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Quantification of plasma *hTERT* DNA in hepatocellular carcinoma patients by quantitative fluorescent polymerase chain reaction

Abstract

Purpose: To investigate the levels of human telomerase reverse transcriptase (*hTERT*) DNA in the plasma of patients with hepatocellular carcinoma (HCC), and to evaluate the diagnostic value and correlation of *hTERT* DNA with clinical parameters in HCC.

Methods: A real-time quantitative fluorescent polymerase chain reaction (FQ-PCR) system was designed and evaluated. Plasma samples were collected from 60 HCC patients, 21 patients with hepatitis B virus (HBV) and 29 healthy controls. Plasma DNA was extracted and quantified by FQ-PCR. The diagnostic value of plasma *hTERT* DNA levels and their relationships with clinical characteristics were analyzed statistically.

Results: Plasma levels of *hTERT* DNA in HCC patients were significantly higher than in HBV patients ($4.18 \times 10^4 \pm 4.94 \times 10^4$ copies/ μ l vs $1.21 \times 10^4 \pm 6.63 \times 10^3$ copies/ μ l, $P=0.003$) and healthy controls ($4.18 \times 10^4 \pm 4.94 \times 10^4$ copies/ μ l vs $1.44 \times 10^4 \pm 6.61 \times 10^3$ copies/ μ l, $P<0.001$). Receiver operating characteristic curve analysis indicated a sensitivity of 64% and a specificity of 90% for the ability of *hTERT* DNA levels to detect malignancy at a cutoff value of 1.87×10^4 copies/ μ l. Association analysis revealed that plasma *hTERT* DNA levels were closely related to tumor size, portal vein cancer embolus and TNM stage ($P=0.013$, $P=0.010$, and $P=0.029$, respectively), but were not associated with lymph node metastasis, hepatitis B surface antigen, or α -fetoprotein (AFP) (all $P>0.05$). The levels of plasma *hTERT* DNA in HCC patients with AFP ≤ 20 ng/ml were significantly higher than in HBV patients ($4.59 \times 10^4 \pm 4.98 \times 10^4$ copies/ μ l vs $1.44 \times 10^4 \pm 6.63 \times 10^3$ copies/ μ l, $P=0.016$) and in healthy controls ($4.59 \times 10^4 \pm 4.98 \times 10^4$ copies/ μ l vs $1.21 \times 10^4 \pm 6.63 \times 10^3$ copies/ μ l, $P=0.001$).

Conclusions: Quantitation of plasma *hTERT* DNA by FQ-PCR may provide a novel complementary tool with potential clinical applications for the screening and detection of HCC. Plasma *hTERT* DNA has the potential to be a broad tumor marker for common cancers.

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Telomerase is a ribonucleoprotein DNA polymerase that synthesizes telomeric TTAGGG repeat sequences that are critical for genomic stability and integrity [1]. Studies of telomerase activation have shown that its expression is a critical step in cellular immortality and human carcinogenesis [2,3]. Human telomerase reverse transcriptase (*hTERT*) is a single-copy gene encoding a catalytic subunit of telomerase, which plays an important role in telomerase activation. Several studies have revealed that amplification of the *hTERT* gene is closely associated with telomerase activation, and contributes to the transformation of human malignancies [4-6]. In addition, *hTERT* is upregulated in almost all human malignant tumors, but not in most benign or normal tissues with the exception of germline cells, proliferative stem cells and certain benign tumors [7,8].

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [9], and the oncogenesis of HCC is associated with *hTERT* expression. A lack of early diagnostic markers means that most HCC patients are diagnosed at a late stage [10], and the overall prognosis of patients with HCC thus remains poor, with 5-year survival ranging from 0-10%. If diagnosed early, however, the prognosis for HCC is better, with a 5-year disease-free survival rate ranging from 50-80% after prompt surgery and other treatments [11]. α -Fetoprotein (AFP) is the most commonly available laboratory test for the diagnosis and surveillance of HCC, but this test still lacks adequate sensitivity and specificity [11,12]. Novel, non-invasive tests are thus required for the early detection of the disease and for monitoring its progression. HCC was used as a representative tumor in the present study.

