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Diagnostic role of serum glypican-3 in differentiating hepatocellular carcinoma from non-malignant chronic liver disease and other liver cancers

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Abstract

Background and Aims: The role of glypican-3 (GPC3), a novel serum marker, in differentiating hepatocellular carcinoma (HCC) from non-malignant chronic liver disease and other malignant space-occupying lesions in the liver is largely unknown. The aims of this study were to evaluate its diagnostic role and clinical correlations in patients with HCC.
Methods: Six groups were studied which included 40 healthy subjects, 50 patients with chronic hepatitis (CH), 50 patients with liver cirrhosis (LC), 100 patients with HCC, 50 patients with intrahepatic cholangiocarcinoma (ICC) and 50 patients with metastatic carcinoma (MCA). Serum GPC3 levels were measured by using a sandwich enzyme-linked immunosorbent assay method.
Results: Fifty-three percent of HCC patients had elevated serum GPC3 levels with values ranging 35.5–7826.6 ng/mL. The serum marker was undetectable in other groups except one patient (2%) with LC and another patient (2%) with MCA. In most cases of HCC, elevated GPC3 values did not correlate with α-fetoprotein (AFP) levels. Detectable GPC3 was significantly correlated with the presence of viral hepatitis markers but was not correlated with tumor size and stage of HCC. Serum GPC3 was superior to AFP in detecting small HCC (56.3% and 31.3%, respectively). A combination of serum GPC3 and AFP yielded an improved sensitivity for detecting small HCC to 75%.
Conclusion: Serum GPC3 is highly specific for detecting HCC. The combined use of serum GPC3 and AFP provides a potentially promising tool to better differentiate HCC from benign liver disorders, as well as from other liver cancers.

Introduction

Hepatocellular carcinoma (HCC) represents one of the most common cancers worldwide, particularly in Southeast Asia, where hepatitis B virus (HBV), and to a lesser extent, hepatitis C virus (HCV) infection are prevalent.[1] In Thailand, HCC is the most common malignant tumor, with an incidence of 6.8/100 000 in men and 2.3/100 000 in women per year.[2] Most patients with HCC are diagnosed at advanced stages and thus the prognosis is generally poor. The diagnosis of HCC could be achieved at an earlier stage by regular screening programs among high-risk populations by using imaging studies and serum tumor markers. Currently, serum α-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 not always elevated to a diagnostic level in all patients, particularly in small HCC, and considerable numbers of patients with more advanced stages would be missed unless another diagnostic tool is used.[3,4] Moreover, its level may be elevated in non-malignant chronic liver diseases, including chronic hepatitis and cirrhosis, as well as in other primary and secondary liver cancers.[3,4] Therefore, the identification of alternative serum markers of HCC is needed.

Glypican-3 (GPC3) belongs to the glypican family of glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans, which plays an important role in cellular growth, differentiation and migration.[5] GPC3 has been reported to be increased in HCC in comparison with pre-neoplastic lesions and cirrhotic tissues at the mRNA and protein levels.[6–12] Interestingly, GPC3 mRNA levels are more frequently elevated than those of AFP, with the difference even greater in small HCC.[13] In contrast, GPC3 has been shown to be downregulated in various cancers, including breast cancer, ovarian cancer, lung adenocarcinoma and

cholangiocarcinoma.[14–17] Given the detection of circulating GPC3, it appears that this oncofetal protein can serve as a potential serum marker for the diagnosis of HCC.[6,9,18] However, data on its clinical correlations in patients with HCC are currently unknown. Furthermore, the role of this marker in differentiating HCC from other malignant space-occupying lesions in the liver has not yet been investigated. To address these issues, we examined whether serum GPC3 represented a useful diagnostic tool for differentiating HCC from benign chronic liver disease and from other liver cancers, including intrahepatic cholangiocarcinoma (ICC) and metastatic carcinoma (MCA). We also examined whether, in combination with AFP, serum GPC3 improved the diagnostic accuracy of HCC.

Methods

Patients and blood samples

Serum samples for the measurement of GPC3 and AFP levels were obtained from 100 consecutive patients (75 men, mean age 58.8 ± 12.7 years) who were diagnosed with HCC for the first time at King Chulalongkorn Memorial Hospital between October 2005 and August 2007. The diagnosis of HCC was based on typical imaging studies and/or histopathology (fine-needle aspiration, core liver biopsy or surgical resection) according to American Association for the Study of Liver Diseases (AASLD) guidelines.[19] Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic computed tomography (CT) or magnetic resonance imaging (MRI). In cases without typical imaging features, liver biopsy was performed to confirm the diagnosis of HCC. In this study, 39 patients were diagnosed with HCC based on histology and the remaining 61 cases were diagnosed by typical imaging patterns.

