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RESEARCH

Quantitation of Plasma Circulating DNA Using Quantitative PCR for the Detection of Hepatocellular Carcinoma

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Abstract Circulating DNA is a potential biomarker for tumor diagnosis and prognosis. This study was aimed to quantify the circulating DNA in plasma from patients with hepatocellular carcinoma (HCC) using quantitative PCR and evaluate its potential clinical value. Blood samples were collected from 72 patients with HCC, 37 with liver cirrhosis or chronic hepatitis and 41 healthy volunteers. Plasma DNA was extracted and quantified by a real-time quantitative PCR method. The diagnostic and prognostic value of plasma DNA analysis for HCC was evaluated. DNA levels in the HCC plasma (median: 173 ng/mL) were significantly higher than those in the healthy controls (9 ng/mL) or control benign patients (46 ng/mL) ($P<0.001$). The area under the receiver-operation characteristic (ROC) curve (AUC) assessing plasma DNA was 0.949 for healthy controls and 0.874 for control patients. Plasma DNA detection could discriminate HCC from normal controls with 90.2% sensitivity and 90.3% specificity at the cut-off value of 18.2 ng/mL. Combined ROC analyses using plasma DNA and serum AFP revealed an elevated AUC of 0.974 with 95.1% sensitivity and 94.4% specificity in discriminating HCC from normal controls. The plasma DNA levels were positively associated with tumor size ($P=0.012$), and were significantly elevated in HCC patients with intrahepatic spreading or vascular invasion ($P=0.035$). The overall survival time of patients with high plasma DNA levels showed a shortened tread when compared with that of patients with low plasma DNA concentrations ($P=0.071$). Plasma DNA may be a valuable noninvasive tool for the detecting and predicting the metastasis potential of HCC; and the prognostic value of plasma DNA needed further investigation.

Keywords Hepatocellular carcinoma · Plasma · Circulating DNA · Quantitative PCR

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of death from cancer worldwide. The regions of high incidence of HCC are Eastern and South-Eastern Asia, Middle and Western Africa. In China, the incidence of HCC is about 30 cases per 100,000 population; and HCC cases in China alone account for more than 40% of all cases in the world [1]. The risk factors for HCC, such as hepatitis virus infection, exposure to aflatoxin B and heavy consumption of alcohol, are well documented [2]. Chronic hepatitis B virus (HBV) infection is prevalent in China and appears to be an important risk factor for HCC; and more than 70% of HCC patients were infected with HBV. There are about 130 million cases infected with HBV in China. The poor

prognostic feature of HCC is largely attributive to a high rate of relapse after surgery or intra-hepatic metastases and difficulties in early detection of HCC. Alpha-fetoprotein (AFP) is most widely used clinically for the detection of HCC; however, the diagnostic sensitivity of AFP for HCC is relatively low and only about half of HCC patients have typical AFP concentrations [3].Therefore, there is urgent need for the development of new biomarkers with diagnostic and prognostic significance for HCC.

Circulating cell-free DNA (cfDNA) is defined as extracellular DNA major occurring in blood [4]. Despite its identification over 60 years ago, research on cfDNA has lagged considerably due to the lack of robust and sensitive quantitative methods. Recently, advances on isolation methods of cfDNA and quantitative procedures using novel fluorescent dyes or quantitative PCR (qPCR) techniques have enabled progression in this active research area.

The increased levels of blood cfDNA in a number of diseases indicated that cfDNA can be used as a non-invasive, rapid, sensitive and accurate method of diagnosis of human diseases, including cancer. The potential diagnostic, prognostic, and monitoring significance of plasma/serum DNA has been clearly demonstrated in breast cancer [5, 6], colorectal cancer [3], lung cancer [7–9], ovarian cancer [10, 11], prostate cancer [12], esophageal cancer [13], HCC [14], gastric cancer [15], and rental cell cancer [16]. However, methodological difference in sample processing, DNA extraction, quantification and the date analysis, made it difficult to compare the result of these researches and consequently hold back the application of cfDNA in clinical practices. This study was aimed to quantitative the circulating plasma DNA levels in HCC using a simple qPCR method, and then evaluate the potential utility of circulating plasma DNA as a diagnostic or prognostic marker for HCC.

