World Journal of Medical Sciences 7 (2): 105-112, 2012
ISSN 1817-3055

DOI: 10.5829/idosi.wjms.2012.7.2.1105

The Diagnostic Value of Peripheral Blood Glypican-3 in Patients with Hepatocellular Carcinoma

[1]Asmaa I. Gomaa, [2]Olfat M. Hendy, [2]Gamal Y. Abou Raia, [2]Hatem Attia, [3]Hosam M. El ezawy, [4]Emad Nafie and [5]Sherif El Baz

[1]Department of Hepatology, National Liver Institute-Menoufyia University, Egypt
[2]Department of Clinical Pathology, National Liver Institute-Menoufyia, University, Egypt
[3]Department of Biochemistry, National Liver Institute-Menoufyia, Egypt
[4]Internal Medicine, Faculty of Medicine for Girls, Al Azhar University, Egypt
[5]Tropical Medicine, International Medical Centre, Egypt

Abstract: Glypican-3 (GPC-3) is a heparin sulfate proteoglycan normally expressed in fetal liver and placenta, but not in normal adult liver. Preliminary studies have shown that glypican-3 can be useful for the diagnosis of hepatocellular carcinoma (HCC). This study aimed to examine the utility of GPC-3 mRNA expression in peripheral blood and serum GPC-3 in diagnosis of HCC. Sixty one patients, 31 HCC and 30 liver cirrhosis and 25 normal healthy volunteers were enrolled. The peripheral blood GPC3 mRNA was detected by real time PCR as well as serum levels of GPC-3 by ELISA technique. The serum levels of GPC-3 were significantly increased in HCC patients as compared to liver cirrhosis and healthy controls. Furthermore, the expression of GPC-3 in peripheral blood was significantly higher in HCC than cirrhosis and controls. At cut-off level of 5.41 ng/ml, GPC-3 yielded a sensitivity of 90.3%, specificity of 98%, for diagnosis of HCC. However, AFP gave a sensitivity of 77.4%, specificity of 60%, at a cut-off level of 42.32 ng/ml. The combined AFP & GPC-3 improve the sensitivity and specificity of AFP alone. Significant positive correlations were detected between serum GPC-3 levels and prognostic parameters in HCC patients (AST, serum albumin, prothrombin concentration, tumor size, number and blood vessel invasion) and GPC3 mRNA, but no relation to AFP serum level was detected. On the contrary, GPC-3 mRNA was only correlated with vascular invasion. In conclusion: GPC-3 protein in peripheral blood can serve as a useful non invasive marker for early detection of HCC diagnosis with good sensitivity and high specificity and the level of GPC-3 can be useful in estimating the prognosis of HCC.

Key words: Hepatocellular · Carcinoma · Serum glypican-3

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third cause of death in prevalent malignant tumors all over the world [1], furthermore, its morbidity is increasing especially in Asia and sub-Saharan Africa. Respecting its worse prognosis and survival [2], screening for HCC based on routine ultrasound (US) examination and serum AFP detection every 6 months is strongly recommended by the European Association for the Study of the Liver (EASL) for detection of HCC in early stage [3]. The detection of nodules of 2 cm or less possess a diagnostic challenge as they are difficult to be characterized by pathological examination [4] or radiological examination [5]. However, US is very dependent on the operator's skill, so this limits the use and valuation of US in clinical practice for large scale screening. Similarly, alpha-fetoprotein (AFP) detection alone is not satisfactory because of its low sensitivity [6, 7]. So in recent years, investigators and clinicians have been devoting themselves to find and validate novel markers with higher sensitivity and specificity for early diagnosis of HCC.

Tumor markers have been used for screening, clinical diagnosis, evaluation of prognosis, monitoring response to therapy and monitoring for recurrence after treatment [8]. There are several established tumor markers for HCC: AFP, *Lens culinaris* agglutinin- reactive AFP (AFP-L3) and des-γ-carboxy prothrombin DCP (also known as PIVKA-II) [9]. The serum levels of these tumor markers are generally useful in predicting the prognosis of patients

Corresponding Author: Asmaa I. Gomaa, Department of Hepatology, National Liver Institute-Menoufyia University, Egypt.

with HCC. However, these markers have poor sensitivity and specificity when used as screening markers [10]. Glypican-3 (GPC-3) is a cell-surface glycoprotein that is absent in hepatocytes of healthy subjects and patients with hepatitis, but highly expressed in hepatocellular cancer cells [11, 12]. The GPC-3 gene encodes a 70-kDa-precursor core protein, which can be sliced by furin to make a 40-kDa amino (N) terminal protein and a 30-kDa membrane-bound carboxyl (C) terminal protein, which has two heparan sulphate (HS) glycan chains [13].

