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Detection of α-Fetoprotein and Glypican-3 mRNAs in the Peripheral Blood of Hepatocellular Carcinoma Patients by Using Multiple FQ-RT-PCR

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This work aimed to investigate the correlation of the expression of α-fetoprotein (AFP) and glypican-3 (GPC3) mRNAs in the peripheral blood with primary hepatocellular carcinoma (HCC) and HCC metastasis by using multiple fluorescence quantitative reverse transcriptase-polymerase chain reaction (FQ-RT-PCR). Peripheral blood samples from 100 patients with HCC were collected. The positive expression rates of AFP mRNA of HCC, hepatitis B, and cirrhosis patients were 56, 5, and 10%, respectively. AFP mRNA was not detected in healthy subjects, hepatic hemangioma, or hepatic metastasis patients' samples. Those of GPC3 mRNA of HCC patients were 76%. GPC3 mRNA was not detected in healthy subjects, hepatitis B, cirrhosis, hepatic hemangioma, or hepatic metastasis patients' samples. In HCC patients' samples, the combined positive rate of AFP and GPC3 mRNA expressions was 81%. The relative expression levels of GPC3 mRNA in the metastasis group and nonmetastasis group were 0.98 ± 0.38 and 0.72 ± 0.26, respectively, and showed significantly different ($P = 0.001$). However, no significant difference was observed in the AFP mRNA expression levels ($P = 0.134$). In conclusion, the sensitivity of HCC diagnosis can be improved by combined detection of AFP and GPC3 mRNA expressions. GPC3 mRNA is HCC-specific, and may indicate HCC metastasis. J. Clin. Lab. Anal. 25:113–117, 2011.

Key words: liver tumor; AFP mRNA; GPC3 mRNA; peripheral blood; fluorescence quantitative reverse transcriptase-polymerase chain reaction

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China. HCC is an occult cancer and is characterized by high malignancy and rapid development. At the time of initiation of diagnosis, most of the HCC patients (two-third of HCC patients) are in the late stages of the disease wherein surgery may not be successful. Therefore, the detection of HCC micrometastasis (hematogenous spread) may be useful for the early and accurate detection of metastasis in the peripheral blood, thereby facilitating prompt surgery or prevention of metastasis after surgery. Thus, the relapse rate can be reduced and patient survival can be improved. At present, α-fetoprotein (AFP) is one of the most important serum markers for the diagnosis of early HCC (1). However, because AFP was not HCC-specific, false-positive expression of AFP mRNA was detected in the peripheral blood of patients with hepatitis and cirrhosis. Moreover, serum AFP was absent in about 30% of HCC patients. Therefore, the significance of AFP mRNA in the detection of HCC micrometastasis is still controversial (2), thereby complicating the early diagnosis of HCC. Glypican-3 (GPC3) is overexpressed in HCC patients, whereas it is underexpressed or not expressed in adult normal tissues, tissues adjacent to cancerous cells, or liver tissues from hepatitis or cirrhosis patients (3,4). GPC3 was also found to show the highest expression rate in the serum AFP-negative HCC patients. In this study, we considered AFP and GPC3 mRNAs as markers for the early diagnosis of

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HCC and detected their levels in the peripheral blood samples from HCC patients by using multiple fluorescence quantitative reverse transcriptase-polymerase chain reaction (FQ-RT-PCR) method. We assessed the significance of using AFP mRNA and GPC-3 mRNA as single marker or double marker in the detection of HCC micrometastasis.

MATERIALS AND METHODS

Materials

We selected 100 HCC patients who were hospitalized in the First Affiliated Hospital of Zhejiang University. All the HCC cases were confirmed using computed tomography (CT) or pathological analysis. The patient group comprised 82 men and 18 women (age, 20–84 years; median age, 55 years). We also selected 20 patients with hepatitis B, 20 with cirrhosis, 20 with hepatic metastasis, and 20 with hepatic hemangioma from the First Affiliated Hospital of Zhejiang University. Healthy subjects were selected from among the persons who participated in the baseline survey of chronic diseases in the Zhejiang community. Fasting anticoagulated venous blood samples were obtained from patients on the day after hospitalization and from the healthy subjects during the physical examination. The anticoagulated blood samples were used for the extraction of total RNA. The threshold value for the measurement of the serum AFP concentration was 400 μg/l (1).

