Diagnostic value of glypican-3 in alpha fetoprotein negative hepatocellular carcinoma patients

Li B[1], Liu H[2], Shang HW[3], Li P[1], Li N[1], *Ding HG[1]

1. Department of Hepatology and Gastroenterology, Beijing You'An Hospital affiliated to Capital Medical University, 100069, China.
2. Department of Pathology, Beijing You'An Hospital affiliated to Capital Medical University, 100069, China.
3. School of Basic Medical Sciences, Capital Medical University, 100069, China.

Abstract

Background: The prognosis of patients with hepatocellular carcinoma(HCC) is generally very poor with a 5-year survival rate of less than 15% since most of them are diagnosed clinically at their late stage.However,the differential diagnosis between alpha fetoprotein(AFP) negative HCC and cirrhotic nodules is still difficult.

Objectives: To evaluate the diagnostic value of glypican-3(GPC3) in patients with AFP negative hepatitis B related HCC.

Methods The liver tissue GPC3 (GPC3L) expression was tested from 426 for surgery and 179 of needle biopsies of hepatitis B related HCC patients using immunohistochemistry staining. Serum GPC3 (GPC3S) and AFP were also measured.

Results Among surgical HCC samples, 80.0% of GPC3L expression was positive, however, in paracarcinomatous and cirrhotic nodules were negative. In needle biopsy tissues, GPC3L positively expression was in 74.9%. The sensitivity of AFP>400μg/L was 25.4%. The GPC3S >3.5μg/L was determined as a positive. The area of ROC curve of GPC3S was 0.68(95% CI 0.56-0.79,P<0.05) in all HCC patients,0.81 (95% CI 0.62 -0. 98, P<0.05) in AFP greater or equal to 400μg/L and 0.64 (95% CI 0.51-0.77, P=0.051) in AFP negative patients. The GPC3S was positive in 48.8% of patients with AFP negative. No difference was observed between GPC3L/GPC3S and serum AFP.

Conclusions GPC3 may be helpful in improving diagnosis of HCC and in differentiating diagnosis between AFP negative HCC and cirrhotic nodules.

Key words: Hepatocellular carcinoma; Glypican-3; Differential diagnosis, Alpha fetoprotein, Hepatitis B

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Introduction

The hepatocellular carcinoma HCC is one of the most common malignant tumor in the world, especially in China, because the main etiological factor of HCC in China is HBV infection[1]. The prognosis of patients with HCC is generally very poor with a 5-year survival rate of less than 15% since most of them are diagnosed clinically at their late stage[2]. Therefore, early detection and diagnosis is still the key way to improve the prognosis of HCC[3]. Screening for HCC is considered to be cost-effective in patients with cirrhosis since the population has an expected annual incidence of HCC exceeding 5% per year[4]. However, most of cases of liver mass/nodular lesions are benign, such as cirrhotic regenerative nodules, adenoma, and focal nodular hyperplasia, but they may mimic malignant liver lesions both clinic and radiology[5]. The hepatocellular carcinoma also show a variety of histological patterns. The pathologic diagnosis of HCC using routine histological stains is usually difficult, especially for well differentiated tumors or small biopsy specimens. Immunohistochemistry has been applied extensively to hepatic neoplasms for the differential diagnosis[6,7]. Monoclonal antibodies including HepPar-1, cytokeratin 7(CK7), cytokeratin 19(CK19), cytokeratin 20(CK20), thyroid transcription factor-1 (TTF-1, carcinoembryonic antigen (CEA), CD34, alpha fetoprotein (AFP) were usually used to differentiate HCC, cholangiocarcinoma and other metastatic tumors. However, they are hardly useful to identify hepatic benign or malignant nodules[8,9,10]. Therefore, the differential diagnosis between hepatitis B related HCC and benign cirrhotic nodules is still a problem. Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycan family, which is linked to the

