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ORIGINAL ARTICLE

The Value of GPC3 and GP73 in Clinical Diagnosis of Hepatocellular Carcinoma

Ji-Sheng Jing [1, *], Wei Ye [2, *], Ying-Kui Jiang [3], Jie Ma [4], Meng-Qi Zhu [1], Jiu-Ming Ma [1], Hua Zhou [1], La-Qing Yu [1], Yong-Feng Yang [2], Shun-Cai Wang [1]

[*] *Ji-Sheng Jing and Wei Ye contributed equally to this work*
[1] *Department of Infectious Diseases, Jurong People's Hospital, Jiangsu University, Zhenjiang, China*
[2] *The Second Affiliated Hospital of Southeast University, Nanjing, China*
[3] *Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China*
[4] *School of Medicine, Jiangsu University, Zhenjiang, China*

SUMMARY

Background: **The incidence of hepatocellular carcinoma (HCC) has increased over the past decades in China. Current screening methods of HCC such as detection of α-fetoprotein (AFP) combined with liver ultrasonography remain unsatisfactory. Many HCC patients have already missed the optimal treatment period when diagnosed. Our study aimed to evaluate the value of Glypican 3 (GPC3) and Golgi protein 73 (GP73) in the detection of HCC.**
Methods: **Thirty-nine patients with HCC and 31 patients with liver cirrhosis were enrolled. The level of serum GPC3 and GP73 were determined by ELISA. The expression of GPC3 mRNA and GP73 mRNA in peripheral blood mononuclear cell (PBMC) and liver tissues were also measured with qRT-PCR. Then, receiving operating characteristic (ROC) curves were plotted to detect the sensitivity and specificity of serum GPC3 and GP73 in the diagnosis of HCC.**
Results: **The levels of serum GPC3 and GP73 in the HCC group were significantly higher than in the cirrhosis group ($p < 0.0001$). Patients with GPC3 > 9.3 µg/L and GP73 > 77.68 ng/mL had a risk of HCC of 92.31%. The HCC diagnosis ROC curve analysis indicated that when setting the GPC3 cutoff value > 9.3 µg/L, AUC = 0.956. The sensitivity and specificity of GPC3 were 89.74% and 96.77%, respectively, with a positive predictive value of 97.2%, negative predictive value of 88.2%, + LR of 27.82 and - LR of 0.11. When setting GP73 cutoff value > 77.68 ng/mL, AUC = 0.937. The sensitivity and specificity of GP73 were 92.31% and 83.87%, respectively, with positive predictive value of 87.8%, negative predictive value of 89.7%, + LR of 5.72 and - LR of 0.092. No significant difference ($p > 0.05$) was found between GPC3 and GP73 AUC in ROC curves, indicating that these two biomarkers were equivalent in the prediction of HCC.**
Conclusions: **The expression of serum GPC3 and GP73 was significantly higher in the HCC patients compared with the cirrhosis patients. GPC3 and GP73 might be effective non-invasive diagnostic indicators of HCC.**
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Correspondence:
Shun-Cai Wang
Department of Infectious Diseases
Jurong People's Hospital
Jiangsu University
Zhenjiang
China
Email: wangshun1978@sina.com

KEY WORDS

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) has increased over the past decades in China [1]. The screening methods for HCC in early stages, such as the detection of α-fetoprotein (AFP) combined with hepatic ultrasonography, remain unsatisfactory. Many patients with HCC have missed the optimal treatment period at the time of clinically confirmed diagnosis. If patients could be diagnosed earlier, comprehensive approaches including surgical resection, liver transplantation, transcatheter arterial chemoembolization (TACE), and radiofrequency ablation (RFA) could result in better outcomes [2]. As the early diagnosis and treatment of HCC can improve the outcome of HCC treatment and extend the patients' life spans, new biomarkers with high specificity and sensitivity need to be explored.