Material and Methods

Collecting Samples

This study collected 150 plasma samples from **72** patients with HCC, 37 with benign liver diseases (including 25 patients with cirrhosis and 12 with chronic inactive hepatitis) and 41 healthy volunteers in accordance with the institutional ethical guidelines. The clinicopathologic data of HCC patients at initial diagnosis were listed in Table 1.

Blood specimens were collected before surgery or therapy. Five mL peripheral blood was collected in K_3-EDTA containing tube, and was centrifuged (2,000 g, 10 min at 4°C) within 2 h after venipuncture. The supernatants were carefully transferred into 2 mL microtubes 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.

Table 1 Relationship between plasma DNA levels and clinicopathological features in hepatocellular carcinoma

Characteristics	n	Plasma DNA levels		$\chi 2$	*P* value
		High value	Low value		
Age				0.900	0.343
≥55	40	22	18		
<55	32	14	18		
Gender				2.683	0.189
Man	61	28	33		
Female	11	8	3		
TNM stage				0.077	0.781
I–II	17	9	8		
III–IV	55	27	28		
Tumor size				6.250	0.012
≥5 cm	48	29	19		
<5 cm	24	7	17		
Serum AFP					
≥400 μg/L	32	15	17	0.225	0.635
<400 μg/L	40	19	21		
HBV infection				0.966	0.514
Yes	61	29	32		
No	11	7	4		

DNA Purification

Plasma genomic DNA was isolated from 600 μL plasma using TIANamp Micro DNA Kit (Tiangen, Beijing, China) following the manufacturers' protocol. To improve the extraction efficiency, carrier RNA was added after the proteinase K digestion. Plasma DNA was eluted in a final volume of 30 μL sterile water and was stored at −20°C until use.

Real Time PCR

The DNA concentration was measured using a previously described real time qPCR targeting the human beta-actin gene with minor modifications [17]. The forward and reverse primer was 5′-GCACCACACCTTCTACAATG-3′ and 5′-TCATCTTCTCGCGGTTGGC-3′, respectively. The length of PCR product was 101 bp. PCR was done in duplicate on a DNA Engineer Opticon II (Bio-Rad Laboratories, Hercules, CA, USA). Each 20 μL reaction consisted of 1 × SYBR Premix DimerEraser™(Takara, Dalian, China), 0.2 μM of each primers, 200 μM deoxynucleotide triphosphate mix, and 2 μL DNA sample. PCR was done at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 58°C for 25 s, and 72°C for 10 s. Melting curve analysis

was performed to confirm the specificity of PCR products. For construction of the calibration curve for each amplification, we generated a standard curve using 5-fold serial dilutions of human genomic DNA (10, 50, 250, 1,250, 6,250 pg). The concentration of plasma DNA was calculated using the following equation [5]: $C = Q \times (V_{DNA}/V_{PCR})/V_{ext}$. C = target concentration in plasma (nanogram per millilitre); Q = target quantity (pg) determined by the qPCR, V_{DNA} = total volume of extraction (30 μL); V_{PCR} = volume of DNA solution used for PCR reaction (2 μL); and V_{ext} = volume of plasma extracted (typically 600 μL).

Statistical Analysis

Descriptive statistics were used for clinical characteristics and comparisons were performed using Chi-square tests. Levels of plasma DNA were compared using the Mann–Whitney *U* test or the Kruskall-Wallis test. Area under curve (AUC) of receiver operating characteristic curve (ROC) was calculated to evaluate the diagnostic value of plasma DNA. Survival curves were estimated according to the Kaplan-Meier method and compared with a log-rank test. Continuous variables were transformed into dichotomous variables using the median as a cutoff before the survival analysis. Overall survival (OS) was calculated from the data of diagnosis to the data of death or to that of the last follow-up. Data analysis was performed with SPSS13.0 for windows (SPSS Software, München, Germany). A *P* value <0.05 was considered significant.

Results

Quantitative Analysis of Plasma DNA

The good quality of the extracted DNA allowed successful amplification and quantification of the beta-actin gene in all plasma samples, and the established PCR system could specifically detect as low as 10 pg human genomic DNA with high amplication efficiency(data not shown). The plasma DNA concentrations in HCC patients (median: 173 ng/mL, range 4–1,300 ng/mL) was significantly higher than that in healthy controls (median: 9 ng/mL; range 1–98 ng/mL) or that in benign controls (median: 46 ng/mL, range 2–868 ng/mL) (Mann–Whitney *U* test, $P<0.01$, Fig. 1). The plasma DNA levels in patients with benign live diseases was also significantly elevated in comparison with normal controls (Mann–Whitney *U* test, $P<0.0001$, Fig. 1).