Potential novel tumor markers such as GPC-3[14] can be used in combination with any of the established markers, in the diagnosis of HCC with improved sensitivity and specificity. Of these novel markers, only GPC-3 has been successfully utilized in both serological and immunohistochemical applications for the diagnosis of HCC [15]. To date a little is known about the molecular identification of GPC-3 in peripheral blood as a non invasive technique for HCC prediction.

Aim of the Study: In this study, serum levels and peripheral blood expression of GPC-3 were determined in patients with HCC or liver cirrhosis and normal healthy people, in order to assess the diagnostic values of GPC-3 alone or in combination with AFP for early diagnosis of HCC.

Patient and Methods: Sixty one patients (48 males and 13 females, age range 42- 64 years), 31 of them have proved HCC (25 males and 6 females) with age ranged from 43-65 years and 30 patients with liver cirrhosis (23 males and 7 females) with age range 42-65 years. They were selected from outpatient and inpatient clinics of Hepatology Department-National Liver Institute-Menoufiya University, International Medical Centre and Internal Medicine Department of Al Zahraa University Hospital from March 2010 till May 2011. The group of HCC were diagnosed according to laboratory and radiological investigations including abdominal ultrasonography and triphasic C.T. abdomen. The group of cirrhosis were selected according to clinical examination, abdominal ultrasonography, laboratory investigations and the histopathological features in liver biopsy samples. Any patient with cancer other than hepatocelular carcinoma was excluded. None of the patients had received local or systemic therapy for HCC before (they are newly diagnosed). The study was approved by the local ethical committee in university hospitals and informed consent was obtained from the patients.

Laboratory investigations for patient groups including serum levels of AST, ALT, total protein, albumin, total bilirubin, alkaline phosphatase and gamma glutamyle transpeptidase (GGT) were done using Integra-400 (Roche-Germany). Prothrombin concentration was done by Fibrintimer (Roche-Germany). Complete blood cell counts were measured by Sysmix Kx-21 automatic cell counter (Japan). AFP serum level was measured by an automated Elecyes (Roche-Germany). HBV markers and HCV antibodies were assayed by EIA (COBAS-core, Germany). HCV-RNA levels were analyzed by real time polymerase chain reaction using a commercial kit (Roche Diagnostic, Branchburg, NJ) according to the manufacturer's instructions.

Serum GPC-3[14]: The GPC-3 was done by an ELISA which is a "sandwich" enzyme immunoassay employing two GPC-3 specific Antibodies, the kit provided by BioMosaics (Burlington,), Catalog Number B1500. An antibody, specific for the human GPC-3 protein, has been immobilized onto the surface of microtiter wells provided in the kit. The sample to be assayed, or GPC-3 standards, were added to the wells and allowed to incubate for two hours at room temperature. Unbound material was removed by washing and the biotinylated detector antibody was pipetted into the wells and allowed to incubate for two hours, during which time any GPC-3 present in samples or standards binds to the detecting antibodies. Unbound material was washed away and horseradish peroxidase conjugated streptavidin was added, which binds to the detector antibody. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetramethylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stop reagent), the intensity of which was proportional to the amount of GPC-3 protein in the sample. The colored reaction product was quantified using a spectrophotometer. Quantitation was achieved by the construction of a standard curve using known concentrations of GPC-3 (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of GPC-3 with that obtained from the standards, the concentration of GPC-3 in the sample can be determined.

Glypican-3 (GPC-3) Expression by Real Time PCR
Extraction of RNA and cDNA Synthesis: Total genomic RNA was extracted from EDTA peripheral blood mononuclear cells (PBMCs) with QIAamp RNA mini kit

(Qiagen, France) in accordance with the manufacturer's protocol. The concentration purity and amount of total RNA were determined by measuring absorbance at wave lengths 260/280 nm. cDNA was synthesized using Applied Biosystems.

