

Diagnostic and monitoring values of circulating AFP-mRNA and HS-GGT for hepatocellular carcinoma*

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Received: 21 August 2014 / Revised: 1 September 2014 / Accepted: 25 September 2014

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Abstract *Objective:* Early finding of hepatocellular carcinoma (HCC) and monitoring of its metastasis are of the utmost importance. The objectives of this study were to investigate the values of circulating hepatoma-specific gamma-glutamyl transferase (HS-GGT) fraction and α -fetoprotein mRNA (AFP-mRNA) from peripheral blood mononuclear cells (PBMCs) in diagnosis or monitoring metastasis of HCC. *Methods:* Total RNA was extracted from hepatomas or PBMCs of patients, synthesized to AFP-cDNA through random primers and reverse transcriptase, amplified by using a nested PCR assay, and confirmed by sequencing. Simultaneity, the HS-GGT activities were quantitatively determined in sera of patients. The comprehensive assessments of two markers were investigated in HCC patients. *Results:* The amplified fragments of AFP-mRNA were identical to original designed ones (159 bp) and confirmed by sequencing. The lowest sensitivity was 2 ng/L of total RNA. The incidences of AFP-mRNA and HS-GGT were 60.4% (113 of 187) and 84.5% (158 of 187) in the HCC group, and their significantly higher ($P < 0.01$) than that in any of other groups. The high frequencies of HS-GGT and AFP-mRNA were 78.2% and 56.4% in the HCC patients with AFP < 50 ng/mL. All positive of AFP-mRNA was found in HCC patients with extrahepatic metastasis. No significant correlation was found between tumor size and AFP-mRNA or HS-GGT. The total incidence of combined AFP-mRNA and HS-GGT detection was 93.6% for HCC diagnosis. *Conclusion:* HS-GGT and AFP-mRNA are specific markers for HCC diagnosis or differentiation, especially the former for early finding and the later for monitoring metastasis of HCC.

Key words hepatocellular carcinoma (HCC); AFP-mRNA; hepatoma-specific gamma-glutamyl transferase; diagnosis; metastasis

Hepatocellular carcinoma (HCC) is the 3rd deadliest and 5th most common cancer worldwide [1], it ranks the second incidence in China among all malignancies, and its mortality is almost equal to its morbidity [2]. Carcinogenesis of HCC is a multi-factor, multi-step and complex process, most of which is associated with a background of chronic and persistent infection of HBV and HCV [3–5], along with alcohol and aflatoxin B1 intake are widely recognized etiological agents in HCC [6–7]. However, the underlying mechanisms that lead to malignant transformation of infected cells remain unclear. Most of HCC patients died quickly because of the rapid tumor progression, and

hepatic resection or transplantation is the only potential curative treatment for HCC patients [8–9]. Although the HCC mortality has significantly decreased with development of surgical technique, about 60%–100% patients suffered from HCC recurrence ultimately even after curative resection that limits the survival of HCC patients [10–11]. The most urgent needs are to find sensitive markers for early diagnosis and monitoring postoperative recurrence of HCC, and guide us to take adequate treatment for HCC patients [12].

HCC has many characteristics, such as infiltrating growth, metastasis in early stage, high-grade malignancy, and poorly therapeutic efficacy. Although total AFP is a useful marker for HCC diagnosis, with false-negative or false-positive rate alone may be as high as 30%–40% [13–14], especially for early diagnosis or small size HCC (< 3 cm). However, the analysis of either genetic markers of HCC or tumor specific-protein can monitor carcinogenesis of hepatocytes and diagnose HCC at early stage [15]. If hepa-

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* Supported by grants from the National Natural Science Foundation of China (No. 81200634), the Projects of Jiangsu Medical Science (No. WSW-011, HK201102, BL2012053), the Qinglan Project of Jiangsu higher Education, and the International S&T Cooperation Program of China (No. 2013DFA32150).

toocyte-specific mRNAs are detected in circulating blood, it is possible to infer the presence of circulating, presumably malignant cells and predict the likelihood of haematogenous metastasis [16]. Hepatic γ -glutamyl transferase (GGT) with alteration of methylation status is re-expressed during HCC development [17,5]. Hepatoma specific GGT (HS-GGT, including I, II, and II') is a part of total GGT activity that was only found in sera of HCC patients, and has been confirmed a useful specific HCC marker and its analysis may improve the specificity and sensitivity of HCC diagnosis [18]. In this study, the clinical values of circulating HS-GGT activities by quantitative detection method and AFP-mRNA from peripheral blood nuclear cells (PBMCs) by reverse-transcriptase polymerase chain reaction (RT-PCR) were investigated as tumor markers in diagnosis, differential diagnosis, and haematogenous metastasis of HCC.

