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Significance of Glypican-3 (GPC3) Expression in Hepatocellular Cancer Diagnosis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Primary hepatocellular carcinoma (HCC) is a malignant tumor that is common China. Early diagnosis is of great significance for improving treatment efficiency. GPC3 level is closely related to HCC occurrence. This study investigated GPC3 expression in HCC patient serum and tissue, and assessed the significance of GPC3 combined AFP detection in HCC diagnosis.

Material/Methods: A total of 76 HCC patients in our hospital were enrolled. Immunohistochemistry was applied to test GPC3 expression in cancer tissue and para-carcinoma tissue. ELISA and RT-PCR were used to detect GPC3 and AFP level in serum. The significance of GPC3 single or combined AFP detection in HCC diagnosis was analyzed.

Results: Immunohistochemistry showed that the GPC3 positive expression rate was obviously elevated in HCC tissue ($P<0.01$). Combination detection of AFP and GPC3 presented significantly higher sensitivity and specificity in HCC than single AFP or GPC3 detection. ELISA showed no significant difference in sensitivity, specificity, or accuracy compared with RT-PCR.

Conclusions: Serum GPC3 was overexpressed in HCC patients. Combination detection of serum AFP and GPC3 can enhance accuracy and efficacy of HCC diagnosis.

MeSH Keywords: Antifreeze Proteins, Type II • Antifreeze Proteins, Type III • Genes, rev • Headache Disorders, Primary • MART-1 Antigen

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Background

Primary hepatocellular carcinoma (HCC) is a kind of malignant liver cancer derived from liver cell cancerization [1]. In China, HCC is the second most common cause of cancer-related deaths after lung cancer, with the 5-year survival rate of less than 5% [2]. As with other malignant tumors, the pathogenesis of HCC is unclear. However, it is generally believed that HCC is closely related to liver cirrhosis, viral hepatitis, and chemical carcinogens [3]. Although great progress has been made in HCC treatment, including minimally invasive surgery, interventional embolization chemotherapy, and liver transplantation, tumor recurrence and metastasis still cannot be prevented [4,5]. Studies showed that HCC early diagnosis and treatment can greatly improve the efficiency of HCC treatment and extend patient life [6].

Alpha-fetoprotein (AFP) is abundantly expressed in the development of human embryos and expression stops after birth. However, the synthesis of AFP is restored when liver cells become cancerous. It has been shown that the serum levels of AFP were elevated in 70–80% of liver cancer patients. Therefore, detection of levels of serum AFP for diagnosis of HCC has been widely recognized as being important [7].

However, there are still 30–40% of HCC patients with negative or low concentrations of serum AFP [8]. Multiple tumor markers combined detection is of great significance to improve HCC diagnosis rate, as well as to reduce misdiagnosis and missed diagnosis [9,10]. Glypican-3 (GPC3) is a member of the glypicans family that anchors to the cell surface through glycosylated phosphatidyl inositol. GPC3 protein is closely associated with the body's growth and development through a variety of cytokines. GPC3 mutation leads to Simpson-Golabi-Behmel syndrome (SGBS) [11]. Moreover, GPC3 is also expressed in multiple tumor cells and serum, such as HCC and melanoma [12,13]. Thus, this study aimed to investigate GPC3 expression in HCC patient serum and we discuss the significance of GPC3 detection in HCC early diagnosis.

Material and Methods

Subject selection

Between January 2012 and March 2015, a total of 76 HCC patients in the First People's Hospital of Guiyang were enrolled into the HCC group, including 49 males and 27 females, with mean age of 52.1±7.8 (45–72) years old. All the patients were primary cases without chemotherapy, radiotherapy, or surgery. Patients with liver cirrhosis were selected as the cirrhosis group, including 24 males and 16 females, with mean age of 53.2±7.2 (40–72) years old. Thirty healthy volunteers were enrolled as normal controls, including 16 males and 14 females, with mean age of 51.3±7.3 (42–68) years old. All subjects were diagnosed by clinical medical history combined CT and MRI using the China liver disease diagnosis and treatment management standard and primary hepatocellular carcinoma diagnosis standard. HCC patients were staged according to primary hepatocellular carcinoma diagnosis standard, including 16 cases in stage I, 32 cases in stage II, and 28 cases in stage III. No significant difference was observed in general information among groups.

This study was approved by the Ethics Committee of the First People's Hospital of Guiyang and all enrolled subjects had signed informed consent.