Real-Time RT-PCR: The real-time RT-PCR used for the detection of GPC-3 mRNA was performed as described previously [16]. The sequences of primers and TaqMan probe of GPC-3 were: forward primer (5'-AGAGGCCTTTGAAATTGT-3'), reverse primer (5'-AAATACTTTCAGGTCACGTC-3') and probe (5'-FAM-ATGCCAAGAACTACA CCAATGC-TAMRA-3'). Reverse Transcription Kit with 5 mg of total RNA in 10 ml of TaqMan RT buffer, 22 ml of 25 mM magnesium chloride, 20 ml dNTPs, 5 ml random hexamers, 2 ml RNase inhibitor, 2.5 ml MultiScribe Reverse Transcription and RNA sample plus RNase free water, for a final volume of 100 ml. The conditions for every PCR reaction were 15 min at 95°C, followed by 40 cycles of denaturation for 20 seconds at 95°C and annealing/extension for 60 seconds at 60°C. Data were analyzed with Sequence Detection Software. The level of expression was calculated using the formula [17]:

$$\text{Relative expression} = \frac{\text{copy number of molecule}}{\text{copy number of } \beta\text{-actin}}$$

The RT-PCR assay was repeated twice and performed with using the LightCycler Instrument ROCHE (ROCHE, Diagnostics, Germany).

Statistical Analysis: Data were coded and summarized using SPSS (statistical package for Social Sciences) version 13.0 for Windows. Quantitative variables were described using mean±standard deviation and categorical data by using frequency and percentage. Comparison between groups Mann Whitney (U) test for non normally distributed variable and Kruskal-Wallis test was done to compare three or more of non normally distributed variables. Correlation coefficients (r) were calculated using the Pearson's correlation analysis. P value was significant at <0.05 level. Sensitivity, specificity and the area under the receiver-operating characteristic curve (AUROC) were determined.

RESULTS

The clinical characteristics of the patients in these groups are shown in Table 1. Most of the patients with HCC had single lesion (71%) and about half of them had tumour size <5cm in diameter. Ten cases had vascular spread. Only 25.8% of HCC cases and 30% of patients with cirrhosis had Child-Pugh class C. The serum level of AFP and GPC-3 was significantly increased in HCC patients as compared to liver cirrhosis and controls

Table 1: Clinical and some laboratory data of patient groups

Data	HCC patients (N=31)	Cirrhosis patients (N=30)
Gender (male%)	25 (80.6%)	23 (76.7%)
Hepatomegaly	22 (70.9%)	26 (86.7%)
Splenomegaly	18 (50.1%)	23 (76.7%)
Melena	15 (48.4%)	14 (46.7%)
Hematemesis	20 (64.5%)	15 (50%)
Ascites	9 (29%)	10 (33.3%)
Hepatitis markers:		
HCV antibodies	27 (87.1%)	25 (83.3%)
HBs Ag	4 (12.9%)	5 (16.7%)
Child-Pugh classification:		
Class A	6 (19.4%)	11 (36.7%)
Class B	17 (54.8%)	10 (33.3)
Class C	8 (25.8%)	9 (30%)
Tumor number:		
Single	22 (71%)	--
Multiple	9 (29%)	
Tumor size:		
<5 cm	17 (54.8%)	--
>5cm	14 (45.2%)	
Vascular invasion:		
Absent	21 (67.7%)	
Present	10 (32.3%)	--

Table 2: Comparison between HCC, cirrhotic and control groups as regards AFP and GPC-3 levels

Variables	HCC (n = 31) M±SD	Cirrhosis (n = 30) M±SD	Controls (n = 25) M±SD	P value
AFP (ng/ml)	987.1±752.4	32.7± 19.2	5.61±3.22	<0.001
Serum GPC-3 (ng/ml)	8.13±3.25	3.14±1.16	1.27±0.45	<0.001
GPC-3 mRNA (copy/ml)	9.22 ±2.11	6.57±1.63	3.92±1.17	<0.05

P<0.05 & p<0.001is statistically significant, p>0.05 is not statistically significant.

Table 3: Sensitivity, specificity, PPV (Positive Predictive Value) and NPV (Negative Predictive Value) of serum GPC-3 and AFP in prediction of HCC

Variables	Cut-Off	Sensitivity	Specificity	PPV	NPV
AFP (ng/ml)	42.32	77.4%	60%	66.7%	72%
Serum GPC-3 (ng/ml)	5.41	90.3%	98%	96.6%	93.8%
Combined AFP & GPC-3	AFP= 42.32 ng/ml & GPC-3 = 5.41 ng/ml	84%	90%	89.7%	87%

Table 4: The correlation of both serum GPC-3 and GPC-3 mRNA with the prognostic parameters in HCC group (n=31)

	Serum GPC-3		GPC-3 mRNA	
Parameters	r	p-value	R	p-value
ALT	0.38	>0.05	0.19	>0.05
AST	0.53	<0.05	0.31	>0.05
Serum albumin	0.51	<0.05	0.24	>0.05
Proth. Conc	0.52	<0.05	0.15	>0.05
Serum AFP	0.11	>0.05	0.23	>0.05
GPC-3 mRNA	0.48	<0.05	--	--
Tumor size	0.56	<0.05	0.23	>0.05
Tumor number	0.55	<0.05	0.14	>0.05
Vascular invasion	0.49	<0.05	0.57	<0.05