Instruments

We used ABI 7500 Real-Time PCR (Applied Biosystems, Foster City, CA), Thermo biological safety cabinets (Thermo Fisher Scientific, Waltham, MA). and Nanodrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Total RNA Extraction

Total RNA was extracted from 1 ml of the anticoagulated venous blood samples by using the QIAamp RNA Blood Mini kit (Qiagen, Dusseldorf, Germany), and the extracted RNA was stored at −80°C until analysis. The total RNA concentration was determined using the Nanodrop 1000 UV spectrophotometer. The quality of the extracted RNA was analyzed by subjecting the RNA samples to agarose (1%) gel electrophoresis.

Establishment of Multiple Real-Time FQ-RT-PCR Method for the Detection of AFP and GPC3 mRNA

Primers and probes

RT-PCR primers and Taq-man probes with various fluorescent labeling groups were designed using Primer Express software (PE Applied Biosystems, Foster City, CA) on the basis of the AFP and GPC3 DNA and RNA sequences obtained from the gene library. The sequences were showed in Table 1.

β-actin (internal standard): the RT-PCR primers and Taq-man probe of β-actin gene were supplied by Shanghai GeneCore BioTechnologies Co., Ltd. The probe was dye labeled by FAM.

Reaction program

A one-Step RT-PCR Kit (Perfect Real Time, TaKaRa Bio Group, Otsu, Japan) was used for one-step quantitative PCR amplification. The reaction mixture had the following composition: 12.5 μl of 2 × buffer, 0.5 μl of RNase inhibitor, 0.25 μl of moloney murine leukemia virus reverse transcriptase, 0.5 μl of Taq enzyme, 0.5 μl of upstream and downstream primers (AFP, GPC3, and β-actin), 0.3 μl of probe (AFP, GPC3, and β-actin), and 3 μl of RNA template. Double-distilled water was added to make a total volume of 25 μl. Multiple reactions were performed in the same tube by using ABI 7500 fluorescence quantitative PCR analyzer. Each RNA sample was made in duplicate, and blank control without template was included in each reaction plate. For each reaction plate, one regular sample was designated as both reference sample and corrector, which formed the basis for the result analysis. The PCR program was performed under the following conditions: 42°C for 15 min, 95°C for 2 min, and 40 cycles of 95°C for 5 sec, and 54°C for 35 sec.

Cell line

HepG2, a human hepatoma cell line (maintained by the First Affiliated Hospital of Zhejiang University),

TABLE 1. Primers and Probes

Gene	Primers and probes	Sequences	Dye label
AFP	Forward primer	TCGGACACTTATGTATCAGACATGAA	
	Reverse primer	GCCTCCTGTTGGCATATGAAG	
	Probe	TGACTCCAGTAAACCCTGGTGTTGGCC	HEX
GPC3	Forward primer	CAACCTCGGGAACGTTCATT	
	Reverse primer	AAGAAGCACACCACCGAGATG	
	Probe	CCCGCTGAAGCTTCTCACCAGCA	ROX

was used as the positive control for multiple FQ-RT-PCR.

Calculation of relative expression level

The mean expression levels of AFP mRNA and GPC3 mRNA relative to β-actin mRNA level were calculated using the quantitation-comparative CT (ΔΔ CT) method on the ABI 7500 fluorescence quantitative PCR analyzer. After correction with the corrector sample, the relative quantitation values of the expression levels were used for the statistical analysis.

Statistical Analysis

All the data on the expression levels were processed using SPSS 13.0 software. The measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Comparisons of the measurement data were analyzed using the t test. Comparisons of the enumeration data were analyzed using the χ^2 test.

RESULTS

RNA Purity and Concentration

Gel electrophoresis of the total RNA samples yielded two bands corresponding to 18S and 28S RNA. The 28S RNA band showed twice the intensity of the 18S band, thereby confirming the integrity of the total RNA. The A_{260}/A_{280} values of the RNA samples ranged from 1.9 to 2.1.

