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The Diagnostic Role of Serum Glypican-3 in Hepatocellular Carcinoma

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ABSTRACT

The differential diagnosis between hepatocellular carcinoma (HCC) and benign chronic liver disease (CLD) is sometimes difficult and new biochemical markers for HCC are required. Glypican-3 (GPC3) has been reported to be a novel tumor marker for the diagnosis of HCC. In this study, we evaluated GPC3 level by a sandwich ELISA method in sera of 10 healthy subjects, 39 patients with CLD and 60 patients with HCC. Our data showed that 47% of HCC patients had elevated levels of serum GPC3 with values ranging from 35.5 to 6,547.9 ng/mL, whereas the marker was undetectable in the other groups. In most cases of HCC, elevated GPC3 values did not correlate with elevated alpha-fetoprotein (AFP) values. The simultaneous determination of GPC3 and AFP (at a cutoff value of 20 ng/mL) significantly increased the sensitivity of the diagnosis to 80%. In conclusion, serum GPC3 elevation is highly specific for HCC. The combined use of serum GPC3 and AFP may significantly increase the sensitivity for differentiating HCC from CLD.

Key words : Glypican-3, HCC, Hepatocellular carcinoma

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INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, particularly in Southeast Asia, where hepatitis B virus (HBV), and a lesser extent, hepatitis C virus (HCV) infection are prevalent[(1)]. Most patients with HCC are diagnosed at advanced stages and thus the prognosis is generally poor. The diagnosis of HCC could be achieved at earlier stage by regular screening programs among high-risk populations by using imaging studies and serum tumor markers. Currently, serum alpha-fetoprotein (AFP), a fetal-specific glycoprotein, has undoubtedly been the most widely used tumor marker for the detection and monitoring of HCC. However, serum AFP is

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not always elevated to a diagnostic level in all patients, particularly in small HCC and a considerable numbers of patients with more advanced stages would be missed unless another diagnostic tool is used[2,3]. Furthermore, it may be also elevated in benign chronic liver diseases, including chronic hepatitis and cirrhosis, as well as in other primary and secondary liver cancers[2,3]. As a result, the identification of novel serum markers for diagnosis of HCC is needed.

Glypican-3 (GPC3) belongs to the glypican family of glycosyl-phosphatidylinositol (GPI) anchored heparan sulphate proteoglycans, which plays an important role in cellular growth, cell differentiation, and cell migration[4]. GPC3 has been reported to be increased in HCC in comparison with pre-neoplastic lesions and cirrhotic tissues at the mRNA and protein levels[5-11]. Interestingly, GPC3 mRNA levels are more frequently elevated than those of AFP, with the difference even greater in small HCC[12]. These findings suggest the potential value of GPC3 as a novel tumor marker for the diagnosis of HCC. Thus, the aims of this study have been to investigate whether serum GPC3 represent a useful diagnostic tool for differentiating HCC from benign chronic liver disease (CLD), and whether, in association with AFP, serum GPC3 improves the diagnostic accuracy of HCC.

Materials and Methods

Subjects

Sera for the measurement of GPC3 levels were obtained from 3 groups of subjects who were attending King Chulalongkorn Memorial Hospital from October 2005 to December 2006.

Group 1 consisted of 10 adult healthy subjects (7 males, mean age 33.3 (8.4 years).

Group 2 consisted of 39 patients with CLD (22 males, mean age 48.0 (15.5 years). The diagnosis in this group was based on histopathology and/or clinical features such as the presence of ascites, or esophageal varices.

Group 3 consisted of 60 patients with HCC (46 males, mean age 59.6 (13.5 years). The diagnosis of HCC was based on histopathology (FNA, core liver biopsy or surgical resection) and/or typical imaging studies that show focal lesion with arterial hypervascularization. The clinicopathological data of the patients in this group at initial diagnosis were collected, which included sex, age, liver function tests, Child-Pugh classification, tumor size, number of tumors, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score[13].

All subjects were informed about the objective of the study, and subsequently provided their consents. Blood was obtained during investigation at the initial presentation; sera were separated by centrifugation and stored at -70˚C until tested for GPC3 level. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University.