Patients and methods

Patients

We studied 187 patients with HCC, 39 cases with acute hepatitis (AH), 92 with chronic hepatitis (CH), 91 with liver cirrhosis (LC), 33 with extra-hepatic tumors (including 5 cases with cholangiocarcinoma, 7 with carcinoma of pancreas, 7 with gastric cancer, 8 with colonic carcinoma and 5 with breast carcinoma) and 60 healthy volunteers as a control. Five mL of blood was collected in heparinized tubes and centrifuged within 2 h for HS-GGT. At the same time, PBMCs were isolated from lymphocytes by density gradient centrifugation. After being rinsed twice with normal saline, the nuclear cells were counted and stored at -85°C . The vein blood were collected with trisodium citrate anticoagulant and stored on ice. The diagnosis of HCC and viral hepatitis was based on the criteria proposed at Chinese Collaborative Cancer Research Group and the Beijing Chinese National Viral Hepatitis Meeting [19], respectively.

Hepatoma tissues

Human hepatoma and its noncancerous tissues were obtained from 30 patients who underwent operations for liver cancers at the Affiliated Hospital of Nantong University. The livers were immediately frozen in liquid nitrogen and kept at -85°C until required. The patients included 25 males and 5 females, age ranged from 28 to 74 years. The sizes of liver cancers were 21 cases less than 3 cm and 9 cases more than 3 cm. Serum AFP levels were 18 cases with > 50 ng/mL and 12 with < 50 ng/mL. Pathologic examination of the livers with hematoxylin and eosin staining showed that all cancerous tissues were highly differentiated HCC; and the noncancerous tissues were cirrhosis in 21 cases, chronic hepatitis in 9 cases, and atypical hyperplasia in 11 cases.

Separation of PBMCs and extraction of total RNA

Five millilitre of anticoagulant blood were collected and 2.5 mL of Ficoll added to each sample, after centrifugation at 2000 r/min for 20 min, the PBMCs were collected from the Ficoll/ plasma interface. Then washed three times in normal saline and pelleted by using low-speed centrifugation, and were collected in 2×10^5 /tube then stored at -85°C for total RNA preparation. The cells or 50 mg of each liver tissue (HCC, and their self-control non- cancerous tissues) was homogenized with a Polytron homogenizer after the addition of 1.0 mL of TRIZol reagent (Promega, USA). Then 0.2 mL of chloroform to tubes, mixed by vortex mixing for 15 s, and put at -20°C for 5 min, and centrifuged at 12000 r/min for 15 min at 4°C . The supernatants were collected, and added equivalent isopropanol to a new tube and mixed gently, put at -20°C for 15 min, and then centrifuged at 12000 r/min for 15 min at 4°C . The supernatants were removed, washed the RNA pellets twice with 0.5 mL of 75% ethanol, mixed and centrifuged at 8000 r/min for 8 min at 4°C . The RNA pellets were air dried 5 min at room temperature and reconstituted in 20 μl of RNase- free DEPC water and incubated at 60°C for 10 min. The purity and concentration of the RNA was estimated from the ratio of absorbance at 260 to 280, and RNA samples were kept frozen at -85°C until required.

Synthesis of AFP-cDNA and Nested-PCR amplification

Two sets of primers were designed according to AFP sequence [20] and synthesized with synthesizer (Shanghai Institute of Cell Biology, Chinese Academy Sciences). The sequences of the two external primer pairs used for the initial PCR amplification were AFP-1 (sense), 5'-AAC TAGCAACCATGAAGTGG-3' (nt 37-56) and AFP-2 (antisense), 5'-CATGGCAAAGTTCTTCCAGA-3' (nt 329-358). The sequences of the two internal primer pairs used for the second PCR amplification were AFP-3 (sense), 5'-CCAATGTA CTGCAGAGATAAG-3' (nt 152-172) and AFP-4 (antisense), 5'-TCTAAACACCCTGA AGACTG-3' (nt 291-310). For synthesis of cDNA, 2 μg of total RNAs was denatured in the presence of random hexamers (100 pmol/L, Promega, USA) and reverse-transcriptase (GIBCO, BRL) at 23°C for 10 min, 42°C for 60 min and 95°C for 10 min, then on ice for 5 min, and stored at -20°C for PCR amplification. The PCR amplification consisted of initial denaturation at 94°C for 5 min, followed by 94°C for 25 s, 55°C for 30 s, and 72°C for 90 s for 30 cycles. The final product of nested PCR was 159 bp. Human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genome [21] was used for control. Primer sequence for GAPDH was GAPDH-1 (sense), 5'-ACCACAGTCCATGCCATCA C-3' (nt 601-620) and GAPDH-2 (antisense), 5'-TCCAC

