Detection of Aberrant p16 Methylation in the Plasma and Serum of Liver Cancer Patients

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Abstract

We have studied the feasibility of detecting tumor-associated aberrant p16 methylation in the circulation of patients with hepatocellular carcinoma (HCC). We extracted DNA from the tumor tissues and peripheral blood plasma or serum of 22 HCC patients. p16 methylation was found in 73% (16 of 22) of HCC tissues using methylation-specific PCR. Among the 16 cases with aberrant methylation in the tumor tissues, similar changes were also detected in the plasma/serum samples of 81% (13 of 16) of the cases. No methylated p16 sequences were detected in the peripheral plasma/serum of the six HCC cases without these changes in the tumor, in 38 patients with chronic hepatitis/cirrhosis, or in 10 healthy control subjects. These results suggest that circulating liver tumor DNA may be detected using tumor-associated DNA methylation changes. Because methylation abnormalities have been found in many other genes and tumor types, this approach may have implications for the noninvasive detection of a wide variety of cancers.

Introduction

HCC is one of the most common human malignancies worldwide (1). Although the molecular mechanisms of hepatocarcinogenesis remain poorly understood, an increasing number of genetic abnormalities are being recognized (2). Of relevance to HCC, inactivation of a tumor suppressor gene, p16, which is important in the regulation of cell cycling, has recently been described in a significant proportion of patients with HCC (3). An important mechanism of inactivation is aberrant methylation in the promoter region of the gene (3, 4). We hypothesize that because tumor-derived DNA has been detected in the plasma and serum of patients with various cancers (5, 6), tumor-associated DNA methylation may also be detectable in the peripheral blood. After a demonstration of aberrant methylation of the p16 gene in HCC tissues, we attempted to detect the same methylation change in the plasma or serum of HCC patients using MSP (7).

Materials and Methods

Patients. We recruited 22 HCC patients from the Joint Hepatoma Clinic at the Prince of Wales Hospital, Shatin, Hong Kong and obtained their informed consent. Ethics approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong. Preoperative peripheral blood and resection HCC specimens were obtained. Histological confirmation of the diagnosis of HCC was obtained in all cases. Peripheral blood (5 ml) from each patient was collected into either an EDTA tube or a plain tube for the isolation of plasma or serum, respectively. Control samples were obtained from 38 patients with chronic hepatitis or cirrhosis and 10 healthy volunteers.

DNA Extraction from Blood and Tumor Samples. Blood samples were centrifuged at 3000 × g, and plasma and serum were carefully removed from the EDTA-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. The buffy coat fraction from the EDTA-containing tubes was also collected to study the presence of circulating tumor cells in the peripheral blood. The samples were stored at −70°C or −20°C until further processing. DNA from plasma and serum samples was extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the blood and body fluid protocol as recommended by the manufacturer (8). The plasma/serum samples (400 µl/column) were used for DNA extraction. A final elution volume of 50 µl was used. Buffy coat samples were extracted using a Nucleon BACC2 DNA extraction kit according to manufacturer’s recommendations (Amersham Life Science, Buckinghamshire, United Kingdom).

HCC specimens were stored at −70°C. DNA extraction was performed using a QIAamp Tissue Kit (Qiagen) according to manufacturer’s recommendations.

MSP. MSP was carried essentially as described previously (7) and was based on the principle that treating DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues, on the other hand, would remain unchanged. Thus, the DNA sequences of methylated and unmethylated genomic regions after bisulfite conversion would differ and would be distinguishable by sequence-specific PCR primers.

Bisulfite conversion was carried out using the reagents provided in an Onco CpGenome DNA Modification Kit (Onco, Gaithersburg, MD). Plasma/serum DNA (25 µl) or HCC/buffy coat DNA (1 µg) were treated with sodium bisulfite following the manufacturers’ recommendations. The bisulfite-modified DNA was amplified using primers specific for the unmethylated p16 sequence. The sense and antisense primers for the methylated sequence were 5′-TTATTTAGGGTGCGGATCGC-3′ and 5′-GACCCCGAACCGC-GACCGTAA-3′, respectively (product size, 150 bp; Ref. 7). As a quality control of the bisulfite conversion process, all bisulfite-treated DNA was also amplified using primers specific for the unmethylated p16 sequence. The sense and antisense primers for the unmethylated sequence were 5′-TATAGGCGGTTGGGATCG-3′ and 5′-CAACCGCAAAACACCAACACCATAA-3′, respectively (product size, 150 bp; Ref. 7).

DNA amplification was carried out using reagents supplied in a GeneAmp DNA Amplification Kit using AmpliTag Gold as the polymerase (Perkin-Elmer Corp., Foster City, CA). A total of 35 and 55 cycles were used for tumor and plasma/serum/buffy coat DNA, respectively. The thermal profile consisted of an initial denaturation step of 95°C for 12 min followed by repetitions of 95°C for 45 s, 60°C for 45 s, and 72°C for 60 s, with a final extension step of 72°C for 10 min. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

The identity of the PCR product for the methylated version of the p16 gene was confirmed by nonradioactive Southern blot analysis as described previously (9). The probe was designed to hybridize to the methylated p16 sequence between the primers (probe sequence, 5′-GAGTAGATGAGGTTTTCGGTGATTGGTTGTTG-3′). Sequence information was obtained from the GenBank database (accession number U12818).