The development of new gene and proteomics technologies has greatly assisted in the selection of new tumor markers. Glypican 3 (GPC3) is an extracellular glycoprotein discovered in 1995 and belongs to the family of heparan sulfate glycoprotein (HSPG). It can be used as a tumor suppressor through its activity on regulating cell proliferation [3]. GPC3 does not express in normal liver tissues but is highly expressed in malignant neoplasms, and may play an important role in liver function [4]. Golgi protein 73 (GP73) was first found in 2000 by Kladney et al. [5]. It is a Golgi type II transmembrane protein mainly located in the cis Golgi membranes (Trans) [6,7]. GPC3 and GP73 have already been recognized as biomarkers of HCC in many studies [8,9]. But there are still many contradictions regarding the effects of these two biomarkers in different studies. Especially, the value of GPC3 and GP73 for the differential diagnosis between HCC and cirrhosis has not been confirmed [10]. To evaluate the effect of GPC3 and GP73 in the clinical diagnosis of HCC, we compared the expression of GPC3 and GP73 in HCC patients with cirrhosis patients in this study.

MATERIALS AND METHODS

Subjects

Thirty-nine HCC patients and 31 liver cirrhosis patients admitted to Jurong People's Hospital and Nanjing Second Hospital from December 2012 to December 2013 were enrolled and analyzed retrospectively. The diagnosis of cirrhosis and HCC was based on liver histology or clinical, laboratory, and imaging data according to the Chinese liver disease diagnosis and treatment standard. Peripheral blood of patients was collected through venipuncture and liver tissues were collected from surgical resection or B ultrasound-guided liver puncture. Demographic information and clinical characteristics were collected as well. Tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until total RNA was extracted. This study was approved by the local medical ethics committee of Jurong People's Hospital and Nanjing Second Hospital and all patients provided informed consent.

Preparation of PBMCs

Peripheral blood was collected from HCC patients and liver cirrhosis patients. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll gradient density separation and washed twice with phosphate-buffered saline (PBS). PBMCs were used for the RNA extraction according to the manufacturer's protocol.

Determination of serum GPC3 and GP73

The serum samples from all patients were collected and detected using the Human GPC-3 ELISA Kit and human GP73 ELISA Kit (Shanghai Boyan Biotechnology Co., Ltd, China). All experiments were performed according to the manufacturer's instructions.

RNA isolation and real-time PCR

Real-time PCR was used to measure the expression of GPC3 mRNA and GP73 mRNA in PBMCs and liver tissues. Total RNA from frozen tissues or PBMCs was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One milligram of total RNA was reverse transcribed in a final volume of 20 μF under standard conditions using random primers and PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian, China; RR047A). After the RT reaction, 1 μL of the complementary DNA was used for subsequent qRT-PCR reactions (SYBR Premix Ex Taq, TaKaRa) according to the manufacturer's instructions. The results were normalized to the expression level of β-actin. The primers of GP73, GPC3, β-actin were as follows: GPC3 sense: 5'-GCCCATTCTC AACAACGC-3', antisense: 5'-CACTTTCAAACCCTT CCTCAT-3'; GP73 sense: 5'-CAGCGCTGATTTTGA GATGA-3', antisense: 5'-ATGATCCGTGTCTGGA GGTC-3'; β-actin sense: 5'-CGTACCACTGGCATCG TGAT-3', antisense: 5'-GTGTTGGCGTACAGGTCTT TG-3'. The primers were designed and synthesized by the Shanghai Biological Engineering Co., Ltd. Real-time PCR and data collection were performed using the ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Continuous variables were expressed as median and range. Categorical variables were analyzed by χ^2 test or Fisher's exact test, as appropriate. All statistical analyses were two-sided, and $p < 0.05$ was considered as statistically significant. Statistical analysis was performed with SPSS statistical package version 23.0. ROC curve analysis was performed with MedCalc v15.2.

Table 1. Demographic characteristics and clinical information of the patients.

Group	No. (male)	Age (years)	ALT (U/L)	AST (U/L)
HCC	39 (30)	46.2 ± 10.1	105.2 ± 15.2	128.2 ± 23.8
Cirrhosis	31 (18)	49.2 ± 10.3	104.2 ± 13.1	116.2 ± 30.1
p-value	0.09	0.47	0.61	0.11

RESULTS

Demographic characteristics and clinical information of the patients

A total of 70 Chinese patients were enrolled in the study including 39 patients with HCC and 31 patients with cirrhosis. The average age of patients was 46.2 ± 10.1 years old and 49.2 ± 10.3 years old in HCC group and cirrhosis group, respectively. Demographic characteristics and other clinical information of the 70 patients were summarized in Table 1. No significant difference was found in gender, age, and liver function between the two groups.