CACCCTGTTGCTGTA-3' (nt 1033–1052), and the PCR product was 452 bp. All PCR products were electrophoresed on 2% agarose gels with ethidium bromide staining. The fragment sizes were evaluated using PCR markers (Promega, USA) as molecular weight standards.

Sequencing of PCR products

AFP amplified products (159 bp) from PBMCs or hepatomas were purified with the Montage PCR centrifugal filter devices (Millipore, USA) according to the instruction of protocol. One microgram DNA was used for preparation of sequencing reaction and directly sequenced using the MegaBACE DNA analysis system in MegaBACE DNA sequencer with the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, USA), following their protocol. The sequences were edited using the MegaBACE Sequence Analyzer Version 3.0 program (Amersham Biosciences, USA) and aligned with the amplified AFP fragments.

Detection of HS-GGT activities

Blood was drawn from 442 inpatients, including HCC patients, hospitalized at the Affiliated Hospital of Nantong University (Nantong, China) and 60 healthy persons from Nantong Central Blood Bank as normal controls. Blood was allowed to clot at room temperature and serum was obtained by centrifugation at 2000 g for 10 min. Total GGT activities and circulating HS-GGT were performed within 1 day of blood collection. Total GGT and HS-GGT activities (IU/L) were quantitatively measured according to the previous method as described [5]. Total GGT activity exceeding 30 IU/L, and HS-GGT concentration more than 5.5 U/L was interpreted as an abnormal result.

Statistical analysis

All patients were divided into 6 groups: HCC, acute hepatitis, chronic hepatitis, cirrhosis, extrahepatic tumor, and normal subjects. Human hepatoma tissues were divided into two groups: cancerous and noncancerous tissues. Results are expressed as mean ± standard deviation (SD). Differences between different groups were assessed

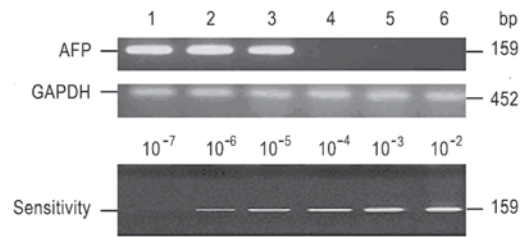


Fig. 1 Amplification of AFP-mRNA from circulating blood samples of HCC and patients with other diseases. AFP-mRNA was synthesized to AFP-cDNA with random hexamers and moloney murine leukemia virus reverse-transcriptase, and detected with different primer pairs by nested PCR (159 bp). The positive fragments of AFP genome were found distinctly in peripheral blood and hepatoma tissues from HCC patients. The amplified fragments (452 bp) of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) genome from peripheral blood as controls. The amplified fragments of AFP genomes in circulating blood samples of HCC patients (1–3), patients with chronic hepatitis (4), patients with acute hepatitis (5), and patients with extra-hepatic tumors (6). The dilution experiment revealed that the lowest sensitivity of the assay was 2 ng/L of total RNA from hepatoma tissues, using total RNA with 10⁻²–10⁻⁸ fold dilution and then amplified by nested PCR.

by the Student's *t* test or the χ^2 test. *P* < 0.05 was considered to be significant.

Results

Amplification of AFP-mRNA and sensitivity

The amplification of AFP genome fragments from HCC tissues and PMBCs by using a nested PCR assay is shown in Fig. 1. The sizes of amplified fragments were identical to the original designed ones, which were 312 bp in single-step PCR, and 159 bp in nested-PCR (Fig. 1). Difference between single-step PCR and nested PCR for amplified AFP-mRNA were compared in 120 peripheral blood samples. The positive frequency of AFP-mRNA was 5.8% (7 of 120) in single- step PCR and 56.7% (68 of 120) in nested-PCR, the later incidence of nested PCR was higher significantly than that in the former one (*P* < 0.05). Total RNA (2 mg/L) extracted from hepatoma tissues were diluted 10⁻²–10⁻⁸ times. The lowest sensitivity