Measurement of serum GPC3 levels

Serum GPC3 levels were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) method. Briefly, Microtiter plates (Maxisorp, Nunc) were coated at 4˚C overnight with 1.6 µg/ml anti-human GPC3 (100 (l/well) in the coating buffer. Uncoated area was then blocked with 1% (w/v) BSA (150 µl/well) for 60 min at 37˚C. After washing, 100 µl of sample or standard (recombinant human glypican-3 9.76-5,000 ng/ml) were added. After incubation for 60 min at 37˚C, plates were washed and added the biotinylated anti-human glypican-3 (100 µl/well; 1:500) and incubated for 60 min at 37˚C. After washing the peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1:2,000) was added and incubated for 60 min at 37˚C. The plates were washed again and then the peroxidase substrate (OPD; 100 µl/well) was added and incubated at 37˚C for 15-20 min to allow the color developed. The reaction was stopped by addition of 50 µl of 4 M H_2SO_4. The absorbance at 492 nm was measured using the Titertek Multiskan M340 multiplate reader.

Measurement of serum AFP levels

AFP levels in parallel serum samples were determined using a commercially available ELISA kit (Cobus®Core, Roche Diagnostics, Basel, Switzerland).

Statistical Analysis

Data are expressed as percentage, mean, and standard deviation. Comparisons between groups were analyzed by the χ^2 or Fisher's exact test for categorical variables and by the Mann-Whitney test or Student's t test when appropriate for quantitative variables. Pearson correlation coefficient was used to find the correlation between the serum levels of GPC3 and AFP. P values <0.05 for a two-tailed test were considered statistically significant. All statistical analyses

were performed using the SPSS software for windows 10.0 (SPSS Inc., Chicago, IL).

RESULTS

Serum levels of GPC3 and AFP

In this study, we found that 47% of patients with HCC (28 of 60) had elevated levels of serum GPC3 with values ranging from 35.5 to 6,547.9 ng/mL, whereas GPC3 was undetectable in the other groups The mean concentration of serum GPC3 in HCC group (374.6 ± 1,212.9 ng/mL) was significantly higher than that in the controls and CLD ($p < 0.05$) (Table 1).

Serum AFP levels were also measured in the same set of serum samples. Using a cutoff value of 20 ng/mL, AFP values were normal in all healthy individuals, whereas values were elevated in 7 of 39 (18%) patients with CLD. Considering the patients with HCC, AFP was elevated in 34 of 60 patients (57%). The mean concentration of serum AFP in HCC group (19,402.8 ± 64,318.5 ng/mL) was significantly higher than that in the controls and CLD ($p < 0.05$) (Table 1).

Serum markers to differentiate HCC from the other groups

In most cases of HCC, elevated GPC3 values did not correlate with elevated AFP values (Pearson correlation coefficient for GPC3 and AFP values, -0.021; P = 0.79). Thus, the simultaneous use of the two markers significantly increased the sensitivity of the diagnosis; at a cutoff value of 20 ng/mL for AFP, 48 of 60 (80%) of the patients have elevated GPC3 or AFP levels. The sensitivity, specificity, and overall accuracy of GPC3, AFP and combined tests in differentiating HCC from CLD are shown in Table 2.

Correlation of serum marker level with disease characteristics

To evaluate the association between serum GPC3 levels and clinical features, the patients with HCC were divided into two groups based on the detection of the marker. Accordingly, there were 28 patients with detectable serum GPC3, and 32 patients with undetectable levels. The correlations between groups and various clinical parameters listed in Table 3 were analyzed. There was no significant correlation between serum GPC3 level and patient age (P = 1.000), gender (P = 0.770), etiology of liver disease (P = 0.128), tumor size (P = 0.312), tumor type (P = 0.398), the presence of venous invasion (P = 1.000), extrahepatic metastasis (P = 0.069), and CLIP score (P = 0.330).

Likewise, the patients with HCC were divided into two groups based on the levels of serum AFP (a cutoff value of 20 ng/mL). There was no significant correlation between serum AFP level and patient age (P = 0.800), gender (P = 0.235), etiology of liver disease (P = 0.795), tumor type (P = 0.321), the presence of venous invasion (P = 0.157) and extrahepatic metastasis (P = 0.719). However, high serum AFP levels were significantly associated with tumor size (P = 0.015)

Table 1 Clinical characteristics of the subjects in this study

Group	No	Age (yr)	Sex (M/F)	AFP (ng/mL)	GPC3 (ng/mL)
Controls	10	33.3 ± 8.4	7/3	4.7 ± 2.3	0
CLD	39	48.0 ± 15.5	22/17	33.2 ± 134.0	0
HCC	60	59.6 ± 13.5	46/14	19402.8 ± 64318.5	374.6 ± 1212.8

Controls = healthy volunteers; CLD = chronic liver disease; HCC = hepatocellular carcinoma. Data express as mean ± standard deviation.

