Frequent p15 Promoter Methylation in Tumor and Peripheral Blood from Hepatocellular Carcinoma Patients

Ivy H. N. Wong, Y. M. Dennis Lo, Winnie Yeo, Wan Y. Lau, and Philip J. Johnson

ABSTRACT

We prospectively analyzed p15 methylation patterns in 25 surgically resected tumors and 130 plasma, serum, and buffy coat samples from hepatocellular carcinoma (HCC) patients, controls with chronic hepatitis/cirrhosis, and healthy subjects. Using methylation-specific PCR, we demonstrated for the first time p15 promoter methylation in 64% of tumors and 25% (4 of 16) of patients’ plasma and serum samples. Concurrent p15 and p16 methylation was shown in 48% of tumors, and p15/p16 methylation was detected in the plasma/serum of 92% (11 of 12) of patients. Of note, 75% of 12 patients with concurrent tumor methylation developed clinical metastasis/recurrence (P = 0.027). In buffy coat samples, p15 methylation was detected in all eight patients with tumor p15 methylation, suggesting the presence of circulating tumor cells. None of the control samples were methylation positive. Our data underscore the frequent presence of tumor methylation with the development of recurrence or metastasis.

INTRODUCTION

HCC is one of the most rapidly fatal human neoplasms. Treatment outcome after surgery is generally poor because of the frequent presence of recurrence or metastasis, which is a major limitation of long-term survival among HCC patients. Hematogeneous dissemination is presumably the main route of metastasis; circulating tumor cells may persist for a long period of time before the formation of clinical metastasis or recurrence. Chronic hepatitis infection and cirrhosis are well-documented risk factors for HCC. However, patients with chronic hepatitis/cirrhosis may develop HCC only after many years. The long-sought goal is therefore the development of sensitive, specific, and noninvasive blood tests or early detection of HCC and disease monitoring.

Although the molecular mechanisms of hepatocarcinogenesis remain unclear, an emerging number of genetic lesions have been identified (2). Different spectra of p53 and Rb alterations have been found in HCCs. Hypermethylation of p16, a cyclin-dependent kinase inhibitor gene that regulates cell cycling, has been detected frequently in human cancers including HCC. The p15 gene, another cyclin-dependent kinase inhibitor gene adjacent to p16 on chromosome 9p21, is also aberrantly methylated in several human neoplasms, especially among hematopoietic malignancies. Among solid and soft tissue tumors, p15 hypermethylation has occasionally been found in plasmacytoma, brain lymphoma, non-Hodgkin’s lymphoma, Burkitt’s lymphoma, and mantle cell lymphoma. To improve the understanding of the molecular and cell biology of HCC, we prospectively analyzed p15 promoter methylation profiles in tumor, plasma/serum, and buffy coat samples from HCC patients using MSP, which can detect methylated CpG sites critical for transcriptional silencing. To explore the potential clinical implications, we studied the association of p15 and p16 methylation with the development of recurrence or metastasis.

MATERIALS AND METHODS

Patients and Control Subjects. With informed consent of patients and ethics board approval, we recruited 25 HCC patients (median follow-up time, 14 months postsurgery) from the Joint Hepatoma Clinic at Prince of Wales Hospital. In this prospective study, preoperative peripheral blood and surgically resected HCC specimen were collected from each HCC patient. The diagnosis of HCC was confirmed histologically in all cases. Eighty plasma/serum samples were obtained from 25 HCC patients, 35 non-HCC patients with chronic hepatitis/cirrhosis, and 20 healthy volunteers. Fifty buffy coat samples were collected from 15 HCC patients, 15 non-HCC patients with chronic hepatitis/cirrhosis, and 20 healthy volunteers.

DNA Extraction from Tumors, Plasma, Serum, and Buffy Coat Samples. DNA was extracted from HCCs using the QIAamp Tissue Kit (Qiagen, Hilden, Germany). Peripheral blood samples were centrifuged at 3000 × g, and plasma and serum samples were carefully collected from the EDTA-containing and plain tubes, respectively. DNA was extracted from 400 μl of plasma/serum using the QIAamp Blood Kit.
The amount of plasma/serum DNA was subjected to MSP.