Using radioimmunoassay, Leon and coworkers first discovered higher levels of circulating DNA in cancer patients, compared with those with nonmalignant diseases and with healthy controls [13]. This finding opened up a new area in traditional cancer research. Many studies have investigated the genetic changes in DNA extracted from the blood of patients with various types of cancers. These changes include mutation of oncogenes or tumor suppressor genes, alterations in microsatellite DNA and changes in methylation status [14-18]. The results of these studies suggest that circulating DNA may provide a useful marker of tumor presence and aggressiveness, based on its easy accessibility and simple manipulation.

The high sensitivity of quantitative fluorescent polymerase chain reaction (FQ-PCR) means that it has been increasingly used to measure mRNA expression levels and DNA and transgene copy numbers [19]. In the present study, FQ-PCR was used to detect and quantify plasma *hTERT* DNA in patients with HCC. The relationships between *hTERT* DNA and

clinical characteristics of HCC were analyzed, including tumor size, portal vein cancer embolus, lymph node metastasis, TNM stage and the results of routine laboratory test for AFP and hepatitis B surface antigen (HBsAg).

Materials and Methods

Patients and controls

60 HCC patients (44 males and 16 females, median age 51 years) with histologically-confirmed HCC were recruited into this study. A further 21 patients with hepatitis B virus (HBV) (15 males and 6 females, median age 45 years), in whom HCC and other malignancies had been excluded by clinical and laboratory examinations, were enrolled as disease controls, and 29 individuals (20 males and 9 females, median age 47 years) determined as healthy by clinical and laboratory examinations were included as healthy controls. Peripheral blood was collected from HCC patients at the First Affiliated Hospital of Chongqing Medical University between June 2009 and June 2010. The samples were collected before any invasive procedures or therapies were initiated. Peripheral blood was also collected from HBV patients and healthy individuals at the same hospital in June 2010. Informed consent was obtained from all patients and controls, and the study was approved by the ethics committees of the First Affiliated Hospital of Chongqing Medical University.

Sample collection and plasma DNA extraction

Venous blood (2.0 ml) was collected from each participant into tubes containing EDTA-K₂. Plasma was immediately separated from the cellular fraction by centrifugation at 2,000 *g* for 10 min, followed by 12,000 *g* for a further 10 min at 4°C, and then stored at -80°C for further use. DNA was extracted from 200 μ l of plasma using a QIAamp Blood Mini Kit based on affinity columns (Qiagen, Hilden, Germany), following the manufacturer's recommendations.

Quantitation of plasma hTERT DNA by FQ-PCR

hTERT gene primers were designed according to a report by Sozzi *et al.* [20] as follows: forward, 5'-GGCACACGTGGC TTTTCG-3'; reverse, 5'-GGTGAACCTCGTAAGTTTAT GCAA-3'. The amplicon length was 98 bp. An *hTERT* DNA plasmid was constructed using TA cloning kit: after linking the purified PCR products into pMD18-T vector, the connection product was transformed to DH5 α competent *Escherichia coli*. The positive cloning *Escherichia coli* was screened and enriched, then the *hTERT* DNA was extracted from the plasmid and

FIGURE 1. Distribution of plasma *hTERT* DNA levels in HCC and HBV patients and in healthy volunteers.

FIGURE 2. ROC curve of plasma *hTERT* DNA as a tumor marker for HCC. AUC=0.78(95%CI: 0.69-0.88); while cutoff was 1.87×10^4 copies/ μ l, the sensitivity and specificity were 64% and 90% respectively.

measured. A standard curve was generated from nine dilutions of DNA (range 10^1 - 10^9 copies/ μ l) using an ABI 7500 sequence detection system (ABI, USA) and SDS software.