Relationship between Plasma DNA Levels and Clinical Characteristics

Among HCC patients, no significant association was observed between plasma DNA levels and age, gender, TNM stage, serum AFP or HBV infection. However, patients with large tumor size (≥5 cm) showed significantly elevated plasma DNA concentrations in comparison with those with small tumor size (<5 cm) ($P=0.012$, Table 1). In addition, the plasma DNA levels in patients with vascular invasion and/or intrahepatic metastasis (median, 261 ng/mL) were higher than that in those patients without (median, 142 ng/mL) ($P=0.035$).

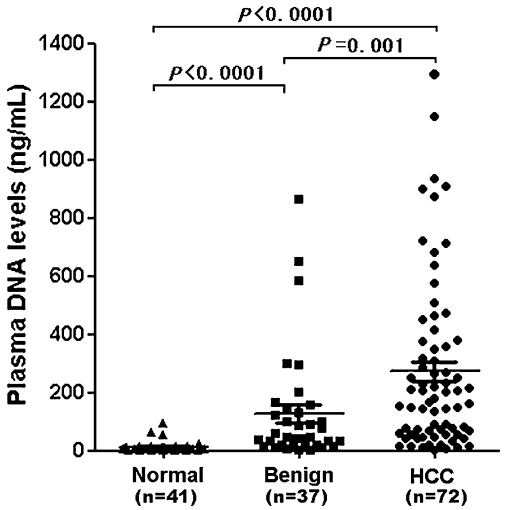

Fig. 1 The plasma DNA levels in normal controls, patients with HCC and benign liver diseases

Sensitivity and Specificity for Plasma DNA as a Diagnostic Marker

ROC curve analysis was performed to investigate the diagnostic potential of circulating plasma DNA as a tumor marker. The AUC for plasma DNA in discriminating HCC from normal control was 0.949 (95% confidence interval (CI): 0.910–0.988). At the cutoff value of 18.2 ng/mL, the optimal sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were 90.3%, 90.2%, 94.2%, and 84.1%, respectively (Fig. 2a). The AUC for plasma DNA in discriminating HCC from benign control was 0.705(95% CI: 0.601–0.806) with 59.7% sensitivity, 78.4% specificity, 82.7% PPV, and 52.3% NPV at the cutoff of 143.0 ng/mL (Fig. 2b). In addition, plasma DNA also showed diagnostic potential between benign liver diseases and normal control, and revealed an AUC of 0.874 (95% CI: 0.931–942) with 83.8% sensitivity, 90.2% specificity, 88.6% PPV, and 86.0% NPV at the cut point of 18.0 ng/mL (Fig. 2c).

No significant association was observed between plasma DNA levels and serum AFP; so a combination analysis was performed to assess the diagnostic value of these two factors. The results revealed that addition of