Concentration of serum GPC3 and GP73

To determine the concentration of serum GPC3 and GP73, ELISA was used to detect the level of the serum GPC3 and GP73 in both of HCC patients and cirrhosis patients. The results showed that the average concentration of serum GPC3 was 16.81 ± 0.56 μg/L in the HCC group, which was significantly higher than in the cirrhosis group (7.41 ± 0.25 μg/L, $p < 0.0001$) (Figure 1A). The average concentration of serum GP73 was 115.92 ± 7.01 ng/mL in HCC group compared with 64.63 ± 3.07 ng/mL in the cirrhosis group ($p < 0.0001$) (Figure 1B). Furthermore, the level of serum GPC3 and GP73 in both the HCC and cirrhosis group showed a significant positive correlation (Figure 1C, D).

The expression of GPC3 mRNA and GP73 mRNA in PBMCs and liver tissues

The GPC3 and GP73 gene expressions were evaluated in PBMCs and liver tissues of HCC and cirrhosis patients by Real-time PCR. The transcripts of the housekeeping gene *β-actin* was also detected in every samples. Then the ratios of the GPC3 or GP73 to *β-actin* were calculated. The level of each indicator mRNA was expressed by ($2^{\Delta ct}$ x 10^{-3}). The results showed that the expression of GPC3 and GP73 mRNA were 16.94 ± 8.67 and 12.51 ± 2.97 in the HCC group, respectively, compared with 15.63 ± 7.35 and 14.25 ± 3.67 in cirrhosis group, respectively. No significant difference was found between the two groups (Figure 2A, B).

The expression of GPC3 mRNA in liver tissue in the HCC group was significantly higher than that in the cirrhosis group (8.91 ± 3.70 vs. 3.04 ± 0.58, $p = 0.028$), while the expression of GP73 was 68.41 ± 32.86 and 2.32 ± 0.25 in HCC patients and cirrhosis patients, respectively ($p = 0.011$) (Figure 2C, D).

Correlation between the expression of GPC3/GP73 and the clinicopathologic characteristics in HCC patients

The clinicopathologic characteristics of 39 HCC patients was summarized and the correlations with the serum concentration of GPC3/GP73 protein and mRNA level in liver tissue were analyzed. The results showed that there were no statistical differences for the expression of GPC3/GP73 in different age, gender, tumor size, and HCC stage in HCC patients ($p > 0.05$). But there was a significant difference for the expression of GPC3 in different histological grades in HCC patients. HCC patients in histological grade 3 had lower levels of serum GPC3 protein and tissue GPC3 mRNA compared with the patients in histological grade 1 and 2 (Table 2).

Diagnostic utility of serum GPC3 and GP73 for HCC

Patients with serum GPC3 > 9.3 μg/L and GP73 > 77.68 ng/mL had a risk of HCC of 92.31% as shown in Figure 1A, B. The HCC diagnosis ROC curve analysis indicated that when setting the GPC3 cutoff value > 9.3μg/L, AUC = 0.956. The sensitivity and specificity of GPC3 were 89.74% and 96.77%, respectively, with positive predictive value of 97.2%, negative predictive value of 88.2%, + LR of 27.82 and - LR of 0.11. When setting GP73 cutoff value > 77.68 ng/mL, AUC = 0.937. The sensitivity and specificity of GP73 were 92.31% and 83.87%, respectively, with positive predictive value of 87.8%, negative predictive value of 89.7%, + LR of 5.72 and - LR of 0.092. No significant difference ($p > 0.05$) was found between GPC3 and GP73 AUC in ROC curves, which indicated that these two indicators were equivalent in the prediction of HCC (Figure 3).

In addition, the results also indicated that the diagnostic value of serum GPC3 and GP73 was significantly higher than that of MR/CT in sensitivity ($p < 0.05$), but not in specificity ($p > 0.05$) (Data not shown). Compared with AFP, which showed 60.78% positive predictive value and 82.76% negative predictive value in our patients, serum GPC3 and GP73 displayed better diagnostic values in our study (Figure 3).

Table 2. The relationship between the expression of GPC3/GP73 and the clinicopathologic characteristics in HCC patients.