Immunohistochemistry

Cancer tissue and para-carcinoma tissue in the HCC group were obtained from surgery and fixed in formalin. The tissue was embedded by paraffin and sectioned at 0.5 μm. Paraffin sections were dewaxed by xylene and hydrated by gradient ethanol and distilled water. After blocking by 3% H_2O_2 solution, slices were washed by distilled water and PBS. Then the slices were blocked by 5% BSA at room temperature and GPC3 specific primary antibody was added at 37°C for 2 h. After washing with PBS, secondary antibody was added, followed by development at 37°C for 30 min. Slices were the developed in DAB for 10 min and observed under a microscope after re-staining, washing, dehydration, hyalinization, and mounting. Five random fields at 400× were selected to calculate cell number.

Result judgement: GPC3 is expressed on cell surface. Cells were regarded as positive when brown particles appeared on the cell surface. GPC3 positive was determined as positive cells >40% of total cell number.

ELISA

A total of 5 ml fasting venous blood was extracted and centrifuged to isolate serum. Serum AFP and GPC3 levels were detected by ELISA (Shanghai Yifeng Biological Technology Co., LTD). A 100-μl sample was added to the 96-well plate together with 100 μl PBS (pH=7.4) and kept at 4°C overnight. Next, the well was blocked by 2% H_2O_2-ethanol solution after being washed by PBS. After blocking by 1% BSA, we added AFP and GPC3 antibody (1:1000) at 37°C for 2 h. Then the well was treated by biotin-tagged secondary antibody (1:200) at 37°C for 1.5 h. After being developed by OPD substrate solution at room temperature for 6 min, the reaction was stopped by 0.2 mM H_2SO_4. The plate was read on a microplate reader (BioTek) to analyze AFP and GPC3 content.

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RT-PCR

A total of 2 ml blood was put into an EDTA anticoagulation tube together with 2 ml PBS. After adding with 4 ml lymphocytes separation medium, the tube was centrifuged at 1500 rpm for 20 min. The white layer was peripheral blood mononuclear cells (PBMC). Total RNA was extracted from PBMC to test GPC3, AFP, and β-actin levels. RT-PCR primers were as follows: GPC3-F, 5'-AGAGGCCTTTGAAATTGT-3'; GPC3-R, 5'-AAATACTTTCAGGTCACGTC-3'; actin-F, 5'-CGTAC CACTGGCATCGTGAT-3'; actin-R, 5'-GTGTTGGCGTACAGGTCTTTG-3'; AFP-F, 5'-TGGAATAGCTTCCATATTGGGATTC-3'; AFP-R, 5'-CCAGTTT GTTCAAGAAGCCACTT-3'. PCR reaction was 93°C for 2 min, followed by 40 cycles of 93°C for 30 s and 60°C for 1 min [14].

GPC3 combined AFP detection on HCC diagnosis analysis

An ROC curve was used to determine the critical value of HCC diagnosis. The minimum value of false-positive cases and false-negative cases was the cut-off value. The result ≥ cut-off value was regarded as positive and result < cut-off value was regarded as negative. Either one index ≥ cut-off value was determined as positive [14].

Statistical analysis

SPSS 20.0 software was applied for data analysis. Measurement data are presented as mean±standard deviation and compared by the Mann-Whitney U test. Correlation analysis was performed by Pearson rank correlation analysis. Inspection level α=0.05. P<0.05 was considered as statistical significance.

Results

GPC3 immunohistochemistry result

Immunohistochemistry was applied to test GPC3 expression in HCC cancer tissue and para-carcinoma tissue. Brown particles appearing on cell membranes indicated GPC3 positive expression (Figure 1). As shown in Figure 1A, cell staining was not significant in para-carcinoma tissue. Cell membrane color was deeper in HCC cancer tissue compared with para-carcinoma tissue, suggesting that GPC3 level was elevated in HCC cancer tissue.

Five random fields (400×) were selected to calculate total number of cells and number of positive cell (Figure 2). The GPC3-positive rate in para-carcinoma tissue was only 3.95% (3/76), while it achieved 72.4% in cancer tissue. The positive rate of stage I was 37.3% (6/16), stage II was 71.9% (23/32), and stage III was 92.9% (26/28). These results indicated that GPC3 expression was upregulated in HCC cancer tissue, and its positive rate obviously increased following histological upstaging.

ELISA result

To determine serum GPC3 concentration in HCC patients, we used ELISA to test serum AFP and GPC3 level (Table 1). We found that the concentration of serum GPC3 and AFP in HCC patients was 272.5±13.3 ng/mL and 404.2±12.6 ng/mL, respectively, which was obviously different from that in controls (P<0.01). No significant difference was observed between the cirrhosis group and chronic liver disease group. Correlation analysis demonstrated that the correlation coefficient of GPC3 concentration with histological grade was r=0.89 (P>0.05), suggesting that the correlation was not significant. The correlation coefficient of serum GPC3 concentration with AFP was r=0.96 (P<0.01), indicating significant correlation.