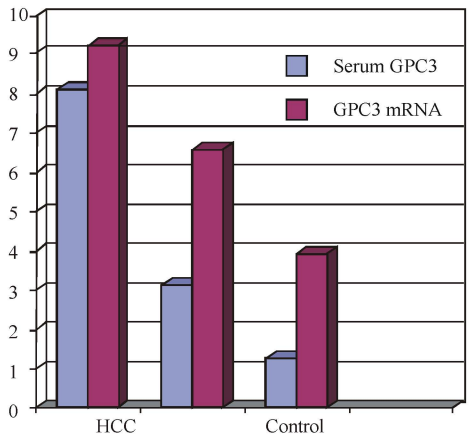

Fig. 1: Level of serum GPC-3 and GPC-3 mRNA in the studied groups

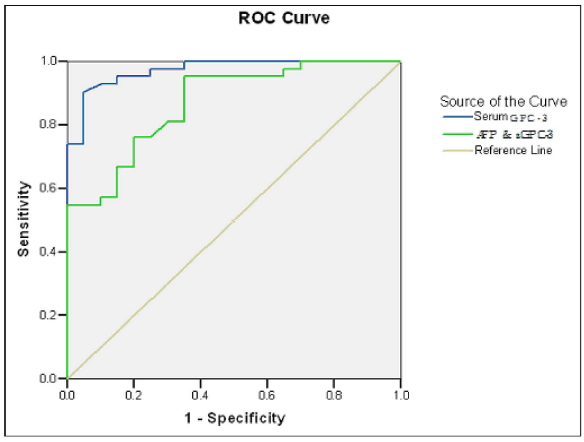

Fig. 2: ROC curve of serum GPC-3 and combined AFP & sGPC-3 for HCC prediction.

(p<0.001) for each. Also, the expression of GPC-3 in peripheral blood was significantly higher in HCC cases compared to liver cirrhosis and control group (p<0.05) (Table 2 & Fig. 1). At cut-off 5.41 ng/ml, serum GPC-3 gave a sensitivity of 90.3%, specificity of 98%, for HCC diagnosis and AUROC of 0.876. However, AFP gave a sensitivity of 77.4%, specificity of 60%, at a cut-off level of 42.32 ng/ml. The combined AFP & GPC-3 gave a sensitivity of 84% and specificity of 90%, which is higher than AFP alone and AUROC of 0.701 (Table 3 & Fig. 2). Significant positive correlations were detected between serum GPC-3 levels and each of AST, serum albumin, prothrombin concentration and GPC-3 mRNA. Also, it was positively correlated with the prognostic parameters in HCC patients (tumor size, number and blood vessel invasion), but not correlated with AFP serum level. On the contrary, GPC-3 mRNA was not related except to vascular invasion (blood metastasis) only (Table 4).

DISCUSSION

AFP and DCP [18] are well known major tumor markers for HCC. Serum AFP levels are often increased in patients with benign liver diseases, such as chronic hepatitis and liver cirrhosis. AFP levels at a value of 20 ng/ml showed good sensitivity but low specificity, whereas at higher cut-offs of 200 ng/ml the sensitivity drops to 22% with high specificity [19]. The AASLD recommended cut off level for diagnosis of HCC is 200 ng/ml, although lower levels, particularly if rising, should be followed very carefully [8]. Furthermore, only a small proportion of tumors at an early stage (10–20%) present with abnormal AFP serum levels [20]. The sensitivity of AFP is not good enough for it to be used alone for diagnosis of HCC, as over a third of cancers will be missed [8]. *Lens culinaris* agglutinin-reactive fraction of a-fetoprotein (AFP-L3%) is a described marker of HCC. AFP-L3% shows a much higher specificity than AFP, but a lower sensitivity. On the other hand, DCP shows a lower false-positive result, but is not always sensitive enough to detect small HCC [21]. Capurro *et al.* [14] reported that GPC-3 can serve as a useful serum marker for HCC diagnosis with good sensitivity. Since then, GPC-3 has attracted attention because of its high specificity independent of serum AFP levels. This finding was supported by Nakatsura *et al.* [22] and Hippo *et al.* [23].