Patients' characteristics	n	GP73		GPC3		P1	P2	P3	P4
		Protein (ng/mL)	mRNA	Protein (μg/L)	mRNA				
Gender									
Male	30	115.65 ± 8.06	78.06 ± 37.41	17.29 ± 0.51	10.49 ± 3.14	0.923	0.171 *	0.123	0.961 *
Female	9	117.74 ± 4.25	4.01 ± 3.13	13.64 ± 1.90	5.14 ± 3.61				
Age									
≤ 50	15	111.29 ± 8.12	130.48 ± 75.81	16.81 ± 0.44	5.42 ± 3.11	0.469 *	0.108 *	0.990	0.850 *
> 50	24	118.90 ± 10.42	28.50 ± 20.37	16.82 ± 0.88	11.15 ± 5.77				
Tumor size (cm)									
≥ 5.0	13	114.75 ± 11.51	40.83 ± 22.24	17.43 ± 0.77	11.18 ± 5.28	0.854	0.121 *	0.214	0.264 *
< 5.0	26	117.45 ± 6.77	104.26 ± 70.42	16.02 ± 0.77	5.95 ± 5.21				
Histological grade									
Grade 1 - 2	31	119.21 ± 8.70	86.45 ± 41.19	17.42 ± 0.57	9.77 ± 4.63	0.386	0.074 *	0.035	0.012 *
Grade 3	8	104.09 ± 6.23	3.44 ± 1.34	14.63 ± 1.13	5.80 ± 3.92				
HCC stage									
Stage I - II	30	110.87 ± 5.81	94.97 ± 53.83	16.28 ± 0.59	7.87 ± 4.34	0.424	0.121 *	0.285	0.457 *
Stage III - IV	9	122.49 ± 14.46	33.87 ± 28.51	17.51 ± 1.02	10.26 ± 6.65				

Note: P1 represents the p-value of GP73 protein, P2 represents the p-value of GP73 mRNA, P3 represents the p-value of GPC3 protein, P4 represents the p-value of GPC3 mRNA. * - Data were analyzed by Mann-Whitney while the other data were analyzed by *t*-test.

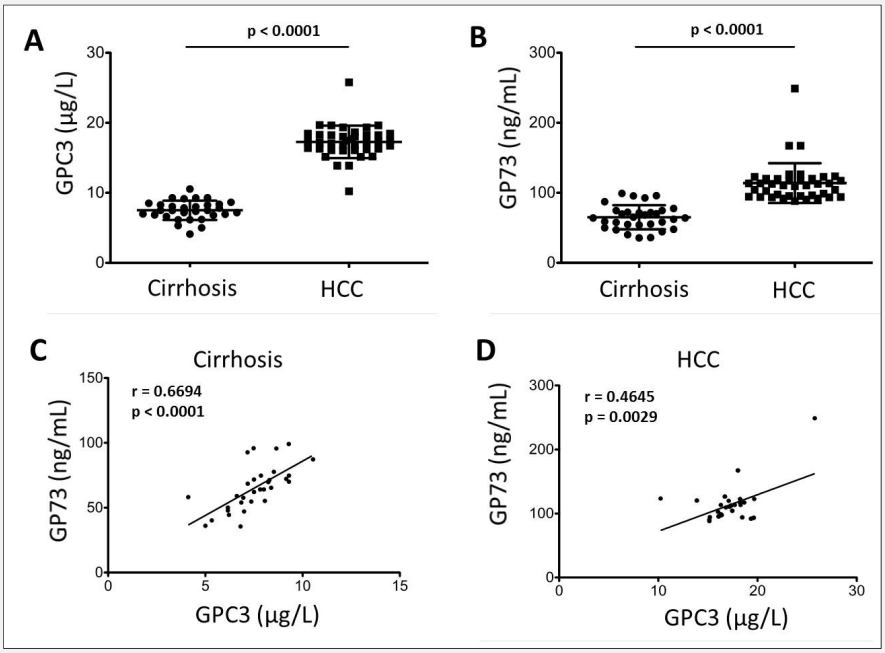

Figure 1. The level of serum GPC3 and GP73 in different patient populations.

(A) Serum level of GPC3 in cirrhosis and HCC patients. (B) Serum level of GP73 in cirrhosis and HCC patients. (C) The relationship between the level of serum GPC3 and GP73 in cirrhosis patients. (D) Correlation analysis between the level of serum GPC3 and GP73 in HCC patients.