Combined analysis of GPC3 and AFP detection in HCC diagnosis

To analyze the significance of combined serum GPC3 and AFP detection in HCC early diagnosis, we investigated sensitivity, specificity, and accuracy of different indices (Table 2). The sensitivity and specificity of single AFP detection was 77.6% and 81.3%, respectively, and the sensitivity and specificity of single GPC3 detection was 75.0% and 81.8%, respectively. However, the sensitivity and specificity of combined detection reached 85.5% and 91.5%, respectively. ELISA showed no significant difference in sensitivity, specificity, or accuracy with RT-PCR (P>0.05).

Discussion

HCC early diagnosis and treatment can effectively improve treatment effect and quality of life. Thus, screening molecular markers with high specificity and good sensitivity is of great significance to improve HCC early diagnosis and reduce misdiagnosis [15]. This study tested cancer tissue GPC3 expression in HCC patients at different clinical stages by immunohistochemistry. The results showed that the GPC3 positive expression rate was obviously elevated in HCC tissue, and it gradually increased following clinical upstaging.

GPC3 is a member of the glypicans family that can anchor on the cell surface by glycosylated phosphatidyl inositol. GPC3 protein is closely associated with growth and development [16]. It was reported that GPC3 is a potential biomarker for malignant tumors because it is upregulated in hepatocellular carcinoma and melanoma [12]. However, GPC3 protein level is reduced in ovarian cancer and breast cancer [17]. In addition, it was also reported that GPC3 function had organizational dependence. It can suppress tumor cell division in some organizations, but becomes the oncofetal protein in the other parts [18].

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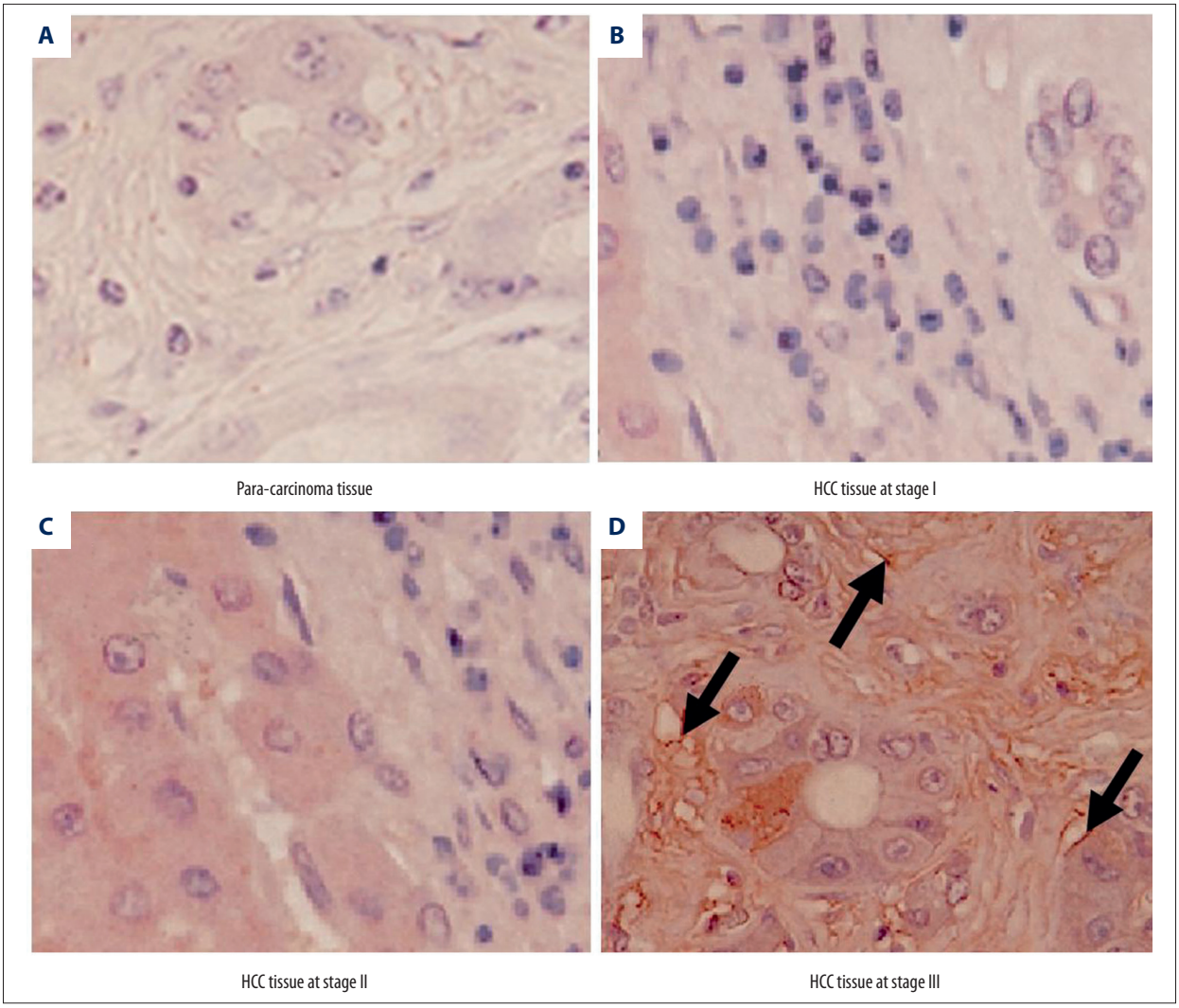

