INK4A} Methylation with mRNA and Protein Expression. Among the 48 HCCs in which p16\textsuperscript{INK4A} mRNA expression was analyzed, the correlation between the p16\textsuperscript{INK4A} methylation status and mRNA expression was strongly significant ($P < 0.001, \chi^2$ test). Thirty-six (75%) tumors showed an inverse relationship between p16\textsuperscript{INK4A} methylation and mRNA expression. Of the six methylated non-cancerous liver tissues exhibiting cirrhosis or chronic hepatitis, five showed undetectable levels of p16\textsuperscript{INK4A} mRNA expression. A strong relationship between p16\textsuperscript{INK4A} methylation and protein expression was also observed ($P = 0.003, \chi^2$ test; Table 1). When the mRNA and protein expression results were combined to evaluate the functional significance of p16\textsuperscript{INK4A} methylation, four of the eight tumors, which did not exhibit p16\textsuperscript{INK4A} methylation but were negative for mRNA expression, exhibited normal p16\textsuperscript{INK4A} protein expression. In these four cases, the absence of detectable p16\textsuperscript{INK4A} mRNA may have been caused by the long-term storage of the sample materials and the inherent instability of mRNA. Additional alterations, such as gene mutations or deletions, may account for the other four tumors, in which p16\textsuperscript{INK4A} protein expression was also negative. As to the four tumors exhibiting p16\textsuperscript{INK4A} promoter methylation and mRNA expression, positive p16\textsuperscript{INK4A} protein expression was also observed. The unexpected concurrence of methylation and the positive expression of p16\textsuperscript{INK4A} may be attributable to the low levels of methylation, as illustrated in a previous study on HCC (10).

Association of p16\textsuperscript{INK4A} Methylation with Clinicopathologic Features and Survival. p16\textsuperscript{INK4A} hypermethylation was more frequently observed in HCCs from older patients, and in tumors from women ($P = 0.02$ and 0.04, $\chi^2$ test; Table 2). p16\textsuperscript{INK4A} hypermethylation was only detected in hepatitis B virus (HBV) or HCV-related tumors, whereas none of the five virus-negative tumors exhibited aberrant p16\textsuperscript{INK4A} methylation ($P = 0.006, \chi^2$ test; Table 2). The frequency of p16\textsuperscript{INK4A} hypermethylation tended to be higher in HCV-related tumors (23 of 32, 72%) than in HBV-related tumors (6 of 13, 46%), although this difference was not statistically significant ($P = 0.1, \chi^2$ test; Table 2). p16\textsuperscript{INK4A} hypermethylation was more frequently observed in tumors from patients with normal serum levels of protein-induced by vitamin K absence or antagonist II (PIVKA-II; $P = 0.04, \chi^2$ test; Table 2). p16\textsuperscript{INK4A} methylation was not related to Tumor-Node-Metastasis staging, tumor differentiation, intrahepatic metastasis, portal involvement, hepatic vein involvement, capsular infiltration, tumor size, background liver disease, or serum $\alpha$-fetoprotein levels (Table 2). Among the clinicopathologic factors, HCV infection was significantly associated with older patients ($P = 0.001, \chi^2$ test), compared with HBV infection. Normal PIVKA-II levels were related to advanced age and HBV or HCV infection ($P = 0.04$ and 0.02, respectively; $\chi^2$ test).

Survival was analyzed in 49 patients, excluding one patient with recurrent HCC. The median follow-up period was 46 (range, 6–71) months. The 5-year overall survival for the 49 patients was 63%. As shown in Fig. 3, no difference in disease-free survival (DFS) was found with regard to p16\textsuperscript{INK4A} methylation status in the 49 HCCs ($P = 0.48$, log rank test; Fig. 3A); the 5-year DFS for patients with or without p16\textsuperscript{INK4A} methylation were 26 and 40%, respectively. When we did the analysis using data for stage I/II tumors only, the 5-year DFS for patients

<table>
<thead>
<tr>
<th>Expression</th>
<th>Total no.</th>
<th>Unmethylated (%)</th>
<th>Methylated (%)</th>
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<td>mRNA</td>
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<td>31</td>
<td>8 (26)</td>
<td>23 (74)</td>
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<td>17</td>
<td>13 (76)</td>
<td>4 (24)</td>
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</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>7 (24)</td>
<td>22 (76)</td>
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</tr>
<tr>
<td>Positive</td>
<td>21</td>
<td>14 (67)</td>
<td>7 (33)</td>
<td>0.003</td>
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Fig. 2 Immunohistochemical analysis of p16\textsuperscript{INK4A} expression in HCC. A, a tumor with unmethylated p16\textsuperscript{INK4A} showing positive p16\textsuperscript{INK4A} expression. About 40% of the neoplastic cells express nuclear p16\textsuperscript{INK4A} immunoreactivity. B, a p16\textsuperscript{INK4A}-methylated tumor showing negative p16\textsuperscript{INK4A} expression. No specific nuclear immunoreactivity was detected in this tumor (×350 magnification).
with or without p16INK4A methylation was 34 and 60%, respectively, but the difference did not reach statistical significance (P = 0.15, log-rank test; Fig. 3B).