Sensitivity of Multiple FQ-RT-PCR in the Detection of Peripheral Blood AFP and GPC3 mRNA

After subculturing the HepG2 cells, we prepared a single cell suspension with a concentration of 1×10^4 cells (stock). The stock suspension was serially diluted ten fold and added to the tubes containing 1 ml of whole blood from healthy subjects. The tubes contained 10^3,10^2, 10, 1, and 0 HepG2 cells. The total RNA of the HepG2 cells was extracted, and multiple FQ-RT-PCR was then performed to detect the AFP mRNA and GPC3 mRNA expression levels. Our results indicated that the lowest levels of AFP mRNA and GPC3 mRNA were observed in the sample containing ten HepG2 cells per ml of whole blood. The results of the typical positive samples are shown in Figure 1.

Specificity of Multiple FQ-RT-PCR in the Detection of AFP and GPC3 mRNAs

AFP mRNA expression

AFP mRNA was detected in the peripheral blood of 56 HCC patients (56/100; positive expression rate, 56%), 1 hepatitis B patient (1/20; positive expression rate, 5%),

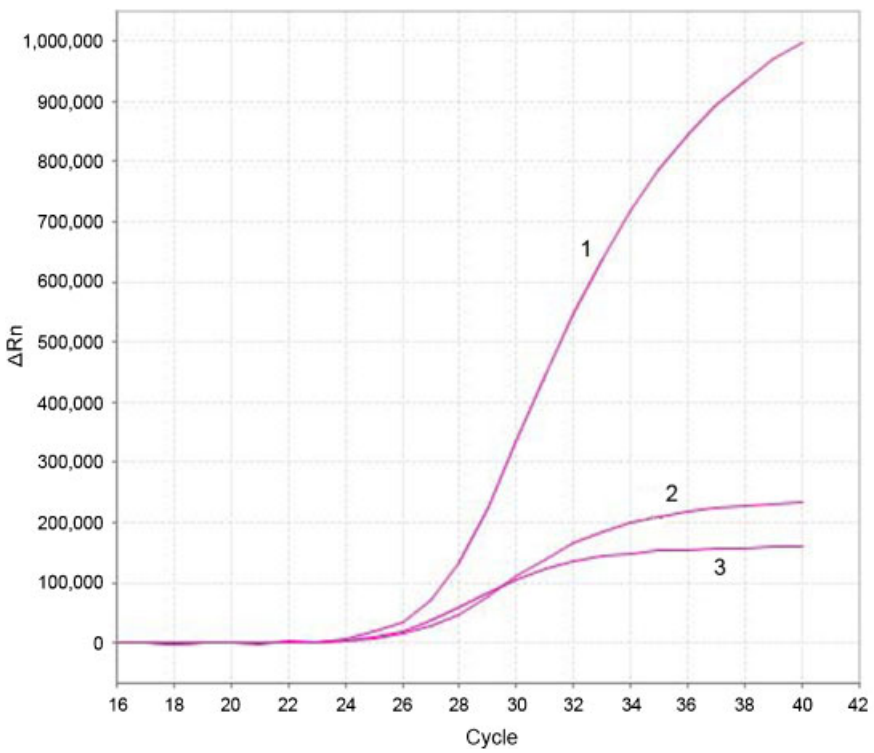

Fig. 1. The positive expression curves of AFP mRNA and GPC3 mRNA in peripheral blood. Curve 1 represents the GPC3 gene; Curve 2, AFP gene; and Curve 3, β-actin gene. We used Applied Biosystems 7500 Real-Time PCR System.

and 2 cirrhosis patients (2/20; positive expression rate, 10%). AFP mRNA was not expressed in the peripheral blood of healthy persons, patients with hepatic hemangioma, and patients with hepatic metastases.