***Corresponding author**:
Dr. Huiguo Ding
Department of Gastroenterology and Hepatology
Beijing You'an Hospital affiliated to Capital Medical University
Beijing 100069
China
Telephone: +86-10-83997155
E-mail: dinghuiguo@medmail.com.cn

cell surface through a glycosylphosphatidylinositol anchor[11]. Glypicans play an important role in cell growth, differentiation, and migration[12,13,14]. It has been reported many times that GPC3 messenger RNA (mRNA) and protein level are increased in a large proportion of HCC[15,16,17,18,19]. Capurro et al. found overexpression of GPC3 protein in 72% of HCC, but no overexpression was detected in focal nodular hyperplasia and cirrhotic liver[18]. GPC3, therefore, seems useful to differentiate preneoplastic from well-differentiated neoplastic hepatocellular disorders or benign cirrhotic nodules and HCCs. However, the relationship between hepatic GPC3 expression and serum GPC3 and AFP is unclear. The differential diagnostic value of GPC3 between AFP negative HCC and cirrhotic nodules is still not reported. Therefore, the aim of this study was to assess GPC3 for the differential diagnostic value in patients with AFP negative hepatitis B related HCC.

Methods

Liver tissue and Serum samples

Total 605 patients with hepatitis B related HCC proved by hepatic histology at Beijing YouAn Hospital, Capital Medical University were recruited from January 2007 to May of 2012. The ages of patients with HCC ranged from 32 to 84 years (mean, 49.8 years), and the male/female ratio was 3.3:1. All of these patients were HBsAg positive. The liver tissue samples were obtained from 426 HCC patients by surgery resection liver tissue including carcinoma and paracarcinomatous (far away edging of carcinoma more than 2cm), and 179 by needle biopsies from liver mass/nodular lesions. All the specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. Four-ìm thick tissue slides were made from whole tissue blocks. The slides were reviewed to confirm the HCC diagnosis as the guidelines of WHO's criteria[20]. All tissue samples were reviewed by 2 experienced pathologists. The serum samples from these patients, including age and gender matched 25 health volunteers, were kept in the Medical Bioinformation Research Center Serum Bank at -80°Cfor measurement of serum GPC3 and AFP. The health volunteers were defined as liver function test and B-ultrasonography normal and HBsAg negative. The study protocol was approved by the Ethical Committee of Beijing YouAn Hospital, Capital Medical University. Investigators explained in detail to all the patients and/or their relatives. Consent forms were obtained from all participants before recruitment.

Immunohistochemistry (IHC) staining

The expression of liver tissue GPC3 (GPC3L) was measured using IHC staining. Monoclonal anti-GPC3 antibody (clone number: sc-65443 1G12, Santa Cruz Inc, USA) were used in the IHC staining. Mouse negative control was also used as control. In briefly, tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by using 3% hydrogen peroxide for 10 minutes. Following heat-induced in 0.1 mol/L of citrate buffer at pH 6.0 using microwaves for 8 minutes, the slides were incubated in pepsin for 7minutes and subsequently incubated with 1:50 diluted primary antibodies overnight at 4!. The secondary antibody used was horse-radish peroxidase (HRP)-labeled antibody (Zhongshan Goldenbridge Company, Beijing, China) for 30 minutes at room temperature. The reaction product was shown using 3, 39-diaminobenzidine tetrahydrochloride and the sections were counterstained with hematoxylin. The results were evaluated by board certificated pathologists. The area of GPC3 staining >5% was positively considered.

Serum AFP and GPC3

Serum AFP was tested by electrochemiluminescence (reagents from Abbott Ltd, USA). The serum AFP<400μg/L was regarded as negative according to Chinese and Asia guideline[21,22]. Serum GPC3(GPC3S) was determined by enzyme linked immunosorbent assay (ELISA) (GPC3 ELISA Kit, Catalog No. CSB-E11333h (96T), Cusabio biotech co. Ltd, Shanghai, China) following the protocol provided by manufacturer. The cutoff value of GPC3S was determined by receiver-operator characteristic curve (ROC) curve, which is 2SD above the average of the healthy individuals. The optical density was measured using spectro-photometer (Bio-Rad xMark, USA).

Statistical analysis

All analyses performed using the SPSS statistical software pack for windows (version 11.5).The differences of the mean of GPC3S between the subgroups were determined by the unpaired sample t test. The ROC curve of GPC3S was performed to determine the diagnostic accuracy and two by two tables for sensitivity and specificity. The categorical variable was compared using the X^2 test with Pearson correction. The difference was considered as significant when P <0.05.