DISCUSSION

Despite remarkable improvements in the diagnosis and treatment of patients with HCC, the long-term prognosis remains generally poor, because of the high incidence of recurrence in the liver remnant. The possible effect of p16INK4A methylation on the malignant behavior and disease outcome of HCC has not been thoroughly investigated to date. Our results showed that p16INK4A hypermethylation was present in 58% (29 of 50) of the examined HCCs and 16% (6 of 38) of the precancerous lesions (including chronic hepatitis and cirrhotic tissues) and that p16INK4A hypermethylation was strongly related to both mRNA and protein expression. In the overall series of HCCs, we failed to find any association between p16INK4A hypermethylation and clinicopathologic factors related to tumor progression and patient survival. Among patients with stage I/II tumors, however, patients with p16INK4A methylation had a higher frequency of recurrence than those without p16INK4A methylation, although the difference was not statistically significant. As only a few stage I/II tumors were included in this study, additional large-scale studies are necessary to obtain conclusive evidence. Taken together, our observations suggest that aberrant p16INK4A methylation may contribute to hepatocarcinogenesis from early stages even in precancerous lesions and may play a more important role in low-stage tumors than in high-stage tumors during the multistage process of hepatocarcinogenesis. p16INK4A hypermethylation has also been shown to be an early event in the development of lung and stomach cancers (21, 22). Positive data about the prognostic significance of p16INK4A methylation has been limited thus far. Consistent with our preliminary findings, however, one study investigating a relatively large number of lung adenocarcinomas reported that p16INK4A methylation was an independent risk factor in the prediction of a poor prognosis for patients with stage I tumors but not for patients with other tumor stagings (23).

Chronic infection with hepatitis B or C virus is a major risk factor for HCC worldwide, although the prevalence of these two viruses varies geographically. In Japan, 60 to 80% of HCC patients are infected with HCV, and 10 to 20% are infected with HBV. In the present study, we showed that p16INK4A hypermethylation was significantly related to HBV or HCV infection; none of the virus-negative but 46% of the HBV-related and 72% of the HCV-related tumors showed hypermethylation of p16INK4A (Table 2).
of the HCV-related tumors exhibited p16INK4A hypermethylation. Furthermore, all six p16INK4A-methylated noncancerous lesions were positive for viral infection. Recently, Kaneto et al. (14) reported that p16INK4A methylation was detected in virus-associated chronic hepatitis and cirrhosis but not in normal liver and other nonneoplastic liver lesions, such as primary biliary cirrhosis and autoimmune hepatitis. Taken together, these observations suggest that a persistent hepatitis virus infection may play a role in the induction of p16INK4A-promoter methylation in hepatocarcinogenesis, possibly starting at an early stage. Consistent with our findings, Kang et al. (25) have reported recently that hypermethylation of tumor suppressor genes SOCS-1, APC, and p15 was associated with HCV-positive HCC relative to virus-negative HCC. Little is known about the molecular basis of virus-associated methylation. The insertion of viral DNA into hamster cells has been reported to increase cellular DNA methylation (26). In the present series, we further observed that HCV-related HCCs were more likely to contain p16INK4A hypermethylation than HBV-related tumors. Considering the different mechanisms that occur for HBV- and HCV-related hepatocarcinogenesis (27), it seems reasonable to hypothesize that the two viruses may have different effects on p16INK4A methylation. Alternatively, because the HCV-infected patients were significantly older than the HBV-infected patients, the relatively higher frequency of p16INK4A hypermethylation in HCV-related HCCs may be related to the aging process.

Among the limited data available about the cause of DNA hypermethylation, aging was recently considered to be a prime candidate for aberrant methylation of multiple genes involved in human malignancies (28–31). Significantly, our data showed that hypermethylation of the p16INK4A-promoter region was more frequent in HCCs from older patients. In agreement with our finding, another study on a series of HCCs from patients living in a high HBV endemic area also showed an association between p16INK4A-promoter methylation and age (15). According to a previous study on colorectal cancer (30), however, p16INK4A methylation was not related to age. As described by Ahuja et al. (30), it is possible that age-related methylation may function not only through gene-specific pattern but through tumor-specific pattern as well.

The third factor with a potential influence on the p16INK4A methylation status in our study was gender; p16INK4A hypermethylation was more frequently observed in females than in males. A higher frequency of p16INK4A methylation in women was also found in studies examining cancers of the colon and the head and neck region (32, 33). In addition, p14ARF hypermethylation has been shown to be related to female gender in colorectal cancers (34). The mechanism by which gender influences DNA methylation remains unknown.

An interesting association was observed between p16INK4A hypermethylation and patients with normal levels of PIVKA-II. PIVKA-II has been widely used as a serum marker for HCC recently. Under the action of vitamin K-dependent carboxylase, prothrombin is normally synthesized in hepatocytes by carboxylation on the γ position of specific glutamic acid residues of the precursor. As a result of a deficiency of vitamin K or a decrease in vitamin K-dependent carboxylase activity in HCC cells, the immature prothrombin without γ-carboxyglutamic acid residues (i.e., PIVKA-II) is secreted into the blood. Vitamin K-dependent carboxylases are considered to be essential for the posttranslational modification of a select number of proteins (35). It is possible that the normal PIVKA-II levels may reflect the functional maintenance of such posttranslational modification systems and that p16INK4A methylation may require participation of related proteins.

A recent study has shown geographic variations in the methylation status of multiple genes involved in HCC (16). Weihrauch et al. (13) have also shown a higher frequency of p16INK4A hypermethylation in HCCs obtained from workers exposed to vinyl chloride, compared with nonexposed controls. These observations suggest that multiple risk factors, including exogenous factors, like viral infections and vinyl chloride exposure, and endogenous factors, like aging and female gender, may be involved in the generation and maintenance of p16INK4A hypermethylation in hepatocarcinogenesis.

In conclusion, our data suggest that p16INK4A hypermethylation is a common and early event during hepatocarcinogenesis and that p16INK4A hypermethylation is significantly associated with hepatitis virus infection, aging, and the female gender.

REFERENCES


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