The clinicopathological data of the patients in this group at initial diagnosis were collected, which included sex, age, liver function tests, Child–Pugh score, tumor size, number of tumors, venous invasion, extrahepatic metastasis, and HCC staging classified by the CLIP score.[20] The CLIP score includes the variables of Child–Pugh classification, tumor morphology, AFP level and the presence of vascular invasion. In this study, 87 patients with HCC had underlying liver cirrhosis, 59 patients were positive for serum hepatitis B surface antigen (HBsAg), 11 patients were positive for HCV antibody (anti-HCV), and 17 patients were associated with alcohol-dependence but negative for HBsAg and anti-HCV. For the remaining 13 patients, the underlying etiology of the liver could not be determined. According to the CLIP score at initial presentation, there were 21 patients in the score 0 subgroup, 13 patients in score 1, 24 patients in score 2, 10 in score 3, 17 in score 4, nine in score 5 and six in score 6. Thirty-three patients had venous invasion, while extrahepatic metastasis was found in 16 patients.

The control group was comprised of 40 healthy volunteers with no apparent liver disease (26 men, mean age 32.8 ± 8.2 years). The non-malignant chronic liver disease (CLD) group included 50 patients with chronic hepatitis (CH) (34 men, mean age 43.3 ± 14.3 years), and 50 patients with liver cirrhosis (LC), who had no evidence of HCC (30 men, mean age 48.6 ± 9.1 years). The diagnosis of CH was based on persistent elevation of alanine transaminase (ALT) levels and confirmed by histopathology. The diagnosis of LC was based on histopathology and/or clinical features such as the presence of ascites, or esophageal varices. HBsAg was positive in 36 and 32 patients with CH and LC, respectively, while anti-HCV was positive in six and seven patients with CH and LC, respectively.

The other liver cancer groups comprised 50 patients with ICC (28 men, mean age 60.8 ± 11.4 years), and 50 patients with MCA (29 men, mean age 51.8 ± 14.9 years). The ICC was diagnosed based on liver tumor features detected by ultrasound/CT scan and confirmed by histology. The diagnosis of MCA was established by clinical settings and confirmed by histopathology. In this study, there were 15 patients with colorectal cancer, seven patients with gastric carcinoma, seven patients with ovarian cancer, 10 patients with lung cancer, three patients with pancreatic carcinoma, one each with lymphoma, renal cell carcinoma and gallbladder cancer, while the primary sites of five patients were unknown.

All subjects were informed about the objective of the study, and subsequently provided their consent. 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 as described previously.[6] Briefly, Microtiter plates (Maxisorp; Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were coated at 4°C overnight with 1.6 mg/mL antihuman GPC3 (100 mL/well) in the coating buffer. The uncoated area was then blocked with 1% (w/v) bovine serum albumin (150 mL/well) for 60 min at 37°C. After washing, 100 mL of sample or standard (9.76–5000 ng/mL recombinant human glypican-3) were added. After incubation for 60 min at 37°C, plates were washed and added to the biotinylated anti-human glypican-3 (100 mL/well; 1:500) and incubated for 60 min at 37°C. After washing, the peroxidase-mouse monoclonal anti-biotin (100 mL/well; 1:2000) was added and incubated for 60 min at 37°C. The plates were washed again and then the peroxidase substrate (OPD; 100 mL/well) was added and incubated at 37°C for 15–20 min to allow the color to develop. The reaction was stopped by addition of 50 mL 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

Serum AFP levels 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-test or Fisher's exact test for categorical variables and by the Mann–Whitney test or Student's *t*-test when appropriate for quantitative variables. Receiver–operator curves (ROC) were constructed to evaluate the diagnostic performance of the serum markers in discriminating HCC from other groups. Sensitivity, specificity,

Table 1 Clinical characteristics and serum marker levels of the subjects

Group	*n*	Age† (years)	Sex (M/F)	HBsAg⁺/anti-HCV⁺	GPC3‡ (ng/mL)	AFP‡ (ng/mL)	AFP (ng/mL)		
							≤ 20	21–99	≥ 100
Controls	40	32.8 ± 8.2	26/14	0/0	0 (0–0)	6.1 (4.1–11.5)	40	0	0
CH	50	43.3 ± 14.3	34/16	36/6	0 (0–0)	8.0 (4.5–155)	43	5	2
LC	50	48.6 ± 9.1	30/20	32/7	0 (0–43.6)	11.2 (4.7–224)	34	13	3
HCC	100	58.8 ± 12.7	75/25	59/11	46.3 (0–7826.6)	105.5 (5.7–75000)	27	23	50
ICC	50	60.8 ± 11.4	28/22	0/0	0 (0–0)	10.0 (4.0–50.5)	45	5	0
MCA	50	55.9 ± 12.7	29/21	2/0	0 (0–202.5)	10.7 (4.7–155)	39	9	2

†Data express as mean ± standard deviation. ‡Data express as median (ranges). CH, chronic hepatitis; controls, healthy volunteers; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICC, intrahepatic cholangiocarcinoma; LC, liver cirrhosis; MCA, metastatic carcinoma.

positive and negative predictive values and diagnostic accuracy were calculated in accordance with standard methods. Pearson's correlation coefficient was used to find the correlation between the serum levels of GPC3 and AFP. The logistic regression analysis was performed to evaluate the clinicopathological parameters associated with elevation of serum GPC3 and AFP levels. $P < 0.05$ for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for Windows ver. 14.0 (SPSS, Chicago, IL, USA).