GPC-3 was found to be frequently expressed in hepatocellular carcinoma (HCC) and, therefore, to possibly function as a diagnostic marker [24]. The expression of GPC-3 is reduced during tumor progression in cancers originating from tissues that are GPC-3 positive in adults as ovary, stomach and breast and this reduction seems to play a role in generation of the malignant phenotype. On the contrary, in the case of HCC, tumors originating from tissues that express GPC-3 only in the embryo, GPC-3 expression tend to reappear with malignant transformation [21, 25]. Capurro *et al.* [26] reported that, hepatocellular carcinomas arising in cirrhotic liver were more likely to be glypican-3 positive (91% vs 57%, $P<0.004$). Whereas all hepatic adenomas and macro-regenerative nodules were glypican-3 negative [27]. Several studies have demonstrated the efficacy of GPC-3 as a diagnostic tool in hepatocellular carcinoma. The reported sensitivity ranged from 75–100%, with figures of 75–85% in larger studies [22, 23, 28]. Nakatsura *et al.* [22] reported that, GPC-3 is not expressed in hepatocytes of healthy subjects and patients with non-malignant liver disease and can be detected in about 50% of HCC patients with high AFP and 33% of HCC patients seronegative for AFP.

Capurro *et al.* [26] confirms that GPC-3 immunohistochemistry has high overall sensitivity for the diagnosis of HCC. Furthermore, Suriawinata *et al.* [21] found that more than 80% of HCC tumors showed a much stronger expression of GPC-3 mRNA than did non-cancerous liver tissue and immuno-histochemical analysis revealed that HCC expressed GPC-3 protein in all HCC patients tested. Some clinical studies have indicated that the simultaneous determination of GPC-3 and AFP could significantly increase the sensitivity in HCC detection, without a reduction in the specificity [14, 29]. Furthermore, it has been shown that soluble GPC-3 (sGPC-3), the NH2-terminal portion of GPC-3, is superior to AFP in the sensitivity of detecting well or moderately differentiated HCC and the simultaneous determination of both markers improves overall sensitivity from 50 to 72% [23]. Thus, it can be seen that GPC-3 could be a good supplementary to AFP in early detection of HCC. Some other investigators have reported that GPC-3 mRNA is upregulated significantly in tumor tissues of HCC compared to para-neoplastic tissues of HCC, liver tissues of healthy adults and liver tissues of patients with non malignant hepatopathy, thus it could also be a good molecular marker for HCC [30-32].

However, the diagnosis of HCC should not rely on positive GPC-3 immunostaining because GPC-3 expression in HCC can be focal and thus, the lack of GPC-3 staining does not exclude the diagnosis of HCC in tissue specimens. Also, focal immunoreactivity can be detected in some cirrhotic nodules [16].

The non invasive techniques for HCC diagnosis were the goal of recent researches. Our study aimed to determine the expression of GPC-3 in peripheral blood and serum levels of GPC-3 in patients with HCC and liver cirrhosis to find its value in relation to serum AFP for early HCC diagnosis. Sixty one patients, 31 HCC and 30 liver cirrhosis and 25 normal healthy volunteers were selected. In the current study, serum levels of GPC-3 were significantly increased in HCC patients as compared to patients with liver cirrhosis and healthy controls. Furthermore, the expression of GPC-3 mRNA in peripheral blood was significantly higher in HCC than cirrhosis and controls. At cut-off level of 5.41 ng/ml, serum GPC-3 gave a sensitivity of 90.3%, specificity of 98%, for early diagnosis of HCC and AUROC of 0.817. However, AFP gave a sensitivity of 77.4%, specificity of 60%, at a cut-off level of 42.32 ng/ml. The combined AFP & GPC-3 gave a sensitivity of 84% and specificity of 90%, which is much better than AFP alone and AUROC of 0.701.Our results are in agreement with the findings of Tangkijvanich *et al.* [33]. Also, Suriawinata *et al.* [21] found that, GPC3 protein

in the sera was detectable only in HCC patients and not in patients with other liver diseases or other kinds of cancers and healthy donors, thereby indicating that the specificity is 100%. Furthermore, they confirmed that GPC3 protein had disappeared from the sera of three patients after surgical treatments for HCC. Sun *et al.* [34] recorded that, plasma GPC-3 levels in HCC group were higher than those in chronic liver disease. The area under the receiver operating characteristics curve value for GPC3 was 0.731 and cut-off value of 75 ng/ml yielded the highest predictive value of diagnosing HCC with sensitivity of 61% and specificity of 85%. They suggested that the plasma GPC-3 is a potential tumor marker for HCC, especially among patients with low serum AFP and DCP.