Table 2 Serum GPC3, AFP and their combination for differentiation between HCC and CLD

	Sensitivity (%)	Specificity (%)	Accuracy (%)
GPC3*	47	100	68
AFP**	57	82	67
Combination	80	82	81

*Detectable serum GPC3; **Serum AFP at a cutoff value of 20 ng/mL

Table 3 Relationship between serum GPC3 and AFP levels and clinical features in patients with HCC

Variables	GPC3		AFP (>20 ng/mL)	
	Positive (%)	P value	(%)	P value
Age (yrs)				
≥60 (n = 31)	14	1.000	17	0.800
<60 (n =29)	14		17	
Gender				
Male (n = 46)	22	0.770	24	0.235
Female (n = 14)	6		10	
Etiology of liver disease				
HBV positive (n = 32)	18	0.128	19	0.795
HBV negative (n = 28)	10		15	
Tumor type				
Uninodular (n = 18)	6	0.398	9	0.321
Multinodular (n = 17)	9		8	
Massive (n = 25)	13		17	
Tumor size				
≤5 cm (n = 10)	3	0.312	2	0.015*
>5 cm (n = 50)	25		32	
Venous invasion				
Presence (n = 18)	8	1.000	13	0.157
Absence (n = 42)	20	21		
Extrahepatic metastasis				
Presence (n = 8)	7	0.069	6	0.719
Absence (n = 52)	21	28		
CLIP score				
Score 0-1 (n = 16)	5	0.330	7	0.024*
Score 2-3 (n = 26)	13		12	
Score 4-6 (n = 18)	10		15	

and high CLIP score (P = 0.024).

DISCUSSION

The progression of HCC is a multistage process with a large proportion of cases involving cirrhosis and viral hepatitis infection[1]. In hyperendemic areas like Thailand, infection rates of HBV have exceeded 50% in patients with chronic hepatitis and cirrhosis, which reflects a potential risk for the future development of HCC[14]. So far, AFP measurement has been the only marker routinely used for detecting and monitoring HCC. AFP is a glycoprotein expressed abundantly in fetal liver but not in normal adult liver and is re-expressed by HCC as it dedifferentiates from a pre-malignant lesion in the cirrhotic liver through well-differentiated and moderately differentiated HCC to poorly differentiated HCC[15]. Generally, AFP shows high sensitivity but also high false-positivity. At a cut-off value of 20 ng/mL, serum AFP shows a 60%-80% sensitivity, although this sensitivity decreases to about 40% for the detection of small tumors[2,3,16]. In addition, a significant increase in serum AFP level (20-200 ng/mL) is detected in a considerable number of patients with chronic liver disease, including approximately 15%-60% of patients with chronic hepatitis and approximately 10%-50% with cirrhosis[2,3]. In this study, the sensitivity and specificity of AFP at a cutoff value of 20 ng/mL were 57% and 82%, respectively.

In this study, we found that whereas GPC3 is undetectable in the serum of healthy subjects and patients with CLD, its levels were increased in 28 of 60 patients (47%) with HCC. Thus, the sensitivity and the specificity of this serum maker from our study were

47% and 100%, respectively. These data were in agreement with previous reports from Canada and Japan[5,8], where chronic HCV infection is the major etiological factor of HCC. Capurro, *et al*.[5] showed that GPC3 was undetectable in sera of healthy donors and patients with hepatitis, but its levels were significantly increased in 18 of 34 patients with HCC. In addition, only 1 of 20 patients with cirrhosis displayed elevated levels of serum GPC3. Therefore, the sensitivity and the specificity of GPC3 were 53% and 95%, respectively. Similarly, Nakatsura, *et al*.[8] detected circulating GPC3 in sera of 40% (16/40) HCC patients using Western blotting and ELISA, but not in those of patients with other benign liver diseases. As a result, the sensitivity and the specificity of GPC3 were 40% and 100%, respectively. Given the recognized heterogeneity of HCC among different geographic areas and etiological factors, the results presented here confirm the very high specificity of GPC3, though its sensitivity is relatively low when using as a single serum marker.