Results

Clinical characteristics

Table 1 compares clinical characteristics of the subjects enrolled in this study. Patients with HCC, ICC and MCA were significantly older than those with CH, LC and healthy controls ($P < 0.001$). However, there was no significant difference in mean age between patients with HCC, ICC and MCA. Patients with LC and CH were significantly older than healthy controls ($P < 0.001$). Likewise, the mean age of patients with LC was significantly higher than that of patients with CH ($P = 0.028$). In this study, there was no difference in sex distribution between groups ($P = 0.155$). The prevalence of HBV and HCV infection was significantly higher in patients with HCC, LC and CH than that of patients with ICC, MCA and healthy controls ($P < 0.001$). However, there was no significant difference in viral hepatitis markers between patients with HCC, LC and CH.

Serum GPC3 and AFP concentrations

In this study, we found that 53 of 100 (53%) patients with HCC had elevated levels of serum GPC3 with values ranging 35.5–7826.6 ng/mL. GPC3 was undetectable in the other groups except one patient with LC (the concentration of GPC3 was 43.6 ng/mL) and another patient with squamous cell carcinoma of the lung (the concentration of GPC3 was 202.5 ng/mL). The level of serum GPC3 in patients with HCC was significantly higher than those of healthy controls, CH, LC, ICC and MCA ($P = 0.001$) (Table 1, Fig. 1a). There was no significant difference in the prevalence of detectable serum GPC3 between cirrhotic HCC (47 of 87 cases; 54.0%) and non-cirrhotic HCC (six of 13 cases; 46.2%) ($P = 0.767$). In addition, there was no significant difference in the median level of serum GPC3 between these two groups (189.0 and 117.5 ng/mL, respectively, $P = 0.581$).

Serum AFP levels were also measured in the same set of serum samples. The level of serum AFP in patients with HCC was significantly higher than those of healthy controls, CH, LC, ICC and MCA ($P = 0.002$) (Fig. 1b). Using the normal upper limit of AFP (20 ng/mL) as a reference, AFP was elevated in 73 (73%) patients with HCC. AFP values were within normal range in all healthy individuals, whereas values were elevated in seven (14.0%) patients with CH, 16 (32.0%) patients with LC, five (10%) patients with ICC and 11 (22%) patients with MCA. When using 100 ng/mL as a cut-off point, AFP concentration was elevated in 50 (50%) patients with HCC, two (4%) patients with CH, three (6%) patients with LC and two (4%) patients with MCA (Table 1).

Serum GPC3 and AFP as diagnostic markers

The ROC for GPC3 and AFP were generated on the same graph to compare the diagnostic accuracies of the two markers. As shown in Figure 2(a), the area under the curve of HCC and non-malignant chronic liver disease (CH and LC) was 0.767 (95% confidence interval [CI] 0.700–0.835] for GP3 and 0.855 (95% CI 0.804–0.906) for AFP. There was no significant difference between these two areas. Similarly, the area under the ROC of HCC and other liver cancers (ICC and MCA) was 0.765 (95% CI 0.697–0.833) for GP3 and 0.859 (95% CI 0.807–0.910) for AFP, which were not statistically different (Fig. 2b).

Based on the ROC analysis, the optimal cut-off points for AFP to differentiate HCC from non-malignant chronic liver disease and other liver cancers were 19 and 22 ng/mL, respectively. To simplify the analysis, we selected an AFP level of 20 ng/mL as the optimal cut-off point. At this concentration, the sensitivity, specificity and accuracy for differentiating HCC from non-malignant chronic liver disease were 73%, 77% and 75%, respectively. At a cut-off value of 100 ng/mL, the sensitivity, specificity and accuracy for differentiating HCC from non-malignant chronic liver disease were 50%, 95% and 72.5%, respectively. To differentiate HCC from other liver cancers, serum AFP at a cut-off value of 20 ng/mL exhibited a sensitivity, specificity and accuracy for diagnosing HCC of 73%, 84% and 78.5%, respectively. Similarly, at cut-off values of 100 ng/mL, the sensitivity, specificity and