In another study, Suriawinata *et al.* [21], the sensitivity of AFP, AFP-L3%, DCP and GPC-3 was 50%, 27.7%, 50% and 40%, respectively. They could not diagnose 30% of HCC patients using AFP and DCP alone, although, they could diagnose an additional 30% of suspicious HCC. Hence, they could diagnose 80% of their patients with HCC using combination of AFP, DCP and GPC-3. They concluded that GPC-3 may be useful for diagnosis of early stage HCC because it is more sensitive for the detection of small tumors. In more recent study by Qiao *et al.* [35] the mean level of GPC-3 in the serum samples of HCC patients was obviously higher than that of other groups. When they used 20.68 ng/ml as the cut-off point in HCC diagnosis, GPC-3 could reach a sensitivity of 69.3% with a specificity of 88.7% and AUROC of 0.892.The prognosis and survival of HCC patients is not only related to tumor size but also related to the integration of tumor size, location, vein invasion and metastasis beside the liver status. In this study, significant positive correlations were detected between serum GPC-3 levels and each of AST, serum albumin, prothrombin concentration and GPC-3 mRNA. Also, it was positively correlated with the prognostic parameters in HCC patients (tumor size, number and blood vessel invasion), but not correlated with AFP serum level. On the contrary, GPC-3 mRNA was not related except to vascular invasion (blood metastasis) only. Capurro *et al.* [14], Nakatsura *et al.* [22] and Hippo *et al.* [23] have reported that GPC-3 is a serologic marker of HCC. They have also found that, due to the lack of correlation between serologic concentrations of GPC-3 and AFP in HCC patients, the simultaneous use of both markers significantly increases the sensitivity of AFP alone.

GPC-3 expression was found to be correlated with poor prognosis in HCC owing to the lower 5-year survival rate in GPC3-positive HCC patients than GPC3-negative HCC patients [36]. GPC-3 is a valuable diagnostic marker and a potential therapeutic target in HCC [34, 37].

Recently Hui-Fen *et al.* [38] reported that the presence of GPC-3 mRNA predicts the possibility of blood metastasis of tumor cells in patients with HCC and plays an assistant role in the diagnosis of HCC in patients with negative or low level of serum AFP. Yan *et al.* [39] reported that, GPC-3 mRNA was detected in HCC patients and not detected in healthy subjects, patients with hepatitis B, cirrhosis or hepatic hemangioma. The sensitivity of HCC diagnosis can be improved by combined detection of AFP and GPC3 mRNA expressions. They concluded that GPC-3 mRNA is HCC-specific and may indicate HCC metastasis. In our study, no correlation between serum GPC-3 or GPC-3 mRNA and AFP serum level was found. Also, in Qiao *et al.* [35] study, there was no correlation between GPC-3 and AFP. Taken together, these results indicate that GPC3, as defined in our study, can be detected in the serum as a secreted protein in a subset of patients with HCC but is undetectable in healthy individuals or patients with cirrhosis. Interestingly, elevated serum GPC-3 levels in most HCC patients do not correlate with their serum AFP values and GPC-3 appears to be more sensitive than AFP as a serologic marker. Thus, GPC-3 may prove to be an appropriate candidate for use in HCC diagnosis and measurement of serum GPC-3 level in combination with ultrasonography can be useful in screening and diagnosis of HCC as well as in follow-up of high-risk population. On the contrary, Ozkan *et al.* [40] reported that the sensitivity, specificity and positive and negative predictive values of GPC-3 was 61.33, 41.82, 58.97 and 44.43%, respectively. The values for AFP were 68.57, 94.55, 94.12 and 70.27%, respectively. There was no correlation between GPC-3 levels and prognostic parameters. They concluded that GPC3 is not a useful diagnostic and prognostic marker for HCC. In addition, Yasuda *et al.* [41] found no elevation of serum GPC-3 level in patients with HCC in comparison with those with chronic liver disease (CLD); and they did not find serum GPC-3 level, measured by a commercially available ELISA kit with GPC-3 antibody, to be useful in the diagnosis of HCC.

CONCLUSION

From the above results, we concluded that, the analysis of GPC-3 expression level and GPC-3 mRNA can serve as a useful non invasive tumor marker for early detection of HCC diagnosis with good sensitivity and high specificity, the combined use of GPC-3 and AFP yields high sensitivity and specificity than AFP alone. In the future GPC-3 can be used as a target immunotherapy for HCC.