One microgram of tumor/buffy coat DNA or one-fourth of the extracted plasma/serum DNA was treated with sodium bisulfite according to the manufacturer's recommendations. Two hundred nanograms of HS-Sultan DNA (corresponding to the patient number in Table 1), patient plasma/serum samples.

Bisulfite Conversion of DNA. Bisulfite modification and MSP were conducted based on the principle that bisulfite treatment of DNA would convert unmethylated cytosine residues into uracil, whereas methylated cytosine residues would remain unmodified (7, 11, 12). Thus, after bisulfite conversion, methylated and unmethylated DNA sequences would be distinguishable by sequence-specific primers. Bisulfite treatment was conducted using the CpGenome DNA Modification Kit (Intergen, New York, NY). One microgram of tumor/buffy coat DNA or extracted plasma/serum DNA was treated with sodium bisulfite following the manufacturer's recommendations. Two hundred nanograms of tumor/buffy coat DNA or one-fourth of the extracted amount of plasma/serum DNA was subjected to MSP.

MSP and Southern Blot Analysis. Bisulfite-modified DNA was amplified using primers specific for the methylated p15 or p16 sequence (7, 11, 12, 14). The sense and antisense primers for the methylated p15 sequence were 5'-GGGTCG-TATTGTGGGCT-3' and 5'-GTGATGTGTTTGTATTTTGTGGTT-3'. The probe designed to hybridize to the methylated p15 sequence was 5'-TAGGC/TGTTTTTTT TAGAAGTAGTATT TAGG-3'. The probe designed to hybridize to the methylated p16 sequence was 5'-GAGTAGTATGGGAGTTTTTGCGT-ATTTAGGT-3'.

Human plasmacytoma cell line HS-Sultan (American Type Culture Collection CRL-1484), which was previously shown to have p15 and p16 methylation by Southern blot analysis using methylation-sensitive restriction enzymes (10), was used as a methylated control for MSP. To determine the sensitivity of MSP, HS-Sultan DNA was serially diluted in water, mixed with normal peripheral blood cell DNA, bisulfite converted, and then amplified by MSP.

Sensitivity of MSP. For methylated p15 alleles, the lower detection limit of MSP using 35 cycles was 1 ng of HS-Sultan DNA in 1000 ng of DNA from normal PBNCs (Fig. 1A). The sensitivity for detecting methylated p15 alleles has reached 2.5 x 10^-4 when using higher-cycle MSP (14). The sensitivity for detecting methylated p16 alleles was 5-fold higher, reaching 5 x 10^-5 with a higher-cycle profile (11).

Statistical Analyses. The association of concurrent tumor methylation with clinical metastasis/recurrence and the correlation between tumor p15 methylation status and methylation positivity/negativity in plasma/serum were analyzed by χ² test or by Fisher's exact test.

RESULTS
Frequent p15 Methylation in HCC and the Association between Concurrent Methylation and Clinical Metastasis/Recurrence. Aberrant p15 promoter methylation was demonstrated in 64% (16 of 25) of HCCs using 35 MSP cycles (Fig. 1A; Table 1). Concurrent p15 and p16 methylation was found in 48% (12 of 25) of tumors (Table 1). Methylation of p15 alone for 10 min. PCR products were loaded onto 2% agarose gels and stained with ethidium bromide. Each sample was analyzed in duplicate in parallel with a methylated cell line control, unmethylated normal controls, and multiple negative water blanks.

The sensitivity for detecting methylated p15 or p16 sequence was confirmed by nonradioactive Southern blot analysis using methylation-sensitive restriction enzymes (10), was used as a methylated control for MSP. To determine the sensitivity of MSP, HS-Sultan DNA was serially diluted in water, mixed with normal peripheral blood cell DNA, bisulfite converted, and then amplified by MSP.

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was demonstrated in 16% (4 of 25) of HCCs, whereas methylation of \( p16 \) alone was found in 28% (7 of 25) of HCCs. Overall, 92% (23 of 25) of HCC patients had \( p15 \) and/or \( p16 \) methylation in tumors. None of the 35 healthy subjects and non-HCC patients with chronic hepatitis/cirrhosis showed methylation in tumors. Among 16 patients with tumor methylation status (Fisher’s exact test, \( P = 0.001 \); Fig. 1B; Table 1). No methylated \( p15 \) sequences were obtained by MSP on plasma/serum from the remaining 9 HCC patients without tumor \( p15 \) methylation or from the 55 healthy subjects and non-HCC patients with chronic hepatitis/cirrhosis. Similarly, no methylated \( p16 \) sequences were obtained by MSP on the plasma/serum from six HCC patients without tumor \( p16 \) methylation or from the control group (\( n = 55 \)). However, unmethylated \( p15 \) and \( p16 \) sequences were detected in all samples by MSP.