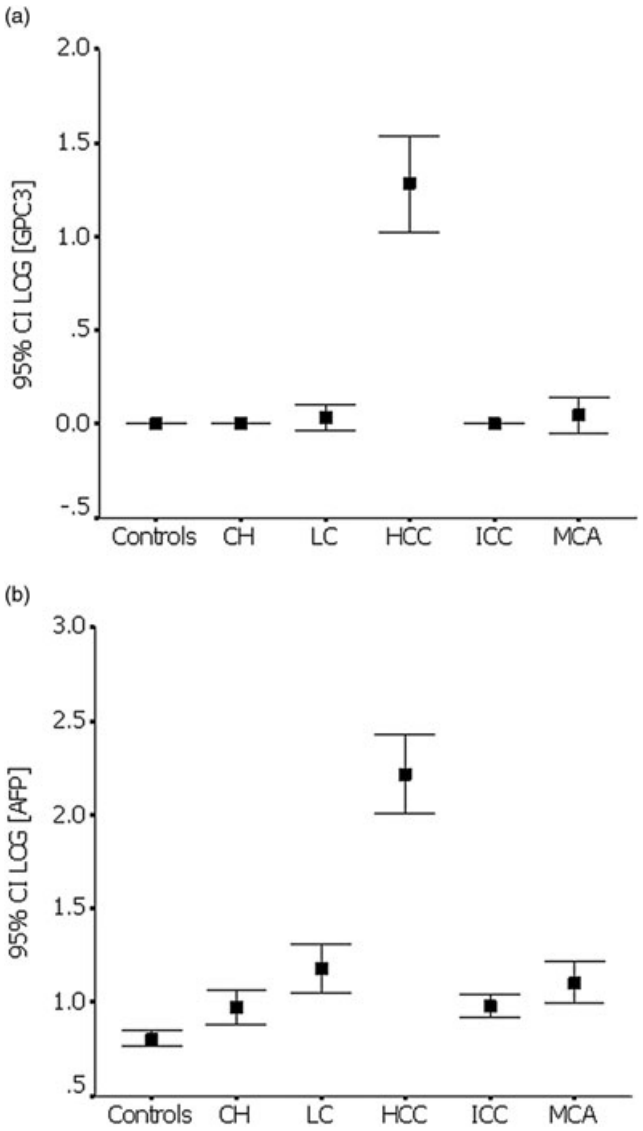

Figure 1 Serum glypican-3 (GPC3) and α-fetoprotein (AFP) levels in each group of patients. (a) 95% confidence interval of $\log_{10}$ (GPC3). (b) 95% confidence interval of $\log_{10}$ (AFP). CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; MCA, metastatic carcinoma.

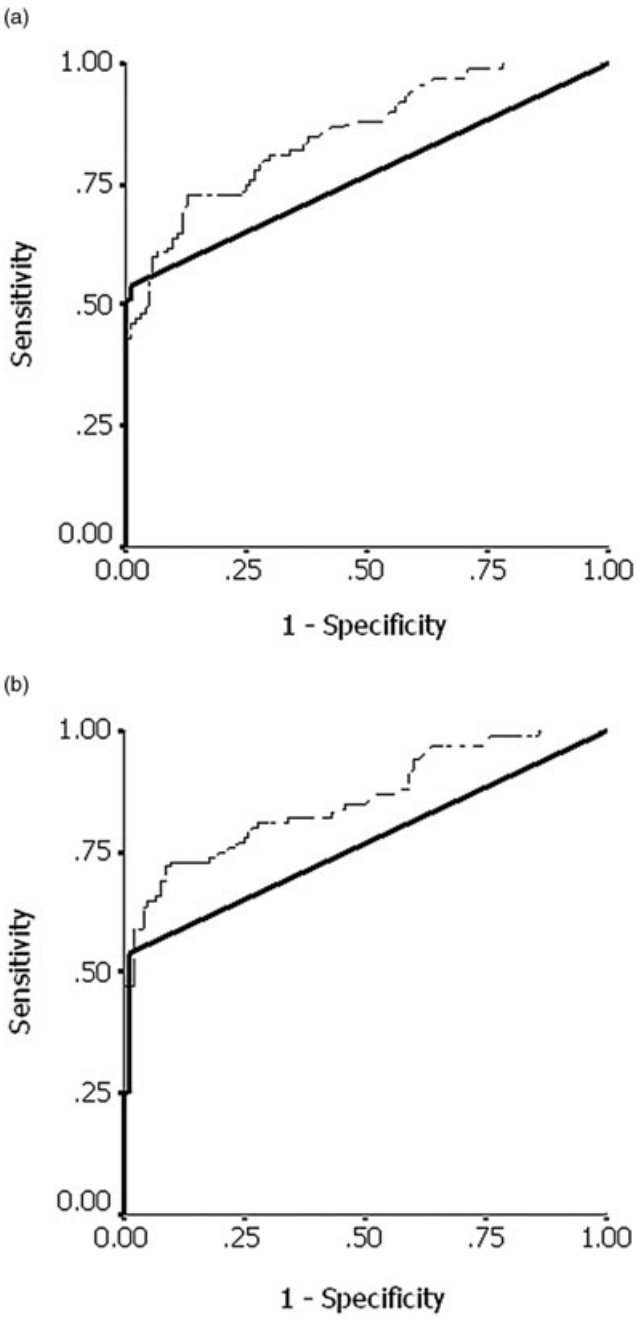

Figure 2 Receiver–operator curves of serum glypican-3 (GPC3) and α-fetoprotein (AFP) in differentiating hepatocellular carcinoma (HCC) from other groups. (a) HCC and non-malignant chronic liver disease (the areas under the curve for GPC3 and AFP were 0.767 and 0.855, respectively). (b) HCC and other liver cancers (the areas under the curve for GPC3 and AFP were 0.765 and 0.859, respectively). (a) — AFP, --- GPC3. (b) — AFP, --- GPC3.

accuracy of serum AFP for differentiating HCC from other liver cancers were 50%, 98% and 74%, respectively (Table 2).