Nonetheless, the simultaneous use of GPC3 and AFP (a cutoff value of 20 ng/mL) significantly increased the sensitivity of the test without compromising specificity. These data were in concordance with previous studies those demonstrated that the combined tests significantly increased the sensitivity for HCC diagnosis[5,8]. These could be explained by the fact that in most cases of HCC there was no correlation between GPC3 and AFP values. Another important issue to be addressed is whether GPC3 will be a better or complementary marker for the detection of small HCC than AFP. Unlike AFP, GPC3 exhibited no significant correlation between its levels and tumor size or tumor stage classified by the CLIP score, as shown in this study. Moreover, it was demonstrated that the expression of GPC3 in liver tissue was significantly greater than that of AFP in small HCC[5,12]. Although our report was a cross-sectional study and was not designed to address this question, we showed that a higher proportion of patients with HCC <5 cm had positive GPC3 values compared with AFP at the cut-off 20 ng/mL (30% and 20%, respectively). In addition, the combination of these markers yielded an improved sensitivity for detecting small HCC to 50%. Studies including a larger number of patients will certainly be required to confirm this observation.

In conclusion, our study showed that serum GPC3 levels were significantly elevated in patients with early and advanced HCC, but not in patients with CLD, and healthy controls. There was no positive correlation of serum GPC3 levels with circulating AFP, indicative of the complementary role of the two markers. Thus, further studies are worthwhile to elucidate the role of these markers in the screening of high-risk groups residing in the endemic area.

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REFERENCES

1. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003; 362: 1907-17.
2. Spangenberg HC, Thimme R, Blum HE. Serum markers of hepatocellular carcinoma. Semin Liver Dis 2006; 26: 385-90.
3. Yuen MF, Lai CL. Serological markers of liver cancer. Best Pract Res Clin Gastroenterol 2005; 19: 91-9.
4. Filmus J, Selleck SB. Glypicans: proteoglycans with a surprise. J Clin Invest 2001; 108: 497-501.
5. Capurro M, Wanless IR, Sherman M, *et al*. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology 2003; 125: 89-97.
6. Libbrecht L, Severi T, Cassiman D, *et al*. Glypican-3 expression distinguishes small hepatocellular carcinomas from cirrhosis, dysplastic nodules, and focal nodular hyperplasia-like nodules. Am J Surg Pathol 2006; 30: 1405-11.
7. Llovet JM, Chen Y, Wurmbach E, *et al*. A molecular signature to discriminate dysplastic nodules from early hepatocellular carcinoma in HCV cirrhosis. Gastroenterology 2006; 131: 1758-67.
8. Nakatsura T, Yoshitake Y, Senju S, *et al*. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003; 306: 16-25.
9. Wang XY, Degos F, Dubois S, *et al*. Glypican-3 expression in hepatocellular tumors: diagnostic value for preneoplastic lesions and hepatocellular carcinomas. Hum Pathol 2006; 37: 1435-41.
10. Yamauchi N, Watanabe A, Hishinuma M, *et al*. The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod Pathol 2005; 18: 1591-8.
11. Zhu ZW, Friess H, Wang L, *et al*. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut 2001; 48: 558-64.

12. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. Cancer Res 1997; 57: 5179-84.
13. Prospective validation of the CLIP score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. The Cancer of the Liver Italian Program (CLIP) Investigators. Hepatology 2000; 31: 840-5.
14. Tangkijvanich P, Hirsch P, Theamboonlers A, *et al*. Association of hepatitis viruses with hepatocellular carcinoma in Thailand. J Gastroenterol 1999; 34: 227-33.
15. Taketa K. Alpha-fetoprotein: reevaluation in hepatology. Hepatology 1990; 12: 1420-32.
16. Tangkijvanich P, Anukulkarnkusol N, Suwangool P, *et al*. Clinical characteristics and prognosis of hepatocellular carcinoma: analysis based on serum alpha-fetoprotein levels. J Clin Gastroenterol 2000; 31: 302-8.