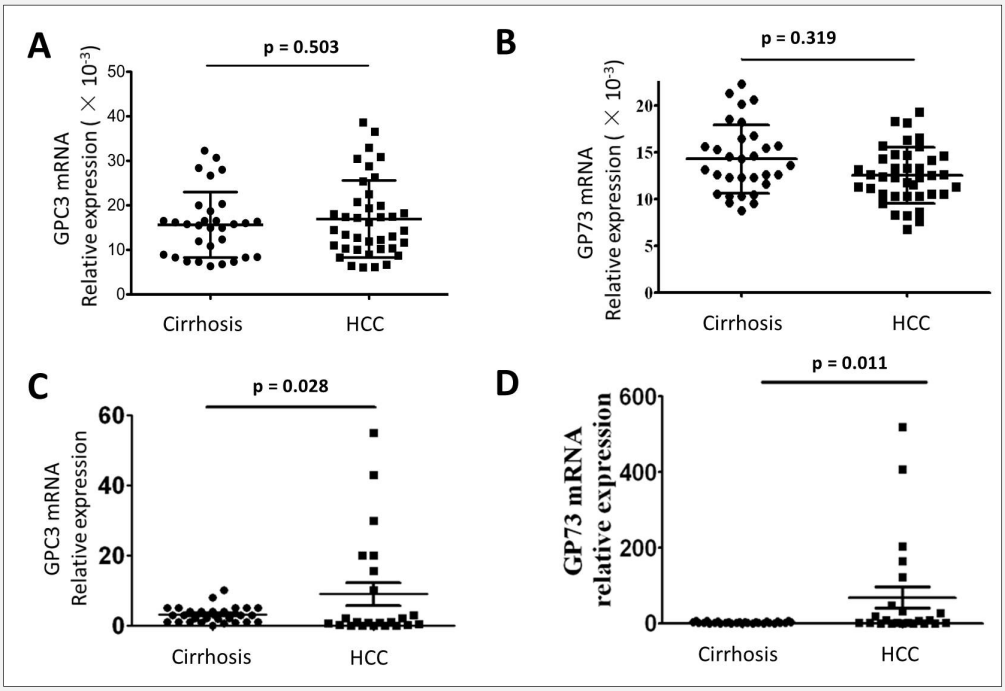

Figure 2. The mRNA expression of GPC3 and GP73 in PBMCs and liver tissues detected by Real-time PCR.

(A) The level of GPC3 mRNA in PBMCs of cirrhosis and HCC patients. (B) The level of GP73 mRNA in PBMCs of cirrhosis and HCC patients. (C) The level of GPC3 mRNA in liver tissues of cirrhosis and HCC patients. (D) The level of GP73 mRNA in liver tissues of cirrhosis and HCC patients.

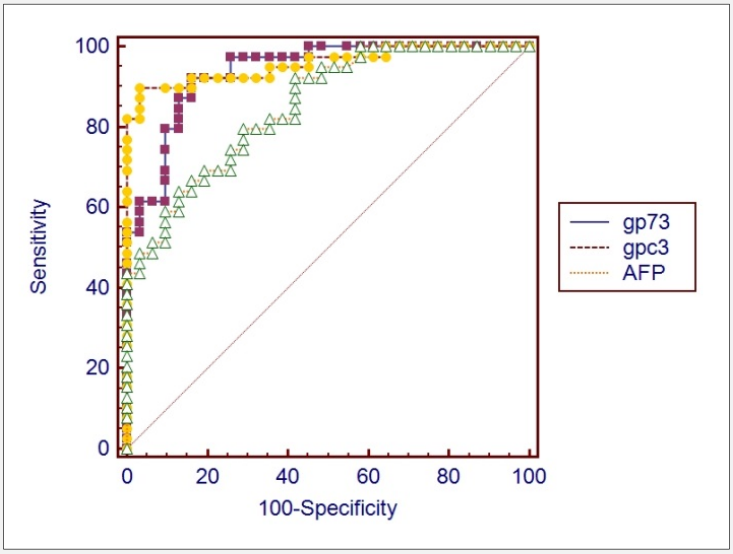

Figure 3. The ROC curves of serum GPC3, GP73, and AFP for diagnosis of HCC.