In this study, there was no correlation between GPC3 and AFP values (Pearson's correlation coefficient for GPC3 and AFP values, –0.028; $P = 0.779$). As a result, the combined use of GPC3 and AFP significantly increased the sensitivity of the diagnosis of HCC. At a cut-off value of 20 ng/mL for serum AFP, 88 (88%) of the HCC patients had either elevated GPC3 or AFP levels, while the specificity were 76–84%. At a cut-off value of 100 ng/mL for serum AFP, the sensitivity and specificity of the combined tests were 78% and 94–97%, respectively. The sensitivity, specificity, positive and negative predictive values, and accuracy of GPC3, AFP and combined tests in differentiating HCC from the other groups are shown in Table 2.

Regarding small HCC (tumor size < 3 cm in diameter), we found that nine of 16 (56.3%) patients had elevated levels of

Table 2 Serum glypican-3 (GPC3), α-fetoprotein (AFP) and their combination for differentiation between hepatocellular carcinoma (HCC) and other groups

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
HCC and CLD					
GPC3	53.0	99.0	98.1	67.8	76.0
AFP20	73.0	77.0	76.0	74.0	75.0
AFP100	50.0	95.0	90.9	65.5	72.5
GPC3 and AFP20	88.0	76.0	78.6	86.4	82.0
GPC3 and AFP100	78.0	94.0	92.9	81.0	86.0
HCC and other liver cancers					
GPC3	53.0	99.0	98.1	67.8	76.0
AFP20	73.0	84.0	82.0	75.7	78.5
AFP100	50.0	98.0	96.2	66.2	74.0
GPC3 and AFP20	88.0	84.0	84.6	87.5	86.0
GPC3 and AFP100	78.0	97.0	96.3	81.5	87.5

AFP100, AFP at a cut-off value of 100 ng/mL; AFP20, AFP at a cut-off value of 20 ng/mL; CLD, non-malignant chronic liver disease (chronic hepatitis and cirrhosis) Other liver cancers (intrahepatic cholangiocarcinoma and metastatic carcinoma); GPC3, detectable serum GPC3; NPV, negative predictive value; PPV, positive predictive value.

Table 3 Sensitivity of serum glypican-3 (GPC3), α-fetoprotein (AFP) and their combination in relation to size of hepatocellular carcinoma

Tumor size	GPC3	AFP20	AFP100	GPC3 and AFP20	GPC3 and AFP100
< 3 cm ($n = 16$)	9 (56.3)	5 (31.3)	5 (31.3)	12 (75.0)	11 (68.8)
3–5 cm ($n = 16$)	8 (50.0)	8 (50.0)	4 (25.0)	13 (81.3)	11 (68.8)
5–10 cm ($n = 33$)	17 (51.6)	24 (72.7)	18 (54.5)	31 (93.9)	27 (81.8)
> 10 cm ($n = 35$)	19 (54.3)	33 (94.3)	23 (65.7)	33 (94.3)	32 (91.4)

Data expressed as *n* (%). AFP100, AFP at a cut-off value of 100 ng/mL; AFP20, AFP at a cut-off value of 20 ng/mL; GPC3, detectable serum GPC3.

serum GPC3, whereas five (31.3%) patients had elevated serum AFP at cut-off values of 20 and 100 ng/mL, respectively. When both serum GPC3 and AFP (at cut-off values of 20 and 100 ng/mL, respectively) were determined in parallel, the sensitivity of the combined tests in detecting small HCC were 75% and 68.8%, respectively. The sensitivity of serum GPC3, AFP and their combination in relation with size of HCC is showed in Table 3.

Correlation of serum marker levels 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 53 patients with detectable serum GPC3 and 47 patients with undetectable levels. The correlations between groups and various clinical parameters listed in Table 4 were analyzed. There was no significant correlation between serum GPC3 level and patient age, sex, Child–Pugh score, tumor size, tumor type, the presence of venous invasion, extrahepatic metastasis and CLIP score. However, detectable serum GPC3 level was significantly found in patients who had positive viral hepatitis markers (HBsAg and/or anti-HCV) ($P = 0.016$).