REFERENCES

1. Parkin, D.M., F. Bray, J. Ferlay and P. Pisani, 2005. Global cancer statistics, 2002. CA Cancer J. Clin., 55(2): 74-108.
2. Wong, L.L., W.M. Limm, R. Severino and L.M. Wong, 2000. Improved survival with screening for hepatocellular carcinoma. Liver Transpl., 6(3): 320-5.
3. Bruix, J. and J.M. Llovet, 2002. Prognostic prediction and treatment strategy in hepatocellular carcinoma. Hepatology, 35(3): 519-24.
4. Kojiro, M., 2004. Focus on dysplastic nodules and early hepatocellular carcinoma: an Eastern point of view. Liver Transpl, 10: S3-S8.
5. Bolondi, L., S. Gaiani, N. Celli, R. Golfieri, W.F. Grigioni, S. Leoni, A.M. Venturi and F. Piscaglia, 2005: Characterization of small nodules in cirrhosis by assessment of vascularity: the problem of hypovascular hepatocellular carcinoma. Hepatology, 42: 27-34.
6. Marrero, J.A. and A.S. Lok, 2004. Newer markers for hepatocellular carcinoma. Gastroenterology, 127(5 Suppl 1): S113-9.
7. Sherman, M. and A. Klein, 2004. AASLD single-topic research conference on hepatocellular carcinoma: Conference proceedings. Hepatology, 40(6): 1465-73.
8. Bruix, J. and M. Sherman, 2005. Management of hepatocellular carcinoma. Hepatology, 42: 1208-1236.
9. Sherman, M. 2001.Alphafetoprotein: an obituary. J. Hepatol., 34: 603-605.
10. Poon, T.C., T.S. Mok, A.T. Chan, C.M. Chan, V. Leong, S.H. Tsui, T.W. Leung, H.T. Wong, S.K. Ho and P.J. Johnson, 2002. Quantification and utility of monosialylated alpha-fetoprotein in the diagnosis of hepatocellular carcinoma with non-diagnostic serum total alpha-fetoprotein. Clin. Chem., 48: 1021-1027.
11. Zhu, Z.W., H. Friess and L. Wang *et al.* 2001. Enhanced glypican-3 expression differentiates the majority of hepatocellular carcinomas from benign hepatic disorders. Gut., 48(4): 558-64.
12. Filmus, J., 2001. Glypicans in growth control and cancer. Glycobiology, 11(3): 19R-23R.
13. Filmus, J. and M. Capurro, 2004. Glypican-3 and alphafetoprotein as diagnostic tests for hepatocellular carcinoma. Mol. Diagn., 8(4): 207-12.
14. Capurro, M., I.R. Wanless, M. Sherman, G. Deboer, W. Shi, E. Miyoshi, *et al.*, 2003. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology, 125(1): 89-97.
15. Shirakawa, H., T. Kuronuma, Y. Nishimura, T. Hasebe, M. Nakano, N. Gotohda, S. Takahashi, T. Nakagohri, M. Konishi, N. Kobayashi, T. Kinoshita and T. Nakatsura, 2009a. Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. Int. J. Oncol., 34: 649-656.
16. Wang, H., F. Anatelli, J. Zhai *et al.*, 2008. Glypican-3 as a useful diagnostic marker that distinguishes hepatocellular carcinoma from benign hepatocellular mass lesions. Arch. Pathol. Lab. Med., 132: 1723-1728.
17. Yang, Y., G.X. Zhang. R.H. Shi *et al.*, 2007. Overexpression of HCCR in hepatocellular carcinoma cells and its clinical significance. Zhonghua Gan Zang Bing Za Zhi, 15(3): 223-4.
18. Moribe, T., N. Iizuka, T. Miura, N. Kimura, S. Tamatsukuri, H. Ishitsuka, Y. Hamamoto, K. Sakamoto, T. Tamesa and M. Oka, 2009. Methylation of multiple genes as molecular markers for diagnosis of small, well-differentiated hepatocellular carcinoma. Int. J. Cancer, 125: 388-397.
19. Trevisani, F., P.E. D'Intino, A.M. Morselli-Labate, G.Mazzella, E. Accogli, P. Caraceni *et al.*, 2001. Serum alpha-fetoprotein for diagnosis of hepatocellular carcinoma in patients with chronic liver disease: influence of HbsAg and anti-HCV status. J. Hepatol., 34: 570-575.
20. Yamashita, T., M. Forgues, W. Wang, J.W. Kim, Q. Ye, H. Jia, *et al.*, 2008. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res., 68: 1451-1461.
21. Suriawinata, A. and S.N. Thung, 2010. Molecular Signature of Early Hepatocellular Carcinoma. Oncology, 78(suppl 1): 36-39.