GPC3 mRNA expression

GPC3 mRNA was detected in the peripheral blood of 76 HCC patients (76/100; positive expression rate, 76%). GPC3 mRNA was not expressed in the peripheral blood of healthy persons, hepatitis B patients, cirrhosis patients, patients with hepatic hemangioma, and patients with hepatic metastases.

Correlation Analysis of Metastasis and the Expressions of AFP and GPC3 mRNAs in the Peripheral Blood of HCC Patients

The expression of either AFP mRNA or GPC3 mRNA or both mRNAs in the peripheral blood was observed in 81 patients. Metastasis was observed in 42 HCC patients (metastasis group). There were no significant differences in the metastasis rate between the AFP mRNA-positive group and AFP mRNA-negative group, the GPC3 mRNA-positive group and GPC3 mRNA-negative group, or the combined detection-positive and combined detection-negative group (Table 2).

Comparison of AFP and GPC3 mRNA Expression Levels Between the Metastasis and the Nonmetastasis Groups

AFP mRNA was expressed in 20 patients of the metastasis group ($n = 42$) and 36 patients of the nonmetastasis group ($n = 58$). The AFP mRNA expression levels in the metastasis and nonmetastasis groups were 0.73 ± 0.26 and 0.69 ± 0.20, respectively; no significant difference was observed in the AFP mRNA expression levels between these two groups ($P = 0.134$). GPC3 mRNA was expressed in 31 patients of the metastasis group and 45 patients of the nonmetastasis group. The GPC3 mRNA expression levels in the metastasis and nonmetastasis groups were significantly different (0.98 ± 0.38 vs. 0.72 ± 0.26; $P = 0.001$, Table 3).

TABLE 2. Correlation Analysis of HCC Metastasis and the Expressions of AFP and GPC3 mRNAs in the Peripheral Blood of HCC Patients

Index	Metastasis	Nonmetastasis
AFP mRNA (+)	20	36
AFP mRNA (−)	22	22
P value	0.151	
GPC3 mRNA (+)	31	45
GPC3 mRNA (−)	11	13
P value	0.663	
Combined detection (+)	34	47
Combined detection (−)	8	11
P value	0.992	

Comparison of the Positive Expression Rates of AFP mRNA and GPC3 mRNA in the Serum AFP-Negative Group

Serum AFP was lower than the threshold level (serum AFP level $< 400\,\mu g/l$) in 53 HCC patients. Of these 53 HCC patients, 13 and 33 patients showed positive expressions of AFP mRNA and GPC3 mRNA, respectively. The positive expression rates of AFP mRNA and GPC3 mRNA ($P = 0.000$) in these 53 patients were significantly different.

DISCUSSION

Primary liver cancer cells can grow rapidly and metastasis often occurs in the early stages of HCC. Therefore, the detection of liver micrometastasis has considerable clinical significance in the early detection and treatment of liver cancer. Normal blood cells do not show mRNA expression; therefore, the detection of the mRNA of the tumor-specific marker in the peripheral blood indicates that tumor cells may have detached from the primary tumor and metastasized into the blood. Matsumura et al. (5) were the first to detect AFP mRNA in the peripheral blood of HCC patients; the presence of AFP mRNA may indicate the micrometastasis of tumor cells in the peripheral blood. The detection of serum GPC3 can increase the sensitivity of the early diagnosis of liver cancer (6,7). In this study, we used multiple FQ-RT-PCR to detect the AFP and GPC3 mRNAs. The positive expression rates of AFP mRNA and GPC3 mRNA in the peripheral blood of the HCC patients were 56 and 76%, respectively, which was consistent with the results obtained in the previous reports (5,8). The combined detection of AFP and GPC3 mRNA improved the sensitivity of HCC diagnosis, and the positive expression rate of this combined detection approach was 81%. The positive expression rates of AFP mRNA in hepatitis B and liver cirrhosis patients were 5 and 10%, respectively; this can be

TABLE 3. Comparison of the Expression Levels of AFP and GPC3 mRNAs Between the Metastasis and the Nonmetastasis Groups (Expressed as Average of Relative Expression Level $\pm s$)