To investigate whether necroinflammatory activity of chronic hepatitis B and C might affect the detection of serum GPC3 in patients with HCC, we examined the histology activity index (HAI) of liver specimens according to the criteria of Knodell *et al.*[21] which comprise two major components, namely, necroinflammation (HAI-I) and fibrosis (HAI-F). In this respect, 28 liver specimens of patients with HCC were available for the analysis. There was no significant difference in HAI-I score between patients with detectable serum GPC3 (16 cases) and those without detectable serum protein (12 cases) (mean HAI-I scores were 6.6 ± 2.4 and 6.2 ± 2.3, respectively, $P = 0.656$). In addition, there was no correlation between serum GPC3 level and HAI-I score of the corresponding liver specimens (Pearson's correlation coefficient, –0.196; $P = 0.318$).

Similarly, the patients with HCC were divided into two groups based on the levels of serum AFP at cut-off values of 20 and 100 ng/mL, which represented the optimal point from the ROC analysis and median value in HCC patients, respectively. At a cut-off value of 20 ng/mL, there was no significant correlation between serum AFP level and patient age, sex, Child–Pugh score, etiology of liver disease, venous invasion and extrahepatic metastasis. However, high serum AFP levels were significantly associated with tumor type ($P = 0.005$), tumor size ($P < 0.001$) and CLIP score ($P < 0.001$) (Table 5.1). At a cut-off value of 100 ng/mL, there was no significant correlation between serum AFP level and patient age, sex, Child–Pugh score, etiology of liver disease, tumor type and extrahepatic metastasis. However, high serum AFP levels were significantly associated with tumor size ($P = 0.005$), the presence of venous invasion ($P = 0.010$) and CLIP score ($P = 0.001$) (Table 5.2).

Table 4 Relationship between serum glypican-3 (GPC3) levels and clinical features of patients with hepatocellular carcinoma

Variables	GPC3 Positive	GPC3 Negative	*P*
Age (yrs)			
≥ 60 (*n* = 48)	23	25	0.423
< 60 (*n* = 52)	30	22	
Sex			
Male (*n* = 75)	41	34	0.646
Female (*n* = 25)	12	13	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	43	27	0.016
Non-viral (*n* = 30)	10	20	
Child–Pugh score			
A (*n* = 45)	21	24	0.470
B (*n* = 40)	24	16	
C (*n* = 15)	8	7	
Tumor type			
Uninodular (*n* = 31)	15	16	0.799
Multinodular (*n* = 30)	16	14	
Massive (*n* = 39)	22	17	
Tumor size			
≤ 5 cm (*n* = 32)	17	15	1.000
> 5 cm (*n* = 68)	36	32	
Venous invasion			
Presence (*n* = 33)	17	16	1.000
Absence (*n* = 67)	36	31	
Extrahepatic metastasis			
Presence (*n* = 16)	10	6	0.430
Absence (*n* = 84)	43	41	
CLIP score			
Score 0–1 (*n* = 34)	18	16	0.605
Score 2–3 (*n* = 34)	16	18	
Score 4–6 (*n* = 32)	19	13	

HBV, hepatitis B virus; HCV, hepatitis C virus.

Table 5.1 Relationship between serum α-fetoprotein (AFP) levels (cut-off value of 20 ng/mL) and clinical features of patients with hepatocellular carcinoma

Variables	AFP (≥ 20 ng/mL)	AFP (< 20 ng/mL)	*P*
Age (yrs)			
≥ 60 (*n* = 48)	39	9	0.074
< 60 (*n* = 52)	33	19	
Sex			
Male (*n* = 75)	55	20	0.615
Female (*n* = 25)	17	8	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	50	20	1.000
Non-viral (*n* = 30)	22	8	
Child–Pugh score			
A (*n* = 45)	30	15	0.326
B (*n* = 40)	29	11	
C (*n* = 15)	13	2	
Tumor type			
Uninodular (*n* = 31)	20	11	0.005
Multinodular (*n* = 30)	17	13	
Massive (*n* = 39)	35	4	
Tumor size			
≤ 5 cm (*n* = 32)	13	19	< 0.001
> 5 cm (*n* = 68)	59	9	
Venous invasion			
Presence (*n* = 33)	28	5	0.058
Absence (*n* = 67)	44	23	
Extrahepatic metastasis			
Presence (*n* = 16)	14	2	0.223
Absence (*n* = 84)	58	26	
CLIP score			
Score 0–1 (*n* = 34)	17	17	< 0.001
Score 2–3 (*n* = 34)	24	10	
Score 4–6 (*n* = 32)	31	1	

HBV, hepatitis B virus; HCV, hepatitis C virus.

Clinical parameters, including age, sex, etiology of liver disease, Child–Pugh score, tumor type, tumor size, venous invasion, extrahepatic metastasis and the CLIP score, were entered into the multivariate logistic regression analysis to evaluate the factors associated with elevation levels of the serum markers. The multivariate analysis revealed that presence of viral hepatitis marker(s) (serum HBsAg and/or anti-HCV positive) was the independent factor of predicting detectable serum GPC3 level. In contrast, large tumor size and high CLIP score represented the independent factors of predicting high serum AFP level in patients with HCC (Table 6).