Table 1 Comparative analysis of AFP-mRNA from peripheral blood nuclear cells with serum total AFP level in diagnosis of HCC

Group	n	Total AFP (ng/mL)				AFP-mRNA		
		Mean ± SD	> 50		PBMCs × 10 ⁴ /μL	Positive		
			n	%		n	%	
HCC	187	1523 ± 1990	128	68.5	0.5–510	113	60.4	
Acute hepatitis	39	41 ± 65*	13	33.3	15–520	0*	0	
Chronic hepatitis	92	86 ± 105*	32	34.8	3.0–120	5*	5.4	
Liver cirrhosis	91	196 ± 179*	34	37.4	5.6–498	15*	16.5	
Extrahepatic tumor	33	60 ± 105*	9	27.3	1.5–340	0*	0	
Normal control	60	8 ± 5.2*	0	0	51–1520	0*	0	

* *P* < 0.001 vs. HCC group. PBMCs: peripheral blood nuclear cells; HCC: hepatocellular carcinoma; SD: standard deviation

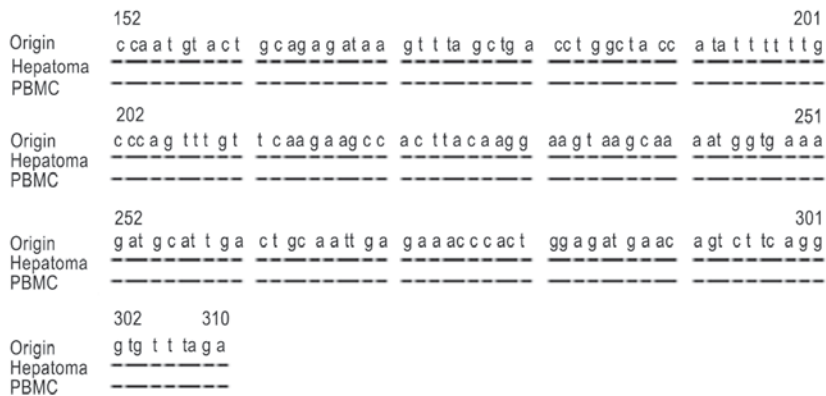


Fig. 2 Alignment of nucleotide sequences of the amplified fragments of AFP genome from hepatoma tissues or PMBCs in HCC patients. Origin: the cited sequence (159 bp, nt 152–310) of human AFP genome; Hepatoma: the amplification fragment of AFP genome from hepatoma tissue; PMBCs: the amplified fragment of peripheral blood AFP genome from patient with hepatocellular carcinoma

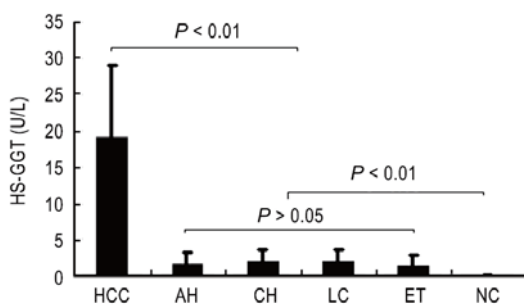


Fig. 3 The diagnostic values of circulating hepatoma-specific gamma-glutamyl transferase activities in sera of patients with different liver diseases or extrahepatic tumors. HS-GGT: serum hepatoma-specific gamma-glutamyl transferase; HCC: patients with hepatocellular carcinoma; AH: patients with acute hepatitis; CH: patients with chronic hepatitis; LC: patients with liver cirrhosis; ET: patients with extrahepatic tumor

of the assay was 2 ng/L of total RNA (Fig. 1) and showed distinctly positive fragments from hepatomas or PMBCs of HCC patients.

Detection of AFP-mRNA and sequence analysis

The total RNA levels were investigated in HCC and their non-cancerous tissues, with 17.90 ± 27.71 μ g/mg wet liver in HCC and significantly lower ($P < 0.01$) than that in their non-cancerous tissues (41.41 ± 50.33 μ g/mg wet liver). However, the incidence of AFP gene in HCC (30 of 30, 100%) was significantly higher ($P < 0.01$) than in their non-cancerous tissues (18 of 30, 60%). The quantitative AFP levels and AFP-mRNA from patients with different diseases and controls are shown in Table 1. The AFP incidence in HCC group was 68.5% and significantly higher than that in the others with benign liver diseases (33%–37%) or extrahepatic tumors (27%). The positive rate of AFP-mRNA was 60.4% in HCC. There were no false-positives among other patients or controls. In patients with chronic hepatitis, 5.4% (5 of 92) had positive AFP-mRNA as well as 16.5% (15 of 91) with cirrhosis. By sequencing, the nucleotide homologies of AFP amplified fragments from hepatomas or PMBCs were identical to

the cited one (Fig. 2) [20].

