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Quantitative analysis of multiple methylated genes in plasma for the diagnosis and prognosis of hepatocellular carcinoma

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ABSTRACT

This study was aimed to evaluate the clinical value of plasma methylation analysis of a panel of four genes (APC, GSTP1, RASSF1A, and SFRP1), which was identified by our previous work, for the noninvasive diagnosis of hepatocellular carcinoma (HCC). The methylation status of these four genes in 150 plasma samples from 72 patients with HCC, 37 benign live diseases and 41 normal controls was detected with methylation-sensitive restriction enzymes-based quantitative PCR (MSRE-qPCR) method. The plasma methylation levels of APC, GSTP1, RASSF1A, and SFRP1 were significantly higher in HCCs than those in normal or benign controls ($P<0.05$). Although the area under the receiver-operation characteristic curve (AUC-ROC) for individual gene was moderate (range, from 0.800 to 0.881), the combination analysis of these four genes resulted in an increased AUC of 0.933 with 92.7% sensitivity, 81.9% specificity, 90.5% positive predictive value (PPV), and 87.2% negative predictive value (NPV) in discriminating HCC from normal control. The combination analysis also indicated an increased AUC of 0.877 when compared with individual gene (from 0.666 to 0.850) in discriminating HCC from benign control, and the consultant sensitivity, specificity, PPV, and NPV was 84.7%, 81.1%, 89.7%, and 73.2%, respectively. Patients with elevated plasma methylation levels of APC or RASSF1A showed poorer overall survival than those with low levels ($P<0.05$). Cox multivariate analysis demonstrated methylated RASSF1A in plasma to be an independent prognostic factor for overall survival (hazard ratio = 3.262, 95% CI: 1.476–7.209, $P=0.003$). These data showed that quantitative analysis of multiple methylated genes in plasma may be a promising tool for noninvasive diagnosis of HCC; and methylated plasma RASSF1A appears to be a prognostic marker of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Despite recent advances in the detection and therapy of HCC, the 5-year survival rate remains low, and late presentation remains the most important obstacle to successful treatment. Most HCC cases have already developed locally advanced disease or distant metastasis by the time of diagnosis. At present, α-fetoprotein (AFP) is the most common used clinically for the detection of HCC; however, the diagnostic sensitivity of AFP is relatively low. Therefore, the development of new biomarkers for early HCC detection is urgently needed.

Aberrant hypermethylation of CpG islands, a well-known epigenetic event, is a hallmark of cancer (Levenson, 2010; McCabe et al., 2009). Silencing tumor suppressor gene (TSG) by promoter hypermethylation has been proved to be an early event in carcinogenesis and is present in the precursor lesions of a variety of cancers, such as HCC. Therefore, it has been intensively attempted to exploit the potential value of tumor associated pattern of DNA methylation as tumor biomarker (Qureshi et al., 2010; Shivapurkar and Gazdar, 2010). These studies revealed that DNA methylation appears to be an emerging tumor biomarker, and frequent promoter methylation of TSGs, such as CDKN2A, RASSF1A, and GSTP1, has been observed in HCC (Lee et al., 2003; Yang et al., 2003). Recent studies clearly demonstrated the advantages of multiple gene hypermethylation analysis in tissue and serum samples regarding diagnostic and prognostic information (Harder et al., 2008; Moribe et al., 2009; Zhang et al., 2007). However, little was known about the value of DNA methylation analysis at multiple gene sites for the detection of HCC in the Chinese population.

Recently, we developed a simple method for the quantitative methylation analysis, which combines the use of methylation-sensitive restriction enzyme digestion (MSRE) and quantitative PCR (qPCR) (Huang et al., 2011). Furthermore, we identified that a combination analysis of four methylated genes (APC, GSTP1,RASSF1A, and SFRP1) in the tumor tissues may be suitable for the diagnosis of HCC (Hua et al., 2011). However, the application of tissue-based method is very limited for clinical diagnostic purposes; and a noninvasive test is desired for tumor early detection and for monitoring disease progression.

Abbreviations: HCC, hepatocellular carcinoma; MSRE, methylation-sensitive restriction enzymes; qPCR, quantitative polymerase chain reaction; AFP, α-fetoprotein; TSG, tumor suppressor gene; ROC, receiver operator characteristic curve; AUC, area under the curve; MP, methylation percentage; PPV, positive predictive value; NPV, negative predictive value; OS, overall survival; HR, hazard ratios; CI, confidence intervals.

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The aim of this study was to evaluate the potential application of this test for HCC detection and prognosis by measuring these methylated genes in plasma samples.

Material and methods

Collection of plasma specimens

This study collected 109 plasma samples from 72 patients with HCC and 37 with benign liver diseases (including 25 patients with cirrhosis and 12 with chronic inactive hepatitis) in accordance with the institutional ethical guidelines. A total of 41 healthy volunteers, who were apparently healthy based on clinical and laboratory examination, were served as healthy controls. Specimens were selected on the basis of sufficient plasma samples and appropriate clinical data. The clinicopathologic data of these HCC patients at initial diagnosis were listed in Table 1.