The human plasmacytoma cell line HS-Sultan (American Type Culture Collection CRL-1484), which was previously shown to have p16 methylation by methylation-sensitive restriction enzymes and Southern blotting techniques.
(10), was used as a positive control for MSP. Serial dilutions of HS-Sultan DNA were made in water. After bisulfite conversion and 55 cycles of amplification using primers specific to the methylated version of the p16 gene, the lower detection limit of the assay was 50 pg of HS-Sultan DNA.

**Results**

A total of 16 of 22 (73%) tumors were found to have methylated p16 sequences (Fig. 1; Table 1). For the 16 cases with methylated p16 sequences in tumors, MSP was able to detect the same change in the plasma and serum samples of 13 cases (81%; Fig. 2; Table 1). For the six cases involving tumors with no detectable p16 methylation, no signal was obtained using MSP on the plasma and serum samples. The association of tumoral p16 methylation status and MSP positivity or negativity in the peripheral plasma/serum was statistically significant (Fisher’s exact test, P < 0.001).

In the 15 cases involving peripheral plasma samples, buffy coat samples were also available. MSP analysis was positive in two buffy coat samples from two patients with tumoral p16 methylation. MSP was negative in the remaining 13 cases.

None of the serum samples from the 38 patients with chronic hepatitis and cirrhosis had detectable methylated p16 sequences. None of the plasma and serum samples from the 10 healthy volunteers showed methylated p16 sequences in the peripheral blood. No signals were observed in any of the multiple water blanks.

As a control for the bisulfite modification process, all bisulfite-treated plasma, serum, and buffy coat samples were amplified with primers specific for the unmethylated p16 gene. All samples were found to have amplifiable sequences, thus demonstrating the success of the bisulfite modification process.

**Discussion**

We have confirmed the presence of aberrant methylation of the p16 gene in a significant proportion of HCC patients and have now shown for the first time that such an aberration can be detected in the peripheral circulation of HCC patients. Using MSP, we detected tumor-associated aberrant p16 methylation in 81% (13 of 16) of cases in which the tumor samples also contained the same methylation abnormality. Clinically, this observation opens up the exciting possibility of noninvasive detection of HCC using peripheral blood analysis. Biologically, peripheral blood methylation analysis may become a powerful tool for studying the pathophysiological basis for tumor DNA liberation into the patient’s circulation.

A review of the clinical parameters of the three patients who possessed tumoral aberrant p16 methylation that was undetectable in peripheral blood plasma did not reveal any distinguishing features from those of patients whose plasma or serum had detectable aberrant p16 methylation. We are currently conducting a larger study that may give us a better chance of detecting potentially distinguishing features in each of these two groups of patients that are not apparent in the relatively small sample size recruited for the present study.

Our approach can also be applied to a wide variety of other tumors that have been shown to exhibit aberrant methylation in a number of genes (11). For example, methylation of the p16 gene has been observed in lung, breast, colon, prostate, renal, bladder, and head and neck cancers (4, 12). Furthermore, genes other than p16 have been reported to be aberrantly methylated in various cancers, such as the E-cadherin gene in breast and prostate cancers (13), the von Hippel-Lindau (VHL) gene in renal cell cancer (14), and the BRCA1 gene in breast cancer (15).
The success of our approach supports previous reports demonstrating the presence of tumor DNA in the plasma and serum of cancer patients (5, 6). The applicability of MSP to a wide variety of tumors will allow one to study the phenomenon of tumoral DNA release into the circulation among different tumor types. This may potentially reveal important differences that may be associated with the varying biological behavior and clinical course of different malignancies.

Previous work concerning the molecular detection of HCC in peripheral blood has relied on the analysis of albumin or α-fetoprotein mRNA by RT-PCR as a marker of circulating HCC cells (9). Using this RT-PCR approach, amplification signals have been detected in individuals without evidence of HCC and attributed to the phenomenon of illegitimate transcription, whereby the transcription of mRNA species may not be absolutely tissue specific (16). For routine clinical use, our DNA-based approach may be more practical, because the handling of RNA materials generally requires much more care than DNA. Biologically, the methylation-based and RT-PCR-based approaches might be studying distinct pathophysiological phenomena; thus, it would be interesting to correlate the results obtained using these methods on the same cohort of patients.

Our detection of methylated p16 sequences in the buffy coat DNA of two HCC patients possessing the same change in tumor tissues suggests that MSP can also be used to detect circulating HCC cells in addition to circulating cell-free DNA. However, the sensitivity of detection in the cellular fraction [2 of 11 cases (18%)] appears to be much less than that in the plasma fraction [8 of 11 cases (73%)]. This observation suggests that there seems to be a relative enrichment of tumor-derived DNA in the plasma fraction compared with the cellular fraction. The mechanism for this phenomenon is unclear at present but may be due to DNA release from the tumor during cell turnover, cellular necrosis, or apoptosis.

A methylation-based approach has certain useful features when compared with several previously described methods for the detection of circulating tumor DNA, such as microsatellite instability (5) and oncogene mutations such as p53 (6). The main disadvantage of the microsatellite approach is its relatively low sensitivity, which is a potential source of false negative results (6). The large number of mutations that have been described for the p53 gene (17) complicate its use for cancer screening from plasma or serum samples. Therefore, the methylation approach outlined here has the advantages of high sensitivity and ease of implementation for the screening of HCC.

It would also be relatively easy for this assay to be converted into a quantitative format for circulating tumor DNA such as has been achieved for circulating fetal DNA in pregnancy (8). Such an assay will allow one to analyze the level of circulating tumor DNA with regard to clinical parameters such as tumor stage. It would also be interesting to study the variation of circulating tumor DNA levels in relation to treatment and to correlate the posttreatment level with the patient’s prognosis.

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References

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