Abnormality of HS-GGT Activity in HCC patients

Serum HS-GGT activities in patients with different diseases are shown in Fig. 3. The quantitative HS-GGT activity showed that only HS-GGT was overexpressed in HCC and much lower in patients with benign liver diseases or extrahepatic tumors. Significant differences ($P < 0.01$) were observed between the HCC group and each of the study groups in HS-GGT. The average HS-GGT activity in the HCC group was 9–12 times higher ($P < 0.01$) than that in the acute hepatitis, chronic hepatitis, cirrhosis, and extrahepatic tumor groups, and 160 times higher ($P < 0.01$) than that in the normal subjects. In the current study, HS-GGT activity exceeding 5.5 IU/L was taken as a positive result; 84.5% (158 of 187) of HCC patients had a positive HS-GGT result (sensitivity 84.5%). There were no false-positives among patients with acute hepatitis or extrahepatic tumor or among normal subjects. The HS-GGT activities at 2 weeks after postoperative patients had decreased to 0.3–6.8 IU/L in sera of 30 HCC patients whose HS-GGT levels were over 5.5 IU/L before surgical operation.

Complementary diagnostic values of HS-GGT and AFP-mRNA

The relationship between two markers and different AFP levels was analyzed in HCC patients. The abnormal frequencies of HS-GGT and AFP-mRNA were 78.2% and 56.4% in 0–50 ng/mL group, 85.7% and 54.2% in 51–499 ng/mL group, 88.0% and 60.0% in 500–999 ng/mL group, and 87.5% and 66.7% in more than 1000 ng/mL group, respectively. The higher positive rates for HCC diagnosis were found in AFP < 50 ng/mL group, and no significant relation was found between two markers. Although the incidence of AFP-mRNA only was lower than that of HS-GGT. However, the positive result of AFP-mRNA could be found in HCC patients with lower HS-GGT activity. A comparative analysis of HS-GGT and AFP-mRNA markers for HCC diagnosis is shown in Table 2. The total incidence of combined two markers was higher up to 93.6%

Table 2 Complementary diagnostic values of combined AFP-mRNA and HS-GGT detection in patients with liver diseases or extrahepatic tumors

Group	<i>n</i>	Positive		Negative		<i>P</i> *
		<i>n</i>	%	<i>n</i>	%	
HCC	187	175	93.6	12	6.4	–
Acute hepatitis	39	0	0	39	100	< 0.001
Chronic hepatitis	92	5	5.4	87	94.6	< 0.001
Liver cirrhosis	91	15	16.5	76	83.5	< 0.001
Extra-hepatic tumor	33	0	0	33	100	< 0.001
Normal control	60	0	0	60	100	< 0.001

* *P*: each group vs. HCC group. HCC: hepatocellular carcinoma; HS-GGT: serum hepatoma-specific gamma-glutamyl transferase

Table 3 Relationship between circulating HS-GGT or AFP mRNA and HCC stage or metastasis

Group	<i>n</i>	HS-GGT		<i>P</i>	AFP-mRNA		<i>P</i>
		<i>n</i>	%		<i>n</i>	%	
HCC							
Stage I–II	115	94	81.7	> 0.05	50	43.5	< 0.01
Stage III–IV	72	64	88.9		63	87.5	
Tumor size (cm)							
< 3	58	46	79.3	> 0.05	20	34.5	< 0.01
≥ 3	129	112	86.8		93	72.1	
Tumor numbers							
Single	125	105	84.0	> 0.05	75	60.0	> 0.05
Multiple	62	53	85.5		38	61.3	
Extrahepatic metastasis							
With	97	84	86.6	> 0.05	97	100	< 0.01
Without	90	74	82.2		16	17.8	
HBV infection							
With	165	139	84.2	> 0.05	99	60.0	> 0.05
Without	22	19	86.4		14	63.6	
Total AFP level (ng/mL)							
< 50	55	43	78.2	> 0.05	31	56.4	> 0.05
> 50	132	115	87.1		82	62.1	

(175 of 187) for HCC diagnosis.