Discussion

The progression of HCC is a multistage process with a large proportion of cases involving underlying cirrhosis.[1] In endemic areas of viral hepatitis like Thailand, infection rates of HBV have exceeded 50% of patients with chronic liver disease, which reflects a potential risk for the future development of HCC.[22] Currently, the measurement of serum AFP level 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 it can be re-expressed by the tumor cells according to their differentiation.[23] Although AFP has high sensitivity in detecting HCC, it exhibits high false-positivity. For example, serum AFP at a cut-off value of 20 ng/mL shows a 60–80% sensitivity, although this sensitivity decreases to approximately 20–40% for the detection of small tumors.[24] 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.[3,4] In the present study, the sensitivity and specificity of AFP for differentiating HCC from benign liver disease at a cut-off value of 20 ng/mL were 73% and 77%, respectively. Apart from AFP, other serological markers such as the *Lens culinaris* agglutinin-reactive α-fetoprotein (AFP-L3) and des-γ-carboxy-prothrombin (DCP) have been developed to improve the accuracy. However, these serum markers are not widely used in clinical practice.

In this study, we found that serum GPC3 levels were increased in 53% patients with HCC but was increased in only one patient with non-malignant chronic liver disease. Thus, the sensitivity and

the specificity of this serum maker for differentiating HCC from benign liver disease were 53% and 99%, respectively. These data were in agreement with previous reports from Canada and Japan,[6,9] where chronic HCV infection is the major etiological factor of HCC. Capurro *et al.*[6] demonstrated 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 one 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.*[9] demonstrated that circulating GPC3 was found in sera of 40% (16/40) of HCC patients, but could not be detected in patients with benign liver diseases. As a result, the sensitivity and the specificity of GPC3 in that study were 40% and 100%, respectively. Collectively, previous data and our results confirm a very high specificity of GPC3 in differentiating HCC from non-malignant chronic liver disease.

Table 5.2 Relationship between serum α-fetoprotein (AFP) levels (cut-off value of 100 ng/mL) and clinical features of patients with hepatocellular carcinoma

Variables	AFP (≥ 100 ng/mL)	AFP (< 100 ng/mL)	*P*
Age (yrs)			
≥ 60 (*n* = 48)	24	24	1.000
< 60 (*n* = 52)	26	26	
Sex			
Male (*n* = 75)	35	40	0.356
Female (*n* = 25)	15	10	
Underlying liver disease			
HBV- or HCV-related (*n* = 70)	32	38	0.275
Non-viral (*n* = 30)	18	12	
Child–Pugh score			
A (*n* = 45)	18	27	0.076
B (*n* = 40)	21	19	
C (*n* = 15)	11	4	
Tumor type			
Uninodular (*n* = 31)	12	19	0.150
Multinodular (*n* = 30)	14	16	
Massive (*n* = 39)	24	15	
Tumor size			
≤ 5 cm (*n* = 32)	9	23	0.005
> 5 cm (*n* = 68)	41	27	
Venous invasion			
Presence (*n* = 33)	23	10	0.010
Absence (*n* = 67)	27	40	
Extrahepatic metastasis			
Presence (*n* = 16)	12	4	0.054
Absence (*n* = 84)	38	46	
CLIP score			
Score 0–1 (*n* = 34)	11	23	0.001
Score 2–3 (*n* = 34)	13	21	
Score 4–6 (*n* = 32)	26	6	

HBV, hepatitis B virus; HCV, hepatitis C virus.

In this study, it should be mentioned that detectable serum GPC3 level in patients with HCC was influenced by the presence of viral hepatitis markers (serum HBsAg and/or anti-HCV positive). These data suggest that serum GPC3 might be more sensitive for detecting HCC in patients with pre-existing chronic viral hepatitis infection than those without viral markers. Thus, it appears that measurement of serum GPC3 may be advantageous for the diagnosis of HCC, particularly in areas where HBV and HCV infections are prevalent. However, it remains in mind that the expression of GPC3 could be detected in benign liver tissue in chronic HCV infection with high-grade necroinflammatory activity, which might potentially lead to a misdiagnosis of HCC.[25] Despite these findings, the staining of GPC3 in liver tissue was invariably cytoplasmic and usually granular, never membranous as in many cases of HCC.[25] Based on our data, there was no correlation between serum GPC3 level and necroinflammatory activity of the liver, suggesting that active hepatitis may play a negligible if any role in the detection of serum GPC3 in patients with HCC.