The FQ-PCR reaction was carried out in 96-well plates with a total volume of 20 μ l/well containing the following reagents: 10.0 μ l of SYBR; 0.8 μ l (10 μ mol/l) of forward primer; 0.8 μ l (10 μ mol/l) of reverse primer; 0.4 μ l of ROX reference dye II; 6.0 μ l of double-distilled H₂O, and 2.0 μ l of plasma DNA from different samples. The reaction was carried out under the following conditions: 95°C for 10 s, followed by 95°C for 5 s and 60°C for 34 s; repeated for 40 cycles. Each sample and DNA standard was analyzed in duplicate, and the mean value was used for quantitation. Only the standard curve, with a coefficient of correlation >0.99, was accepted.

Clinical characteristics

The clinical characteristics of the HCC patients, including tumor size, portal vein cancer embolus, lymph node metastasis and TNM (tumor, nodes, metastases) stage, were collected. The results of laboratory tests for AFP and HBsAg were obtained.

Statistical analysis

The data were analyzed using the Mann-Whitney U-test, the Kruskal-Wallis test and Spearman correlation. A receiver operating characteristic (ROC) curve was plotted to determine the

area under the curve (AUC), sensitivity, and specificity of plasma *hTERT* DNA. Statistical analysis was performed using SPSS 13.0 software.

Results

Methodological evaluation of FQ-PCR

The within-group and between-days coefficients of variation (CV) were 0.86% (n=20) and 1.44% (n=20), respectively. The dissociation curves showed good specificity, with the mean peak at 83.7°C. The negative control was a straight line.

Ten-fold serial dilutions of plasmid *hTERT* DNA from 10^1 - 10^9 copies/ μ l were used to generate a standard curve. The curve showed good linearity from 10^3 - 10^8 copies/ μ l, with a correlation coefficient of 0.997.

Comparison of plasma *hTERT* DNA levels between groups

Plasma *hTERT* DNA levels in the HCC group (mean $4.18 \times 10^4 \pm 4.94 \times 10^4$ copies/ μ l) were significantly higher than in the healthy (mean $1.21 \times 10^4 \pm 6.63 \times 10^3$ copies/ μ l) and HBV groups (mean $1.44 \times 10^4 \pm 6.61 \times 10^3$ copies/ μ l) ($P < 0.001$ and $P = 0.003$, respectively); however, there was no significant difference in levels between the healthy and HBV groups ($P = 0.146$) (Fig. 1).

TABLE 1. Plasma *hTERT* DNA levels in HCC patients

	<i>n</i> (%)	<i>hTERT</i> DNA level ($\bar{x} \pm s$, copies/ μ l)	<i>P</i>
Study population			
Healthy controls	29	$1.21 \times 10^4 \pm 6.63 \times 10^3$	
HBV	21	$1.44 \times 10^4 \pm 6.61 \times 10^3$	0.146 *
HCC	60	$4.18 \times 10^4 \pm 4.94 \times 10^4$	<0.001 *, 0.003 †
Tumor size (cm)			0.013
≤5	17(28)	$2.36 \times 10^4 \pm 2.29 \times 10^4$	
>5 & <10	22(37)	$4.04 \times 10^4 \pm 6.15 \times 10^4$	
≥10	21(35)	$5.79 \times 10^4 \pm 4.72 \times 10^4$	
Portal vein cancer embolus			0.010
Negative (-)	40(67)	$2.83 \times 10^4 \pm 2.50 \times 10^4$	
Positive (+)	20(33)	$6.87 \times 10^4 \pm 7.17 \times 10^4$	
TNM stage			0.029
I-II	27(45)	$2.72 \times 10^4 \pm 2.64 \times 10^4$	0.012*, 0.022 †
III-IV	33(55)	$5.37 \times 10^4 \pm 6.00 \times 10^4$	
Lymph node metastases			0.317
Negative (-)	43(72)	$4.01 \times 10^4 \pm 5.06 \times 10^4$	
Positive (+)	17(28)	$4.62 \times 10^4 \pm 4.72 \times 10^4$	
HBsAg			0.824
Negative (-)	15(25)	$3.37 \times 10^4 \pm 2.98 \times 10^4$	
Positive (+)	45(75)	$4.44 \times 10^4 \pm 5.43 \times 10^4$	
AFP (ng/ml)			0.673
≤20	18(30)	$4.59 \times 10^4 \pm 4.98 \times 10^4$	<0.001*, 0.016 †
>20 & <400	17(28)	$4.17 \times 10^4 \pm 6.68 \times 10^4$	
≥400	25(42)	$3.87 \times 10^4 \pm 3.50 \times 10^4$	