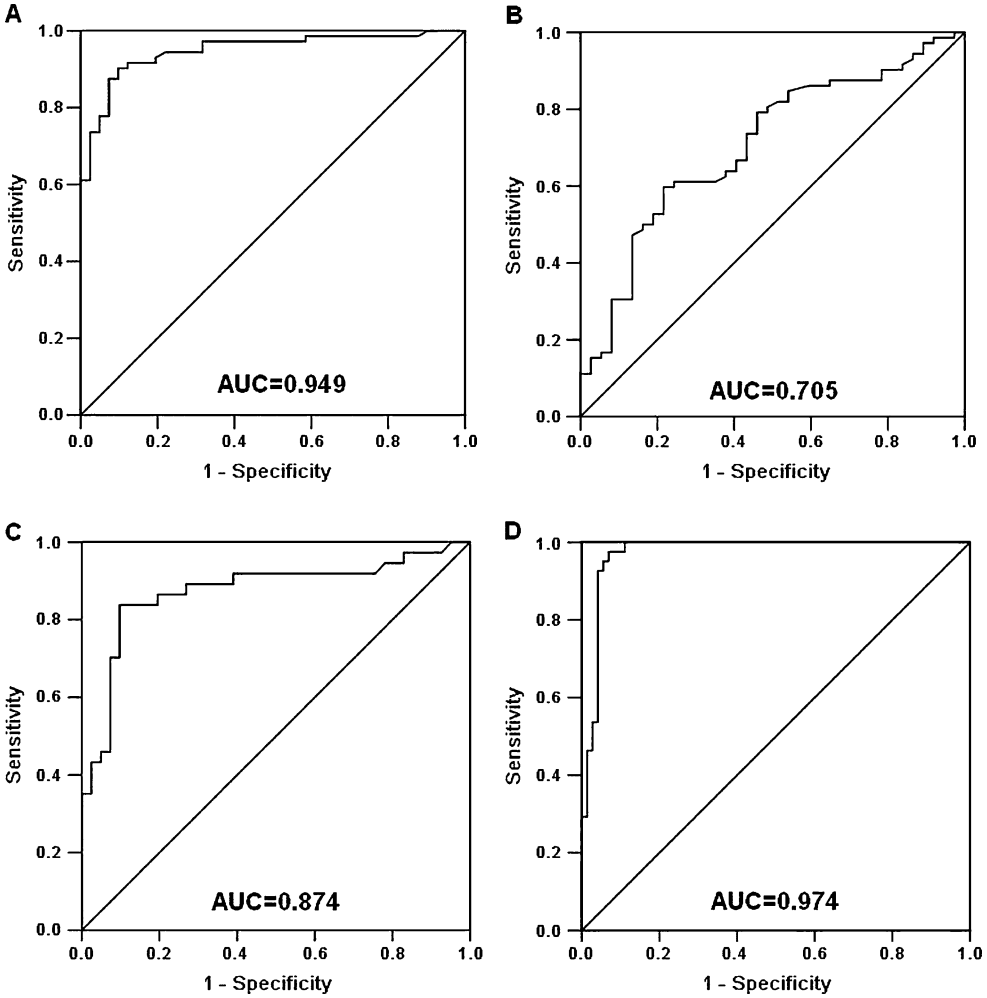

Fig. 2 Receiver operating characteristics (ROC) curve analysis for discriminating HCC using plasma DNA levels. Plasma DNA yielded an AUC (the areas under the ROC curve) of 0.949 (95% CI: 0.910–0.988) in discriminating HCC from normal control (**a**) and an AUC of 0.705 (95%CI: 0.601–0.806) in discriminating HCC from benign control (**b**). Plasma DNA also showed discriminating potential with an AUC of 0.874 (95% CI: 0.931–942) between benign liver diseases and normal control (**c**). A combination analysis of plasma DNA and serum AFP resulted in an increased AUC of 0.974 (95% CI: 0.946–1.002) in discriminating HCC from normal control (**d**)

AFP could improve the differentiation power of plasma DNA between HCC and normal control, resulting in an increased AUC of 0.974 (95% CI: 0.946–1.002) with 95.1% sensitivity and 94.4% specificity, indicating the additive effect of these two assays for HCC diagnosis (Fig. 2d).

Plasma DNA as a Prognostic Marker in HCC

In 20 HCC patients, a second plasma sample was collected 1 to 6 months after surgery to monitor changes in plasma DNA levels during clinical follow-up. The overall median DNA concentration in follow-up plasma samples was 42 ng/mL, showing a clear trend toward reduction, compared with those recorded in the sample before surgery (median, 156 ng/mL)(P=0.030).

To determine whether pre-treatment DNA concentrations correlated with the survival time in 72 HCC patients, we performed a univariate analysis of overall survival for plasma DNA using the median concentration (173 ng/mL) as a cutoff. Patients with high plasma DNA concentration (≥173 ng/mL) showed a tread of prolonged survival when compared with patients with low plasma DNA levels (≤173 ng/mL), but no significant difference was found (log-rank test, P=0.071, Fig. 3).