DISCUSSION

The incidence of HCC has continued to grow all over the world in recent decades. Five-year survival rate of HCC is less than 10%. It has been regarded as one of the most challenging malignant tumors. In 2005, the American Association of Liver Diseases (AASLD) proposed to monitor serum AFP and ultrasonography in high-risk patients with HCC every 6 months [11]. However, the sensitivity and specificity of current approaches are still not satisfactory, as 30% - 40% of HCC patients have low concentrations of serum AFP. In recent years, MR/CT is thought to achieve higher sensitivity and specificity than ultrasound, but the cost of inspection and equipment requirements limits its application. Thus, researchers have not stopped looking for new and better HCC biomarkers [12,13]. This study measured the expression of GPC3 and GP73 in HCC and cirrhosis patients by ELISA and qRT-PCR. The results revealed that the levels of serum protein and tissue mRNA of GPC3 and GP73 in HCC patients were significantly higher than in cirrhosis patients. Serum GPC3 and GP73 had better sensitivity, specificity, positive predictive value, negative predictive value, approximately equal index, AUC, likelihood ratio (+ LR, -LR) in the HCC diagnosis ROC curve analysis. The expression of GPC3 mRNA and GP73 mRNA in PBMCs had no significant difference between the HCC and cirrhosis group.

Ideal tumor markers require high specificity to distinguish HCC from cirrhosis, hepatitis, and regenerative nodules of the liver as well as high sensitivity in early diagnosis of HCC. The effect of serum GPC3 and GP73 in the diagnosis of HCC has been explored in recent years and most of the studies suggested that they could become valuable biomarkers for the diagnosis of HCC. GPC3 was discovered in 1995 and was used as a tumor suppressor, which can regulate cell proliferation [14, 15]. GPC3 could also be considered as a clinically useful biomarker in hepatocellular carcinoma, lung carcinoma, and ARDS, but further research is still needed [16-18]. The expression of GP73 is positively correlated with the degree of liver fibrosis (the more severe, the higher GP73). When cirrhosis develops to HCC, GP73 expression also reaches its peak. GP73 expression in patients with HCC had no significant correlation with AFP, so that GP73 expression may be related to HCC occurrence and development [15,19,20].

There were some contradictory results in different studies about the value of GPC3 in the diagnosis of HCC [21]. Some studies showed that the GPC3 positive expression rate was obviously elevated in HCC tissue. Combination detection of AFP and GPC3 presented significantly higher sensitivity and specificity in HCC than single AFP or GPC3 detection [22]. Yejoo Jeon's group found the plasma GPC3 levels in HCC patients were very low and the plasma level of GPC3 was a poor diagnostic marker for HCC [23]. In this study, we found the average level of serum GPC3 was up to 16.81 μg/L in the HCC group, which was significantly higher than in the cirrhosis group. Mao et al. compared serum GP73 and alpha-fetoprotein (AFP) in a total of 4217 human subjects in multicenter study and claimed that serum GP73 had high sensitivity and specificity in the diagnosis of HCC [24]. However, in another study, researchers found serum GP73 was not a good diagnostic marker for differentiating HCC from cirrhosis [10]. Our study detected the expression of serum GP73 in the 39 HCC patients and 31 cirrhosis patients and confirmed that the difference in the level of serum GP73 was significant. Patients with GPC3 > 9.3 μg/L and GP73 > 77.68 ng/mL had 92.31% risks of HCC, which could support that serum GPC3 and GP73 as valuable biomarkers in the diagnosis of HCC.

GPC3 and GP73 mRNA were overexpressed in tumor tissue of most HCC patients. This was the basis for GPC3 and GP73 to be evaluated as serum markers. Furthermore, the expression of GPC3 and GP73 mRNA in tissue was correlated with the histological grades of liver, which suggested that the elevated production of GPC3 and GP73 mRNA might be linked to the increased hepatocytes' proliferation and regeneration. However, whether the serum GPC3 and GP73 could be used to evaluate the hepatocytes' proliferation and regeneration still needs more studies. There is little research on the expression of GPC3 and GP73 mRNA in PBMCs. In this study, the qRT-PCR assay showed that GPC3 mRNA in PBMCs was in accordance with the level of GP73 mRNA expression and could not be distinguished between liver cancer and cirrhosis.

The analysis of curves indicated that serum levels of GPC3 and GP73 could be used as clinical indicators for the diagnosis of HCC with high specificity and sensitivity. Furthermore, the diagnostic value of serum GPC3 and GP73 was significantly higher than that of MR/CT in sensitivity. When compared with AFP, serum GPC3 and GP73 had better diagnostic value in our study. The expression of serum GPC3 and GP73 was significantly higher in HCC patients compared with cirrhosis patients. GPC3 and GP73 might become effective non-invasive diagnostic indicators of HCC.

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Declaration of Interest:

The authors have declared no conflict of interest in regard to this work.

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