Eleven of 12 patients (92%) with concurrent \( p15 \) and \( p16 \) methylation in HCCs showed methylation of at least one of the two genes in plasma/serum (Table 1; Fig. 2). Among cases with tumor methylation in only one of the genes, 25% (1 of 4) of patients with tumor methylation of \( p15 \) alone exhibited methylated \( p15 \) sequences in plasma. Seventy-one percent (5 of 7) of patients with tumor methylation of \( p16 \) alone demonstrated methylated \( p16 \) sequences in plasma/serum. In other words, circulating tumor DNA was detected in 74% (17 of 23) of plasma/serum samples from patients with tumor methylation.

Detection of Aberrant \( p15 \) Methylation in PBNCs from HCC Patients. Among 50 buffy coat samples analyzed, methylated \( p15 \) alleles were detected in PBNCs from all 8 HCC

<table>
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<th>Patient no.</th>
<th>HBsAg/cirrhosis</th>
<th>Tumor size (cm)</th>
<th>Tumor methylation status</th>
<th>Plasma/serum methylation status</th>
<th>Buffy coat ( p15 ) methylation status</th>
<th>Clinical evidence of recurrence/metastasis (days after surgery)</th>
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\[a\] HBsAg, hepatitis B surface antigen.

\[b\] ●, methylated; ○, unmethylated; n, not analyzed.
methylation in the peripheral blood of 87% (20 of 23) of HCC patients (Table 1). Taken together, we found detected circulating tumor DNA in 74% (17 of 23) of plasma/serum samples and circulating tumor cells in an additional three HCC patients (Table 1). Taken together, we found p15/p16 methylation in the peripheral blood of 87% (20 of 23) of HCC patients with tumor p15/p16 methylation.

**DISCUSSION**

We demonstrate, for the first time, aberrant p15 promoter methylation in a substantial proportion (64%) of HCCs. Epigenetic alteration of p15 has only rarely been detected among solid tumors, suggesting its tumor type specificity in HCC and hematological malignancies (7, 10). On the other hand, the role of p16 inactivation by hypermethylation has been well recognized in many human cancers including HCC (6, 12). In this study, we found hypermethylation of p15 alone without p16 involvement in 16% of HCCs, suggesting a unique role for aberrant p15 methylation in hepatocarcinogenesis.

During cell cycling, differential function of p15 or p16 protein in a selective manner has been indicated (13). Expression of p15 appears to be independent of pRB protein but is induced by extracellular growth inhibitors IFN-α and TGF-β (13, 16). There is evidence suggesting that IFN-α treatment may decrease the rate of hepatocarcinogenesis in cirrhosis patients (17). On the other hand, disruption of TGF-β-mediated apoptosis and growth arrest is crucial in hepatocarcinogenesis (18). Thus, p15 inactivation via hypermethylation as reported here could possibly abrogate cell cycle control and confer resistance to the growth-inhibitory effect of TGF-β that is usually overexpressed in HCC cells (19).

The selective pressure that TGF-β imposes on p15 inactivation might potentially account for the high frequency and tumor type specificity of aberrant p15 methylation in HCC. Of particular interest, this incidence rate is comparable to that seen in acute leukemias, which arise in the bone marrow with highly expressed TGF-β (the potent growth inhibitor of hematopoietic stem cells or early progenitors; Ref. 14). The identification of hepatic oval cells of bone marrow origin and hematopoietic stem cells of liver origin may lend credence to the possibility that HCC and hematological malignancies might have distinctive genetic and epigenetic lesions in common (20, 21). One of the possible abnormalities appears to be aberrant p15 methylation, as reported here.