Figure 1. GPC3 immunohistochemistry result (×400).

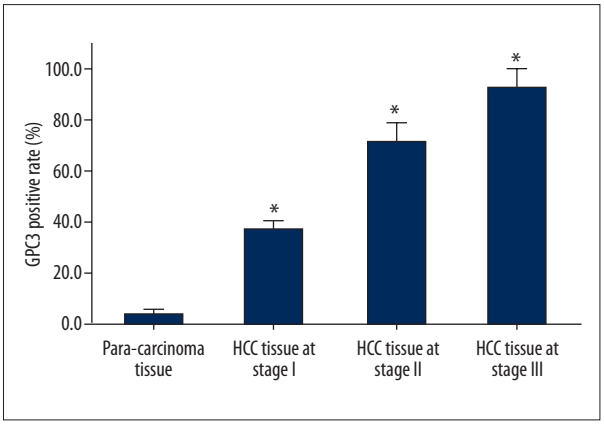

Figure 2. GPC3 expression in HCC tissue. * P<0.01, compared with para-carcinoma tissue.

MiR-219-5p can mediate GPC3 mRNA expression at the post-transcriptional level [19]. microRNA microarray analysis revealed that miR-219-5p was downregulated by more than 50% in HCC cancer cells, leading to GPC3 overexpression [20]. GPC3 expresses in HCC cells but not normal liver cells, which also suggests that GPC3 could be a potential target for HCC treatment. Zhu et al. injected GPC3 monoclonal antibody in advanced HCC patients with different GPC3 levels, and found that tumor progression time in patients with high GPC3 level was longer than that with low expression level [21].

AFP is highly expressed in human embryonic development process, and stops synthesis after birth. However, AFP synthetic ability is recovered during liver cell cancerization. Serum AFP is elevated in about 70~80% of HCC patients; thus, measuring serum AFP level in HCC patients for diagnosis has been widely accepted. However, there are still 20~30% of advanced HCC patients without significant changes of serum AFP level,

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Table 1. Serum GPC3 and AFP level.

Group	n	AFP (ng/mL)	GPC3 (ng/mL)
Control	30	47.2±9.1	56.2±6.1
Chronic liver disease	40	52.5±10.5	57.5±8.3
Cirrhosis group	30	55.3±7.6	661.9±6.8
HCC group	76	404.2±12.6*#&	272.5±13.3*#&

* P<0.01, compared with control; # P<0.01, compared with chronic liver disease; & P<0.01, compared with cirrhosis group.

Table 2. GPC3 combined AFP detection on HCC diagnosis analysis.

Method	Index	Sensitivity (%)	Specificity (%)	Accuracy (%)
ELISA	AFP	77.6 (59/76)	84 (84/100)	81.3 (143/176)
	GPC3	75.0 (57/76)	87 (87/100)	81.8 (144/176)
	AFP+GPC3	85.5 (65/76)	96 (96/100)	91.5 (161/176)
RT-PCR	AFP	69.7 (53/76)	90 (90/100)	86.9 (153/176)
	GPC3	67.1 (51/76)	89 (89/100)	685.2 (150/176)
	AFP+GPC3	78.9 (60/76)	99 (99/100)	90.3 (159/176)

resulting in leak detection [7]. This study discussed the meaning of GPC3 as a molecular marker in HCC early diagnosis. GPC3 showed similar accuracy and sensitivity with AFP in HCC diagnosis, while combined detection can significantly enhance the accuracy and sensitivity [22].

Conclusions

We used total RNA from PBMC to test AFP and GPC3 mRNA level by RT-PCR. RT-PCR had results consistent with ELISA, suggesting that we could use PBMC to test AFP and GPC3 level by RT-PCR. It had higher efficacy, less window phase, and higher throughput compared with ELISA, which is of great significance for early diagnosis of HCC [23].

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