In agreement with previous studies, our data also showed that the simultaneous measurement of GPC3 and AFP significantly increased the sensitivity and accuracy for HCC diagnosis.[6,9] These could be explained by the observation that there was no correlation between GPC3 and AFP values in most cases of HCC. Another important issue to be addressed is whether GPC3 would be a better marker for the detection of small HCC than AFP. Indeed, it has been demonstrated that the expression of GPC3 in small HCC was significantly greater than that of AFP.[6,13] Unlike AFP, our data showed that there was no correlation between serum concentration GPC3 and tumor size or tumor stage. In addition, we showed that a higher proportion of patients with HCC of less than 3 cm had positive GPC3 values compared with AFP at the cut-off 20 ng/mL

Table 6 Factors associated with elevation of serum glypican-3 (GPC3) and α-fetoprotein (AFP) levels in the multivariate logistic regression analysis

Variables	Regression coefficient	Standard error	Odds ratio	95% confidence interval	*P*
GPC3					
Viral marker positive†	1.414	0.525	4.111	1.470–11.497	0.007
AFP20					
Tumor size (> 5 cm)	2.926	0.797	18.652	3.910–88.988	< 0.001
High CLIP score (4–6)	4.213	1.446	27.580	3.974–149.192	0.004
AFP100					
Tumor size (> 5 cm)	1.527	0.621	4.605	1.363–15.560	0.014
High CLIP score (4–6)	2.406	0.841	11.085	2.132–57.624	0.004

†Serum hepatitis B surface antigen and/or anti-hepatitis C positive. AFP20, AFP at a cut-off value of 20 ng/mL; AFP100, AFP at a cut-off value of 100 ng/mL.

(56% and 31%, respectively), and the combination of these markers yielded an improved sensitivity for detecting small HCC to 75%. However, studies including a larger number of patients will certainly be required to confirm this observation.

The exact biological functions of GPC3 on hepatocarcinogenesis are still not well understood. GPC3 expression was, both on immunohistochemistry and by real-time reverse transcriptase polymerase chain reaction (RT–PCR), much higher in small HCC than in cirrhosis, indicating that the transition from premalignant lesions to small HCC is associated with a sharp increase of GPC3 expression in a majority of cases.[7] Recent data have shown that GPC3 promotes the growth of HCC by stimulating the autocrine/paracrine canonical Wnt signaling.[26] In fact, transcriptional profiles of GPC3 were increased in early and advanced HCC compared with normal tissue and dysplastic nodules.[8] In contrast, GPC3 may act as an inhibitor of cell proliferation and thus can induce apoptosis in certain types of tumors.[27] For instance, down-regulation of GPC3 has been reported in several types of malignancies, including mesotheliomas,[28] gallbladder cancer, breast cancer,[17] ovarian cancer[15] and lung adenocarcinoma.[14] It has been shown that GPC3 functions as a potential tumor suppressor in the lung[14] and inhibits invasion and metastasis in a breast cancer model.[29] Collectively, these data suggest that GPC3 can act as a negative regulator of growth in most cancer types. Thus, the observation that GPC3 protein was rarely found in the sera in patients with MCA was not surprising. In the current study, we found that 98% of MCA were negative for serum GPC3. The only case expressing GPC3 was metastatic squamous cell carcinoma of the lung. In fact, it has recently been shown that GPC3 is tended to be overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma of the lung.[30]

Another important finding of this study is that serum GPC3 is also useful for differentiating patients with HCC from those with ICC. Indeed, cholangiocarcinoma represents the second most common primary liver malignant tumor arising from cholangiocytes, which are the epithelial cells lining the bile duct apparatus.[31] Cholangiocarcinoma is an uncommon liver cancer in Western countries but is particularly common in certain parts of the world such as China and Southeast Asia, where liver flukes are endemic. Thus, in the geographic areas where both chronic viral hepatitis and liver flukes are highly prevalent, it remains an essential problem to differentiate HCC from cholangiocarcinoma, particularly ICC, because both tumors may at times present the same clinical characteristics. Although HCC is usually associated with underlying cirrhosis, while conversely, ICC develops in a non-cirrhotic liver, the occurrence of ICC in cirrhosis has been increasingly recognized.[32] Moreover, approximately 10% of patients with HCC presented with obstructive jaundice, a clinical feature that might imitate the hilar type of cholangiocarcinoma.[33] Accordingly, an accurate serum marker would be considered as a valuable adjunct to non-invasive imaging for differentiating these primary liver cancers. In this study, serum GPC3 exhibited a very high specificity (100%), because none of the ICC patients had detectable serum levels. This finding supports the difference of GPC3 expression pattern in the liver between HCC and ICC.[16] When positivity for either GPC3 or AFP ($\geq$ 20 ng/mL) was used, the combined assay provided additional diagnostic sensitivity to approximately 90%, suggesting the complementary role of the two markers in differentiation of HCC from ICC.

In conclusion, our study showed that serum GPC3 levels were significantly elevated in patients with early and advanced HCC, but were rarely detectable in patients with other liver cancers, patients with non-malignant chronic liver disease 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, the combined assay of these two markers provides a potentially promising tool to better differentiate HCC from benign liver disorders, as well as from other malignant space-occupying lesions in the liver. Further studies in large scale are worthwhile to confirm these observations and to elucidate the clinical significance of serum GPC3 in patients with HCC.

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