Blood specimens were collected before surgery or therapy. Five mL sample of peripheral blood was collected in blood collection tube containing EDTA and was centrifuged (2000 *g*; 10 min at 4 °C) within 2 h after venipuncture. The supernatants were carefully collected and centrifuged again (12,000 *g*; 10 min at 4 °C) to prevent potential cellular DNA contamination. The plasma was distributed into aliquots and stored at −80 °C until use.

DNA purification

Genomic DNA was isolated from 600 μL plasma using TIANamp Micro DNA Kit (Tiangen, Beijing, China) and eluted in 30 μL sterile water following the manufacturers' protocol. To improve the extraction efficiency, carrier RNA was added after the proteinase K digestion. Two μL plasma DNA was used for the measurement of DNA concentration using qPCR as described by our previous work (Huang et al., 2010). If a plasma specimen had a DNA concentration less than 10 ng/mL, DNA was purified from 1 to 3 mL of plasma, and was concentrated in a final volume of 30 μL by using Eppendorf Concentrator Plus 5301 (Eppendorf, Germany). DNA samples were finally stored at −20 °C until use.

DNA digestion with MSRE

Digestions were performed with HhaI (Takara, Dalian, China) according to our previous work with some modifications (Hua et al., 2011; Huang et al., 2011). Briefly, twenty picograms of unmethylated pDC316 plasmid DNA was added to each plasma DNA sample (24 μL). Then, each specimen was divided into two equal aliquots. The first one was digested with 10 U HhaI at 37 °C for 16 h in a final reaction volume of 20 μL; and the second aliquot was used as a sham-treated control. After incubation, each digested sample was diluted 5-fold in sterile water and incubated at 65 °C for 15 min to inactivate HhaI. The methylation-sensitive enzyme degrades unmethylated DNA sequences, whereas methylated DNA sequences remain intact and detectable by PCR.

Table 1
The clinical pathological parameters of patients with hepatocellular carcinomas.

Characteristics	N (%)
Total case number	72
Age	
≥55	40(55.6)
<55	32(44.4)
Gender	
Male	61(84.7)
Female	11(15.3)
UICC stage	
I–II	17(23.6)
III–IV	55(76.4)
Tumor size	
≥5 cm	48(66.7)
<5 cm	24(33.3)
HBV infection	
Yes	61(84.7)
No	11(15.3)
Serum AFP levels	
≥400 μg/L	33(45.8)
<400 μg/L	39(54.2)

Quantitative PCR

Quantitative PCR was done in duplicate on a DNA Engineer Opticon II (Bio-Rad Laboratories, Hercules, CA, USA) as described (Hua et al., 2011). Melting curve analysis was performed to confirm the specificity of PCR products. Each run included negative controls, blank controls, and 5-fold dilutions of genomic DNA or pDC316 to construct an external standard. Methylation percentage (MP) was used to represent the methylation level of target CpG sites at a specific location in a promoter. MP was calculated using the following equation: $MP = (Value_{Digested} / Value_{Sham\text{-}digested}) \times 100\%$ where $Value_{Digested}$ was the quantitative result of qPCR for digested DNA sample, and $Value_{Sham\text{-}digested}$ was the quantitative result of the qPCR for the sham-digested sample (Hua et al., 2011).

Statistical analysis

The difference of DNA methylation status between different groups was analyzed using the Mann–Whitney *U* or the chi-square test where appropriate. Optimum cutoff values for the four genes were separately determined by simultaneously maximizing both sensitivity and specificity for the detection of HCC for all values of MP using receiver-operating characteristics (ROC) curves. Overall survival (OS) was defined as the time between diagnosis and either death or the time of the last follow-up. Survival curves were generated by the Kaplan–Meier method and the log-rank test was adopted to compare survival time between patients with different plasma methylation levels. Cox's proportional hazards model was used to estimate Hazard Ratios (HRs) and their 95% confidence intervals (CIs), representing the overall relative risk of death associated with plasma methylation. A p value of less than 0.05 was considered statistically significant. All statistical analyses were conducted with SPSS 13.0 software for windows (SPSS Inc., Chicago, USA).

Results

The reliability of MSRE-qPCR for analysis of plasma methylation

To evaluate the enzyme digestion efficiency of HhaI for plasma DNA, all 150 plasma DNA samples were detected in a separate PCR reaction using pDC316-specific primers. Results of this PCR served as a quality control for the digestion procedure. In this study, the digestion efficiencies for all plasma DNA samples ranged from 99.0% to 99.9%, suggesting the high efficiency of enzyme digestion; and any DNA sample with less than 1% input plasmid sequence after digestion was regarded as qualified sample.

Methylation levels of four genes in plasma

The methylation status of these four genes was evaluated using MSRE-qPCR in 150 plasma samples, including 72 patients with HCC, 37 with benign live diseases and 41 normal controls, and revealed that the MPs of these four genes (APC, GSTP1, RASSF1A, and SFRP1) were higher in HCCs than in benign controls or healthy controls (Mann–Whitney *U* Test, $P<0.05$, Fig. 1).

The diagnostic ability of these four methylated genes and their combination was evaluated using ROC analysis. The AUC for individual gene