Clinical characteristics of HS-GGT and AFP-mRNA

According to HCC stage, tumor size and number, extrahepatic metastasis, and HBV infection, the clinical characteristics of HS-GGT and AFP-mRNA are shown in Table 3. No positive correlation was found between HS-GGT marker and above clinical characteristics of HCC ($P > 0.05$), and between AFP-mRNA marker and tumor number or HBV infection ($P > 0.05$). However, significant difference was found between AFP-mRNA and HCC stage, or tumor size and HCC with extrahepatic metastasis ($P < 0.01$), suggesting that the positive AFP-mRNA predicts a higher level of vessel invasiveness and higher metastasis tendency of liver tumors.

Discussion

Major risk factors for HCC in these areas are exposure to aflatoxin B1 and infection by HBV or HCV [22–24], and

its prognosis is poor and early diagnosis is of the utmost importance. HCC patient has putatively accepted that surgery, including curative resection and liver transplantation, is the only hope for curing this malignant disease. With the advance in surgical techniques, the surgical mortality of HCC has decreased significantly in the past decades. The 5-year survival rate after curative resection of HCC has risen from 16.0% up to 48.6%. But unfortunately, even after curative resection, approximately from 40% to 100% HCC patients will suffer from tumor recurrence [25–26]. However, it is of great importance to find valuable early diagnostic and prognostic markers, which could predict the development and metastases of HCC. With the help of those markers, doctors could perform proper postoperative treatment on those who are at a high risk of cancer recurrence.

AFP-mRNA is a predictor for HCC metastasis and recurrence, but the results are rather controversial. However, the current data revealed that the HS-GGT and AFP mRNA as tumor markers for diagnosing HCC and predicting the distal metastasis of HCC. The high GGT activity

observed at the fetal stage decreases rapidly at birth and is barely detectable in the adult. It is often re-expressed in adult hepatocarcinogenesis, because the gene encoding human GGT is highly expressed in fetal liver or in adult liver-bearing cancers related to the hypomethylation status of GGT gene CCGG sites, 5 but not in the normal adult liver. Experimental studies have shown that GGT is strikingly activated during the course of tumorigenesis in rat hepatoma models, and that it is significantly increased in hepatocytes at the precancerous stage and in HCC [17]. In this study, an elevated HS-GGT activity could occur only in HCC patients, and not in others with benign liver diseases or non-hepatic tumors, and it may improve the specificity of HCC diagnosis and is useful in the differential diagnosis of benign and malignant liver diseases, suggesting that the complementary diagnostic values of HS-GGT and AFP-mRNA simultaneous assay can increase the diagnostic accuracy of HCC.

Both HS-GGT and AFP-mRNA were analyzed as potential HCC molecular biomarkers [27–28]. Although pathological parameters such as tumor size, tumor stage, and tumor metastasis were analyzed, there was marked difference of HG-GGT and AFP-mRNA markers among the groups. For HCC diagnosis, the higher positive rate of HS-GGT was found in any of groups and no correlated with these clinical parameters between different groups in HCC patients, especially for diagnosis of small-size HCC or early finding of HCC with stage-I and -II. Conversely, the higher positive rate of AFP-mRNA was strongly associated with advanced-stage HCC, large-size HCC and HCC with distal metastasis. HS-GGT is a biomarker not only for AFP-mRNA positive HCC but also for AFP-mRNA negative HCC, and no significant relation was found between them. On the other hand, AFP-mRNA positivity was detected in some cases with negative HS-GGT. Furthermore, serum HS-GGT activity could decrease to lower level or normal reference after operation. However, although the positive rate of AFP-mRNA was not so higher in the early diagnosis of patients with small-size HCC, it could monitor the distal mini-metastasis of HCC or the postoperative recurrence of HCC.

HCC exhibit numerous genetic abnormalities as well as epigenetic alterations including modulation of DNA methylation. Molecular factors involved in the process of HCC development and metastasis. Recent findings from several laboratories have implicated constitutive activation of the transcription factor NF- κ B as one of the early key events involved in neoplastic progression of the liver. Further studies will permit us to analyze mechanism of human hepatocarcinogenesis and pay attention to these areas. However, the combination of the pathological features and some of the biomarkers with high sensitivity and specificity for early diagnosis and metastasis of HCC seem to be more practical up to now [29–30].

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-014-0049-5

Cite this article as: Wang L, Yao M, Yao Y, *et al.* Diagnostic and monitoring values of circulating AFP mRNA and HS-GGT for hepatocellular carcinoma. *Chinese-German J Clin Oncol*, 2014, 13: 457–463.