Index	Metastasis	Nonmetastasis
AFP mRNA (+)	20, 0.73 ± 0.26	36, 0.69 ± 0.20
P value	0.134	
GPC3 mRNA (+)	31, 0.98 ± 0.38	45, 0.72 ± 0.26
P value	0.001	

attributed to the hepatitis exacerbation caused by the shedding of heterogeneous hepatocytes into the peripheral blood. These results suggest that AFP mRNA is hepatocyte-specific but not liver cancer-specific. GPC3 mRNA was expressed in the peripheral blood of HCC patients, but not in that of healthy persons, hepatitis B patients, liver cirrhosis patients, patients with hepatic hemangioma, or patients with hepatic metastasis, thereby indicating that GPC3 mRNA is HCC-specific.

In HCC patients, the metastasis rates in the peripheral blood of AFP-positive group and AFP-negative group, GPC3-positive group and GPC3-negative group, and combined detection-positive group and combined detection-negative group were 35.7 and 50%, 40.8 and 45.8%, and 42.0 and 42.1%, respectively. No significant differences were observed between the positive and negative groups, thereby suggesting that detection of AFP mRNA, GPC3 mRNA, or combined detection of both mRNAs in the peripheral blood had little significance in the diagnosis of HCC metastasis. The relative expression level of AFP mRNA in the metastasis group was not significantly different from that in the nonmetastasis group ($P = 0.134$), but a significant difference was observed in the relative expression levels of GPC3 mRNA between the metastasis and the nonmetastasis groups ($P = 0.001$). Therefore, GPC3 is not only HCC-specific, but may also be a sensitive indicator of HCC metastasis.

The serum AFP level, which is the traditional marker of liver cancer, plays an important role in the early diagnosis of HCC and the monitoring of treatment efficacy, tumor recurrence, and metastasis. In this study, the positive expression rate of GPC3 mRNA in the serum AFP-negative HCC samples was 62.3% (33/53). However, this value is lower than that (73.17%) reported by Guanghui Ding et al. (9), which can be attributed to the different sample types. The positive expression rate of AFP mRNA was 24.5% (13/53), which was significantly lower than that of GPC3 mRNA ($P = 0.000$). Our results showed that GPC3 mRNA showed better complementarity for serum AFP than for AFP mRNA. The detection of GPC3 mRNA in the peripheral blood may be considered as an auxiliary diagnosis in serum AFP-negative HCC patients to overcome the inability of the serum AFP levels to reflect HCC metastasis.

The low numbers of tumor cells, which occur due to metastasis and recurrence of HCC, in the peripheral blood, cannot be easily detected using conventional cell morphological examination, but they can be detected using FQ-RT-PCR technology. FQ-RT-PCR was chosen over conventional RT-PCR because this method uses double-labeled fluorescent probes to improve the sensitivity and specificity of detection, thereby allowing early detection of cancer micrometastasis. In multiple real-time FQ-RT-PCR, multiple pairs of primers and probes with different fluorescent labeling groups are added to the same RT-PCR system, multiple target genes are amplified in the same tube, and the expression of various target genes is detected in real-time by continuous monitoring of the signal intensity changes of corresponding fluorescence. Multiple real-time FQ-RT-PCR affords many advantages over single FQ-RT-PCR. First, the approach eliminates the impact of different interference factors in different reaction systems on the amplification efficiency. Second, the effects of sampling error on the quantitative results in different reaction tubes are avoided. Third, this technology is easy to operate. Fourth, it requires less sample, time, and effort. Finally, the method is economical and can be performed with low risks of contamination. Inspite of these advantages, very few studies have reported the application of multiple FQ-RT-PCR in the detection of AFP mRNA, GPC3 mRNA, and other liver micro-metastasis-specific markers in the peripheral blood. In this study, we established multiple FQ-RT-PCR method on the basis of Taq-man probe technology. We used β-actin as an internal standard to obtain more accurate measurements of the AFP and GPC3 mRNAs. We have also obtained repeated measurements from various samples and showed that this method has good specificity and sensitivity by repeatedly measuring various samples. However, as we studied a limited number of cases, our results and conclusions should be validated by further studies.

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