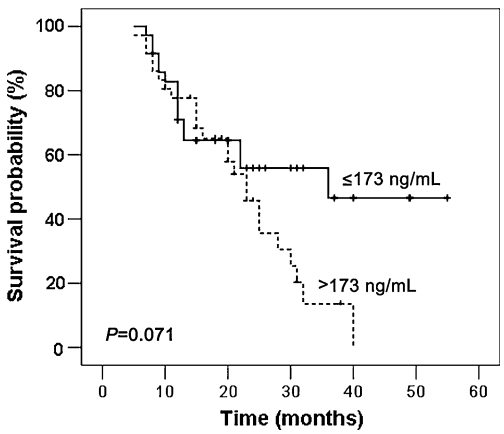

Fig. 3 Overall survival curve for HCC patients evaluated according to plasma DNA levels

Discussion

In this study, we measured the level of cfDNA in the plasma of healthy controls and patients with benign or malignant liver diseases, using a qPCR method. We found that plasma DNA levels were significantly higher in HCC patients than in normal controls or patients with benign live diseases (cirrhosis or chronic inactive hepatitis), suggesting the potential of plasma DNA for HCC screening.

In 1977, Leon et al. [18] firstly reported that cancer patients have much higher concentration of cfDNA than those with non-malignant diseases, and cfDNA levels were decreased in some cases after anti-cancer therapy. However, the lag in the technical and mechanical studies, hamper the advances of cfDNA. Recently, qPCR-based methods greatly promoted the research and application of cfDNA [5, 6, 11, 15, 19–22]. Both plasma and serum samples have been used for cfDNA research. Higher cfDNA levels were previously observed in serum than in matched plasma due to a non-specific release of DNA from the hematopoietic cells during the clotting process [23]. Recent studies also showed that plasma better reflect the in vivo levels of circulating DNA than serum [24, 25]. We accordingly selected plasma instead of serum for cfDNA analysis in this study.

Using a simple, rapid and sensitive qPCR method, we found that plasma DNA levels were significantly higher in HCC patients than in normal controls or patients with benign live diseases (cirrhosis or chronic inactive hepatitis). ROC analysis revealed a powerful association between high DNA concentration and malignancy. Importantly, plasma DNA and serum AFP showed an additive effect for the detection of HCC. In addition, the AUC between the patients with cirrhosis or chronic inactive hepatitis and normal controls was 0.874, suggesting that plasma DNA may be also a valuable marker for discriminating patients with benign liver diseases from normal controls. However, plasma DNA only showed limited value for discriminating benign and malignant liver diseases. In a previous study, Ren et al. [14] also reported an elevated plasma DNA levels in HCC when compared with healthy volunteers using a fluorescence dye-based spectrophotometry. They observed a moderate discriminating power (AUC=0.8) between HCC and normal control, and no significant difference was found between patients with HCC or liver cirrhosis. However, they found that higher level of plasma DNA was associated with poor survival of HCC patients. So they concluded that plasma DNA may not be a valuable diagnostic tool but a prognostic marker for HCC. The diagnostic power of plasma DNA in our study (90.3% sensitivity and 90.2% specificity) is higher than that in Ren's study(51.9% sensitivity and 95% specificity) [14]. The reasons for these discrepancyies may include differences in analysis method and patients population; and the small case size in the control group of Ren et al. (20 patients with liver cirrhosis and 20 normal controls) may be another factor influencing the reliability of their results. In addition, a significant association was observed between plasma DNA concentration and tumor size, vascular invasion and/or intra-hepatic metastasis, which was also found in previous studies [14, 26], suggesting that large or invasive tumor may resulted in more cfDNA and increased plasma DNA may be associated with poor prognosis [14]. In according with these results, we observed a borderline relationship (P=0.071) between plasma DNA levels and OS. In view of the relative small case size both in our and their study, enlarged case number in the future may help us to determine the prognostic value of plasma DNA on the survival of HCC patients.

The elevated levels of cfDNA was found not only in cancer patients, but also patients who suffer from non-malignant diseases, i.e. autoimmune disorders [27], myocardial infarction [28], or pulmonary thromboembolism [29]. So an increased cfDNA level in plasma/serum is not specific for a defined disease; and a quantitative analysis of cfDNA will only have a limited value as a diagnostic marker, and may be valuable for preliminary screening of tumor or in combination with other molecular markers or imageology methods. However, it might be a valuable prognostic tool for evaluation of relapse, therapy response or long-term survival [9, 10, 14, 26]. Due to the small case size of this study, a definite conclusion about the diagnostic and/or prognostic value of cfDNA for HCC requires future multicenter, large case size, prospective studies.

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