Results

Expression of GPC3 in HCC tissues

By using IHC staining, GPC3L was easily detected to express in HCC specific cells, and no expression in paracarcinomatous and cirrhotic nodules tissue (figure 1A-C). Positive cell staining was restricted to the area of HCC differentiation. The non-HCC tissues, including cirrhotic nodules or paracarcinomatous, were consistently negative. It was found GPC3L was positive in 80.0% (341/426) of the HCC excised tissue samples. Of those patients, GPC3L was positively expressed in 81.2% (147/181) well-differentiated, 81.1% (86/106) moderately differentiated and 77.7% (108/139) poor-differentiated HCCs. There was no significant difference of GPC3L among HCC differentiation degree (P>0.05). GPC3L was positive in 74.9% (134/179) of needle biopsy tissues from HCC patients. There was no difference of GPC3L expression between surgery tissue and needle biopsy samples.

No difference between GPC3L and serum AFP

The sensitivity of serum AFP greater or equal to 400μg/L for diagnosis HCC was 25.4% (table 1). The GPC3L expression was detected in 77.4% (328/424) of patients with serum AFP negative and in 81.2% (147/181) of patients with AFP greater or equal to400μg/L in figure 2A. No difference was observed between GPC3L and serum AFP.

The mean of GPC3S in patients with AFP<400μg/L(12.63±2.93μg/L) or AFP greater or equal to 400μg/L (20.20±5.41μg/L) was significantly higher than in controls (1.92±0.95μg/L,P<0.01) (figure2B). According to ROC curve (r^2=0.96, F=162.1, P<0.01), the cutoff value was 3.5μg/L of GPC3S to determine HCC as a positive. The sensitivity and specificity in all, AFP greater or equal to 400μg/L and AFP negative HCC patients were 54.6%, 75%, 54.6% and 80%, 80%, 76%, respectively. Their positive predictive value and negative predictive value were 83.3%, 60%, 77.8% and 43.2%, 86.4%, 46.3%, respectively. The area under a ROC curve of GPC3S in all, AFP greater or equal to 400μg/L and AFP negative HCC patients was 0.68(95% CI 0.56-0.79, P<0.05), 0.81 (95% CI, 0.62 -0. 98, P<0.05) and 0.64 (95% CI, 0.51- 0.77, P=0.051) (figure 3). In this study, the GPC3S was positive in 48.8% of patients with serum AFP negative. The no difference between GPC3S and GPC3L expression was also observed.

Table 1: The sensitivity and specificity of different AFP cut-off value for diagnosis of HCC

Cutoff	Evaluation indicators (μg/L)				
	SEN	SPE	+LR	-LR	OR
20	0.55	0.73	2.01	0.62	3.22
200	0.32	0.96	8.11	0.70	11.52
400	0.25	0.98	10.58	0.76	13.85

SEN, Sensitivity; SPE, specificity; +LR,Positive likelihood ratio; -LR, Negative likelihood ratio; OR, Diagnostic odds ratio.

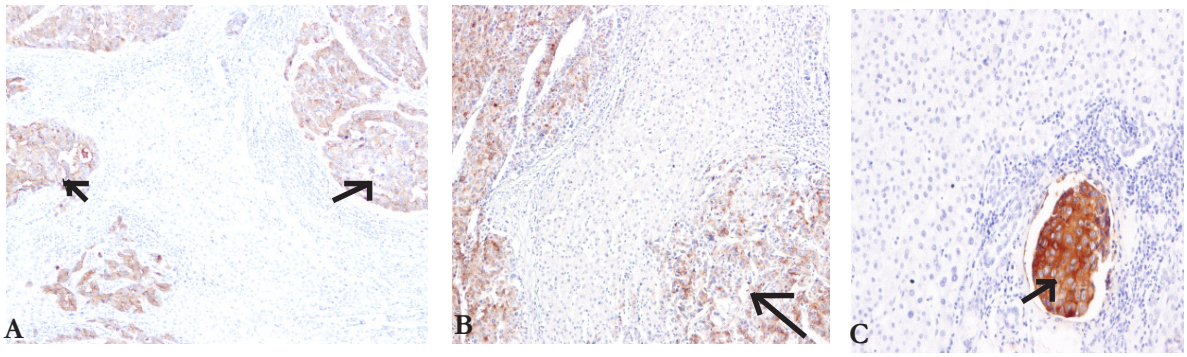

Figure 1: IHC staining of GPC3 in HCC liver samples A-C (10x). The cells stained with brown particles were considered as positive (arrow).