22. Nakatsura, T., Y. Yoshitake, S. Senju *et al*., 2003. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem. Biophys. Res. Commun., 306: 16-25.
23. Hippo, Y., K. Watanabe, A. Watanabe *et al*., 2004. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res., 64: 2418-23.
24. Yamauchi, N., A. Watanabe, M. Hishinuma *et al*. 2005. The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. Mod. Pathol., 18: 1591-1598.
25. Di Tommaso, L., A. Destro, J.Y. Seok, E. Balladore, L. Terracciano, A. Sangiovanni, *et al*., 2009. The application of markers (HSP70 GPC3 and GS) in liver biopsies is useful for detection of hepatocellular carcinoma. J. Hepatol., 50(4): 746-54.
26. Capurro, M.I., Y.Y. Xiang, C. Lobe and J. Filmus, 2005. Glypican-3 Promotes the Growth of Hepatocellular Carcinoma by Stimulating Canonical Wnt Signaling. Cancer Res., 65(14): 6245-54.
27. Shafizadeh, N., L.D. Ferrell and S. Kakar, 2008. Utility and limitations of glypican-3 expression for the diagnosis of hepatocellular carcinoma at both ends of the differentiation spectrum. Modern Pathology, 21: 1011-1018.
28. Filmus, J. and S.B. Selleck, 2001. Glypicans: proteoglycans with a surprise. J. Clin. Invest., 108: 497-501.
29. Nassar, A., C. Cohen and M.T. Siddiqui, 2009. Utility of glypican-3 and survivin in differentiating hepatocellular carcinoma from benign and preneoplastic hepatic lesions and metastatic carcinomas in liver fine-needle aspiration biopsies. Diagn Cytopathol., 37: 629-635.
30. Midorikawa, Y., S. Ishikawa, H. Iwanari, T. Imamura, H. Sakamoto, K. Miyazono, T. Kodama, M. Makuuchi and H. Aburatani, 2003. Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. Int. J. Cancer, 103: 455-465.
31. Sung, Y.K., S.Y.Hwang, M.K. Park, M. Farooq, I.S. Han, H.I. Bae, J.C. Kim and M. Kim, 2003. Glypican-3 is overexpressed in human hepatocellular carcinoma. Cancer Sci., 94: 259-262.
32. Lu, Z.L., D.Z. Luo and J.M. Wen, 2005. Expression and significance of tumor-related genes in HCC. World J. Gastroenterol., 11: 3850-3854.
33. Tangkijvanich, P., T. Chanmee, S. Komtong *et al*., 2010. Diagnostic role of serum glypican-3 in differentiating hepatocellular carcinoma from non-malignant chronic liver disease and other liver cancers. J. Gastroenterol. Hepatol., 25: 129-37.
34. Sun, C.K., M.S. Chua, J. He, S.K. So, 2011. Suppression of glypican 3 inhibit of hepatocellular carcinoma cells through upregulation of TGF-β2. Neoplasia, 13: 735-747.
35. Qiao, S.S., Z. Cui, L. Gong, H. Han *et al*., 2011. Simultaneous Measurements of Serum AFP, GPC-3 and HCCR for Diagnosing Hepatocellular Carcinoma Hepato-Gastroenterology, 58: 1719.
36. Shirakawa, H., H. Suzuki, M. Shimomura *et al*., 2009b. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci. 100(8): 1403-1407.
37. Ho, M. and H. Kim, 2011. Glypican-3: a new target for cancer immunotherapy. Eur. J. Cancer, 47(3): 333-338.
38. Hui-Fen, Y., C.Ji, B. Ke-Chen, S. Jian-Jia, L. Yuan, O. Chao and D. Xiao-Xian, 2006. Detection of glypican-3 mRNA in peripheral blood and its clinical significance. 2011; DOI [CNKI: SUN: ZZLL.0. -06-011].
39. Yan, D., Q. He, Y. Chen, L. Wang and X. Zhang, 2011. Detection of á-fetoprotein and glypican-3 mRNAs in the peripheral blood of hepatocellular carcinoma patients by using multiple FQ-RT-PCR. J. Clin. Lab. Anal., 25(2): 113-7.
40. Ozkan, H., H. Erdal, E. Koçak, H. Tutkak, Z. Karaeren, M. Yakut and S. Köklü, 2011. Diagnostic and prognostic role of serum glypican 3 in patients with hepatocellular carcinoma. J. Clin Lab Anal., 25(5): 350-3. doi: 10.1002/jcla.20484.
41. Yasuda, E., T. Kumada, H. Toyoda, Y. Kaneoka, A. Maeda, S. Okuda, N. Yoshimi and O. Kozawa, 2010. Evaluation for clinical utility of GPC3, measured by a commercially available ELISA kit with Glypican-3 (GPC3) antibody, as a serological and histological marker for hepatocellular carcinoma. Hepatol., Res., 40(5): 477-85.