* compared with healthy controls; † compared with HBV patients

Diagnostic efficacy of plasma *hTERT* DNA for HCC

The diagnostic efficacy of plasma *hTERT* DNA as a tumor marker for HCC was determined on the basis of an ROC curve defining the specificity at 95%, which was determined by the distribution in control subjects. The AUC was 0.78 (95% CI: 0.69-0.88). According to the rule of maximum Youden index, 1.87×10^4 copies/ μ l was set as the optimum cutoff value, and the diagnostic sensitivity and specificity for HCC at this value were 64% and 90%, respectively (Fig. 2).

Associations between patient characteristics and plasma *hTERT* DNA

Table 1 lists the plasma *hTERT* DNA levels in HCC patients, together with their clinical characteristics and the results of laboratory tests. There were no significant associations between plasma *hTERT* DNA levels and lymph node metastasis or HBV infection status.

Elevated plasma *hTERT* DNA levels were significantly associated with increasing tumor size ($P=0.013$). Spearman correlation analysis of plasma *hTERT* DNA levels and tumor sizes revealed a significant positive correlation (Spearman correlation=0.382, $P=0.003$).

Higher levels of plasma *hTERT* DNA were found in HCC patients with portal vein cancer embolus, compared with those without portal vein cancer embolus ($6.87 \times 10^4 \pm 7.17 \times 10^4$ copies/ μ l *vs* $2.83 \times 10^4 \pm 2.50 \times 10^4$ copies/ μ l; $P=0.010$). Regarding TNM staging, plasma *hTERT* DNA levels in stage I-II patients were significantly lower than in those with TNM stage III-IV ($P=0.029$); however, they were still higher than in HBV patients and healthy individuals ($P=0.012$ and $P=0.022$, respectively).

There was no significant association between plasma *hTERT* DNA levels and AFP. Plasma *hTERT* DNA levels in HCC patients with serum AFP ≤ 20 ng/ml were significantly

higher than in healthy controls and HBV patients ($P=0.001$ and $P=0.016$, respectively).

Discussion

Diagnostic assays based on blood samples are attractive because of the simplicity of sample collection and the future potential for their automation for clinical use [21]. Accurate analysis of tumor markers in blood from cancer patients has a potentially significant impact on facilitating the screening, diagnosis and monitoring of malignancies. In 2005, Miura *et al.* reported that serum *hTERT* mRNA is a novel and available marker for HCC diagnosis [22]. In fact, serum *hTERT* mRNA, or endogenous RNA component, does not seem to be stable because of the presence of RNase; therefore, circulating DNA is preferable to circulating mRNA. Elevated levels of total DNA in blood have recently been described in patients with malignant tumors, compared with controls, suggesting that circulating DNA may represent a potential new tumor marker [23-28]. Although both plasma and serum samples have been used for analyzing circulating DNA, several studies have shown higher DNA concentrations in serum samples than in matched plasma samples, generated by the lysing of white blood cells *in vitro*. Plasma samples are thus preferable for quantifying circulating DNA [29-31]. Chiu *et al.* found that plasma, obtained by single centrifugation alone, without subsequent centrifugation, contained abundant cellular components that could lead to the presence of aberrantly high total concentrations of plasma DNA [32]. In this study, plasma was therefore centrifuged for an additional 10 min prior to analysis.

The present results showed a strong linear relationship between the Ct values and the logarithm of the DNA copy number ($r^2>0.99$), with very low assay variability (within-run CV 0.86%, between-days CV 1.44%). This demonstrates that the current method is reliable, and can thus provide a suitable quantitative tool for detecting plasma DNA.