A: The area of cirrhosis liver around the HCC has no GPC3 expression.

B: There was no labeling in area with non-cirrhosis liver around the HCC.

C: The area of portal venous tumor thrombus showed strong GPC3 expression(arrow).

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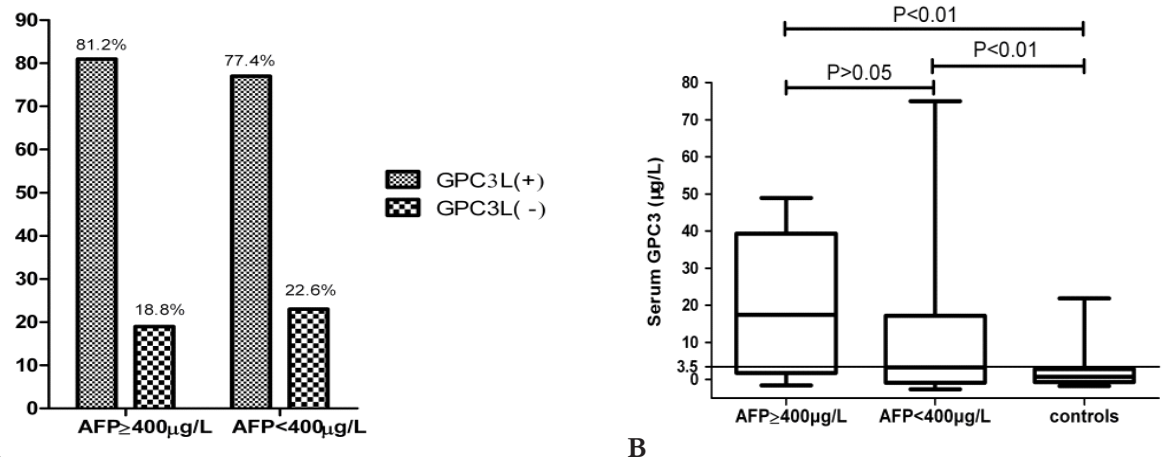

Figure 2 A-B: The relationship between GPC3L/GPC3S with serum AFP

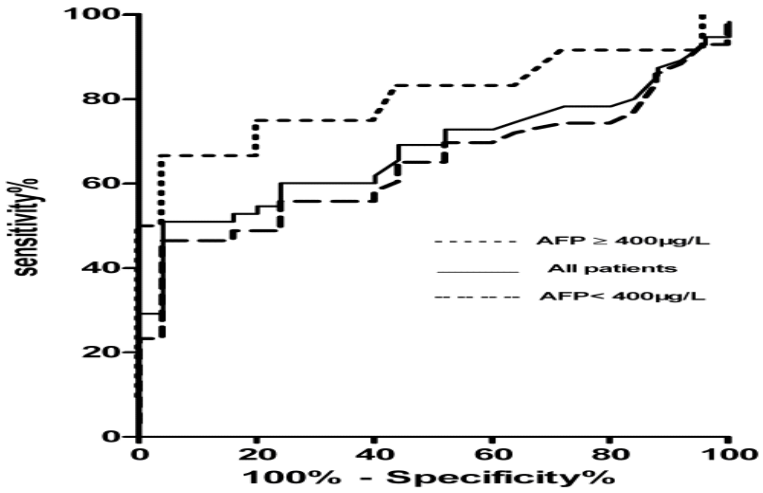

Figure 3: The diagnostic accuracy of GPC3S was performed by ROC curves.
The GPC3S was over 3.5μg/L in 48.8% of patients with AFP<400μg/L. The sensitivity and specificity in all, AFP greater or equal to 400μg/L and AFP negative HCC patients were 54.6%, 75%, 54.6% and 80%, 80%, 76%, respectively. The positive predictive value and negative predictive value were 83.3%, 60%, 77.8% and 43.2%, 86.4%, 46.3%, respectively.