Expression of p16 is repressed in part by pRB protein, whereas Rb expression, in turn, is inhibited by p53 protein (22, 23). There has been evidence indicating that p16 inactivation is closely associated with aberrant p53 expression (24), suggesting a collaborating role for p16 in apoptosis (25). In contrast to Rb and p53 abnormalities that were inconsistently detected in HCC, aberrant p16 methylation has been found frequently (4, 5, 11, 12). In this study, we demonstrated methylation of p16 alone without involving p15 in 28% of the HCCs examined, suggesting an important role for p16 inactivation in hepatocarcinogenesis.

On the other hand, progressive p16 methylation has been associated with metastasis and invasive phenotypes in cancers (26, 28). Dual p15 and p16 methylation has been found almost exclusively in hematological malignancies such as Burkitt’s lymphoma and T-cell acute leukemia (7, 29). The latter two diseases generally have very high proliferative indices. Of note, we detected concurrent p15 and p16 methylation in 48% of HCCs, an unusual phenomenon among solid tumors. It has been shown previously that overall survival was significantly shortened for patients with high proliferative indices and low degrees of apoptosis and necrosis in HCCs (30). To augment the selective growth advantage, p16 methylation may act in concert with p15 methylation during hepatocarcinogenesis. Additional p16 methylation might also contribute to immortalization and inhibition of apoptosis (31). In this first attempt to investigate the clinical relevance of p15 and p16 methylation in HCC, we found a significant association between concurrent tumor methylation and the development of recurrence or metastasis. The functional significance of p15 and p16 methylation may thus be implicated in tumor progression, in that methylation could be an initiating event leading to progressive inactivation of the cell cycle-regulatory genes (10, 26, 32). Impaired p15 and p16 expression, which confers a selective growth advantage to tumor cells capable of clonal expansion, might promote stepwise transformation and neoplastic progression.

Tumors that have metastasized may not shed many cells into the peripheral blood but might release tumor DNA into the circulation. Detection of genetic alterations and methylation abnormalities in the plasma/serum of cancer patients may create a profound impact on noninvasive diagnosis of cancers among high-risk populations (11, 12, 33, 34). In plasma/serum samples, we detected methylated p15 sequences in 25% of HCC patients with p15 methylation in tumor. Of note, nearly all patients (92%) showing concurrent p15 and p16 methylation in HCCs had detectable methylation abnormalities in plasma/serum. The lower detection rate of p15 methylation may be related to the lower sensitivity of p15 methylation detection (2.5 × 10⁻⁵) compared with p16 methylation detection (5 × 10⁻⁵). The detectability for p15 methylation can be enhanced if a larger amount of plasma/serum is used.

The application of dual p15 and p16 methylation markers allowed us to detect circulating tumor DNA in 74% (17 of 23)
of plasma/serum samples from 92% (23 of 25) of HCC patients with tumor p15/p16 methylation. In addition, p15 methylation was shown in PBNCs from three additional cases. Regardless of the tumor size (range, 1.4–11 cm in diameter), we found methylation abnormalities in the peripheral circulation of 87% (20 of 23) of HCC patients with tumor methylation. Our findings may form the basis for noninvasive diagnosis of small HCC among high-risk populations at an early stage and for disease monitoring. The mechanism of DNA release from the tumor into plasma/serum remains unknown but may be related to cellular turnover, necrosis, or apoptosis. The methylation analysis of peripheral blood may prove valuable for studying the pathophysiological basis for cell-free tumor DNA liberation and tumor cell dissemination into the patient’s circulation.

With regard to the molecular detection of circulating HCC cells, albumin and a-fetoprotein mRNAs have been analyzed previously using reverse transcription PCR (1, 15). However, “illegitimate transcription” is a potential problem that needs to be addressed. In PBNCs, we showed methylated p15 sequences in all HCC patients with tumor p15 methylation, in no HCC patients without tumor p15 methylation, and in no healthy subjects and non-HCC patients with chronic hepatitis/cirrhosis. These results indicate that MSP for p15 enables specific detection of circulating HCC cells in addition to cell-free tumor DNA.

Dual p15 and p16 methylation abnormalities as diagnostic and prognostic markers for HCC can be used generally for widespread cancer screening and monitoring of patients. Furthermore, this approach may be applied to many other tumor suppressor genes or metastasis suppressor genes, which are methylated in different tumor types. The peripheral blood MSP analysis is sensitive and specific and can be conducted on the tumor suppressor p15 and p16 INK4B and p16 INK4A characterize the major types of hematological malignancies.

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REFERENCES


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