Comparing the levels of plasma *hTERT* DNA among different groups, this study found that the median plasma *hTERT* DNA levels in HCC patients were significantly higher than in either HBV patients or healthy individuals. This result is supported by those of Divella *et al.*, who demonstrated that plasma *hTERT* DNA levels in breast cancer patients were significantly higher than in those with benign breast disease and in healthy donors [33]. There was no significant difference in plasma *hTERT* DNA levels between HBV patients and healthy individuals, indicating that plasma *hTERT* DNA is not increased solely because of HBV infection. Sozzi *et al.* [20] and Paci *et al.* [25] reported that plasma *hTERT* DNA in lung cancer patients was higher than that in healthy controls. Further stud-

ies by Sirera *et al.* found that plasma *hTERT* DNA level was an independent poor prognosis marker for mean time to progression and overall survival [34]. Altimari *et al.* took plasma *hTERT* DNA as the representative of free plasma DNA and suggested it as a candidate biomarker for early diagnosis and monitoring of localized prostate cancer [35]. Analysis of the ROC curve in present study revealed a sensitivity of 64% and specificity of 90% at a cutoff value of 1.87×10^4 copies/ μ l, indicating a diagnostic efficacy at least as good as that of AFP [12].

The present study demonstrated a significant positive correlation between plasma *hTERT* DNA and tumor size ($r=0.382$, $P=0.003$), suggesting that larger or more invasive tumors may release more DNA into the plasma, and providing indirect evidence that circulating DNA is derived from tumor tissue. Portal vein cancer embolus is another risk factor for tumor invasion and distant metastases in HCC patients. The present results indicated an increased probability of invasion or distant metastases in HCC patients with high plasma *hTERT* DNA levels, suggesting the need to monitor the progression of HCC by measuring plasma *hTERT* DNA. Tumor staging was associated with plasma *hTERT* DNA, possibly indicating that cells in advanced HCC release more DNA into the plasma when they metastasize or invade hepatic vessels. The present study also found that plasma *hTERT* DNA levels were increased in the early stage of HCC, suggesting their potential usefulness as an early marker. These findings are in agreement with those of previous studies showing that circulating DNA was elevated in inchoate cancer. For example, Sozzi *et al.* found higher levels of plasma DNA in stage Ia patients compared to healthy controls [36]. Flamini *et al.* also found significant differences in serum DNA levels between stage A (Dukes stage) patients with colorectal cancer and healthy individuals [37]. In addition, Miura *et al.* measured *hTERT* mRNA, together with *EGFR* mRNA levels, in serum from patients with lung cancer, and showed that serum *hTERT* mRNA correlated with tumor size, the presence of metastasis, disease recurrence and smoking [38]. These results demonstrate that the presence of circulating nucleic acids has good diagnostic value in the early stage of cancer, and has the potential use for cancer screening and monitoring [39].

AFP is currently widely used as a laboratory marker for diagnosing and monitoring the prognosis of HCC; however, its sensitivity is only 60% at a cutoff value of 20 ng/ml [12]. Although no significant association between plasma *hTERT* DNA levels and serum AFP levels were found in this study, the level of plasma *hTERT* DNA in HCC patients with AFP <20 ng/ml was significantly higher than in HBV patients and healthy individuals. This suggests that plasma *hTERT* DNA

might have the ability to identify HCC patients with AFP levels <20 ng/ml; i.e., plasma *hTERT* DNA could make up for the shortcomings of AFP in diagnosing early HCC. A combination of the two markers would thus increase the diagnostic efficacy for HCC.

In conclusion, increased plasma *hTERT* DNA levels in HCC patients were associated with clinical characteristics and progression of HCC. The results of the present study suggest that plasma *hTERT* DNA may provide a noninvasive complementary tool for diagnosing and monitoring HCC in clinical practice. Large-scale population-based studies are needed to confirm this role. Finally, *hTERT* appears to be relevant to the occurrence and progression of most malignancies, future studies in various cancers are warranted, and plasma *hTERT* DNA has the potential to be a broad tumor marker for common cancers.

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