The morbidity of HCC is raised in liver cirrhosis or chronic hepatitis B patients in China[23-24]. The pathological diagnosis of HCC using routine histological stains is usually difficult because of variety of histopathological patterns, especially for well differentiated tumors or small biopsy specimens. Immunohistochemistry has been applied extensively to hepatic neoplasms for the differential diagnosis and a number of immunomarkers have been widely utilized to facilitate the distinction HCCs from benign lesions. However, the currently available immunomarkers have significantly limited[9-10]. For example, monoclonal HepPar-1 antigen does not discriminate benign from malignant hepatocytes and tends to be nonimmunoreactive in poorly differentiated HCCs. AFP, which expressed well in fetal livers, shows low sensitivity in HCC and is seldom used in our routine work for HCC diagnosis. CK19 or CK7 is useful in distinguishing HCCs from cholangiocarcinomas, but there is no use to distinct hepatic cellular benign and malignant lesions either. In a word, all of these antibodies for IHC can't discriminate between benign and malignant hepatocytes.

In this study, we found GPC3L was positive in 80.0% of the HCC patients for surgery excised tissue and 74.9% of needle biopsy samples. However, GPC3L negative expression in paracarcinomatous

and cirrhotic nodules was found. Some recent studies had reached the same conclusion that none of cirrhotic livers and non-neoplastic liver tissues exhibited positive GPC3 immunostaining using different methods[25,26,27].

In recently study on prospective validation of an immunohistochemical panel -GPC3, heat shock protein 70(HSP70) and glutamine synthetase (GS) in liver biopsies for diagnosis of very early hepatocellular carcinoma has been done by Dr. Tremosini S[28]. It was found that the sensitivity and specificity for HCC diagnosis were: GPC3 57.5% and 95%, HSP70 57.5% and 85%, GS 50% and 90%, respectively. However, the sensitivity and specificity of the different combinations were significantly increased. These data support the guideline of AASLD on immunohistochemical staining of GPC3 for the diagnosis of early HCC[29]. Therefore, it is strongly supported that GPC3L may be usefull to differenate between HCC and cirrhotic nodules. The results are different from some previous reports which reported 3%-19% expression in non-neoplastic and preneoplastic disorders[30].

The serum AFP is the widely used tumor marker in the diagnosis of HCC for 40 years. In recently, the diagnostic value of serum AFP is questioned because of very low sensitivity[29,31]. In this study, the sensitivity of serum AFP greater or equal to 400μg/L for diagnosis HCC was 25.4%. However, the data of serum GPC3 for diagnosis HCC are limitation. Ozkan H,et al. had recently reported that the sensitivity, specificity, and positive and negative predictive values of serum GPC3 was 61.33%, 41.82%, 58.97%, and 44.43%, respectively. It is suggested that serum GPC3 is not a useful diagnostic and prognostic marker for HCC[32]. However, in this study, it is firstly reported that the GPC3L expression was detected in 77.4% of patients with serum AFP negative and in 81.2% of patients with AFP greater or equal to 400μg/L. It is interestingly found that there is no difference between GPC3L and serum AFP. GPC3L was highly expressed in patients with serum AFP negative(AFP<400μg/L) HCC. The area under a ROC curve of GPC3S was 0.68 in all HCC patients, 0.81 in serum AFP greater or equal to 400μg/L and 0.64 in patients with serum AFP negative. Our study was also found GPC3S was significantly positive in 54.6% of patients with HCC, especially in serum AFP negative HCC. Therefore, these data is strongly suggested that combinations of serum AFP and GPC3S may increase diagnostic sensitivity, especially for early diagnosis of HCC.

Conclusion

We confirmed the high and specificity expression of GPC3L in HCC tissue as a diagnostic marker. GPC3 may be very helpful in improving diagnosis of HCC and in differentiating diagnosis between AFP negative HCC and cirrhotic nodules. The combination test of serum AFP and GPC3S may increase the early diagnosis of HCC. It would be mentioned for clinicians in routine clinical practice.

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