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Role of serum glypican-3 in the diagnosis and differentiation of small hepatocellular carcinoma from hepatitis-C virus cirrhosis

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KEYWORDS

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Abstract *Background:* Serum alpha-fetoprotein (AFP) has insufficient sensitivity and specificity for detection of hepatocellular carcinoma (HCC). Recently, glypican-3 (GLP-3) was suggested as a new biomarker for the detection HCC.
Objectives: To determine the role of serum GLP-3 levels in the early diagnosis and differentiation of small (3 cm or less in diameter) HCC from liver cirrhosis. Also, to correlate GLP-3 levels to clinico-laboratory data.
Methods: The study included sixty patients; 30 of them with hepatitis C virus (HCV) cirrhosis, and 30 patients with proved HCC. In addition, 20 healthy subjects were included as a control group. Clinical and radiological features (abdominal ultrasonography and/or abdominal triphasic computed tomography) were recorded. Liver function tests, complete blood cell count, and serum AFP were measured. Serum GLP-3 values were determined by an ELISA technique.
Results: Serum levels of GLP-3 were significantly elevated in patients with HCC compared with HCV cirrhosis group ($p < 0.001$). Also, these levels were significantly elevated in these two patients' groups versus controls ($p < 0.001$). Also, serum GLP-3 levels with cut-off value of $\geqslant$240 ug/L, had a higher sensitivity (100%) and same specificity (93.3%), than AFP with cut-off value of $\geqslant$200 ng/ml, for detection of HCC. Moreover, GLP-3 levels showed a higher sensitivity than AFP (50% vs.41.7%), for detection of small HCC. The combined use of both markers (i.e. when either one of the two markers positive) improved the specificity to 88.9%. Regarding unicentric HCC,

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GLP-3 at cut-off value of ⩽580 ug/L had better specificity than AFP at cut-off value of ⩽765 ng/ml (57.1% vs. 42.9%). The combined use of both markers improved the sensitivity and specificity to 82.6% and 71.4%, respectively.

Conclusion: Serum GLP-3 levels are higher in HCC versus HCV cirrhosis, which can differentiate HCC from liver cirrhosis. Also, serum GLP-3 is highly sensitive and specific for detecting HCC. Moreover, GLP-3 is more sensitive than AFP for the detection of small HCC. Furthermore, a combination of both serum markers yielded an improved specificity and both sensitivity and specificity for the diagnosis of small and unicentric HCC, respectively.

1. Introduction

Hepatocellular carcinoma (HCC) represents the fifth most common cancer worldwide and the third most common cause of cancer death.[1] It constitutes about 70% of all liver tumors among Egyptians.[2] The HCV infection is the most common risk factor of HCC in Egypt which leads to cirrhosis and severe liver damage.[3,4] The HCC is frequently diagnosed at late stages.[5,6] HCC is usually asymptomatic in the early stages, and most patients present with incurable disease at the time of detection, so early diagnosis of HCC is critical for a good prognosis.[7]

Although ultrasonography (US) has been widely used in clinical screening of HCC,[8] it is very operator dependent and less reliable in obese and cirrhotic subjects.[9] Alpha-fetoprotein (AFP) is the only serological marker currently widely used for the diagnosis of HCC.[10] AFP is not secreted in all cases of HCC and may be normal in as many as 40% of patients with early HCC. On the other hand, the AFP level can reach high levels (2500 μg/L) in around one fourth of patients with chronic hepatitis, and liver cirrhosis.[11] In contrast, GLP-3 is a 60 KDa cell surface-linked heparin sulfate proteoglycan, which is not expressed in adult liver, was first introduced as a possible tumor marker of HCC by observing significantly high levels of this protein in the serum of HCC patients.[12,13]

As patients with cirrhosis may develop HCC only after many years, emphasis has been placed on the early detection of HCC when it is small, asymptomatic and potentially curable.[12] So, this study aimed to estimate serum GLP-3 levels in patients with cirrhosis and HCC, versus healthy controls. Also, to define the role of serum GLP-3 levels in the early diagnosis and differentiation of small (diameter of 3 cm or less) HCC from liver cirrhosis, and to correlate these levels with clinico-laboratory data.

2. Patient and methods

2.1. Patients' selection

During the period from June 2012 to June 2013, we selected sixty patients from the tropical, and internal medicine departments, as well as the oncology center, of the university hospital and faculty of medicine, Menoufiya University, Egypt.

Thirty of these patients were diagnosed as HCC. All HCC patients were newly diagnosed cases and did not receive prior chemotherapy. Tumor biopsy was carried out in twelve patients (40%) and showed that eight of them had poorly differentiated HCC, and the other four had well differentiated HCC. Tumor biopsy was not done in the remaining eighteen HCC patients, because either the tumor site was difficult for the biopsy to be taken and/or there was contraindication to biopsy. HCC was diagnosed according to history, clinical examination, classic radiological investigations [abdominal ultrasonography (US) and/or triphasic computed tomography], serum AFP levels above 200 ng/ml, and/or histopathological examination of tissue biopsy when available.[14]

The remaining thirty patients have HCV liver cirrhosis. Liver cirrhosis was diagnosed by history, clinical features of cirrhosis, abdominal US features, laboratory investigations and/or liver biopsy.

Twenty healthy subjects matched for gender and age were included and served as controls. All controls had normal liver function tests, and were seronegative for hepatitis B markers (HBs Ag, HBeAg and HBc-Ab) and HCV antibodies. The study was approved by our local ethics committee of the university hospitals and informed oral consent was obtained from all participants.

2.2. Laboratory assays

10 ml of fasting venous blood for at least 10 h was withdrawn from all subjects. Plasma was obtained from 1.8 ml of whole blood, was added to 0.2 ml sodium citrate, centrifuged at 4000 rpm for 5 min, and then plasma was used for measuring prothrombin concentration by using Fibrintimer II instrument of Behring, Germany Using Sysmex K-21, Japan. Two ml of whole blood was added to EDETA containing tubes with good mixing then automated homogram was done for all samples, including hemoglobin estimation (HB), red cell count (RBCs), total leukocytic count (TLC) and platelet count. These parameters were determined by colter counter model Beckman750, Int, L.S.A. Six ml of venous blood was transferred slowly into a plain tube, allowed to clot, and then centrifuged for 10 min. The clear supernatant was separated in several aliquots, kept frozen at −20 °C, till analysis. The following investigations were carried out by auto analyzer (SYNCHRON CX5 from Beckman): liver function tests including: alanine transaminase (ALT), aspartate transaminase (AST), serum albumin and total bilirubin. Kidney function tests including; serum urea and creatinine were done. Hepatitis markers (HBsAg, and HCV antibodies) were done by the ELISA technique[15,16] while anti-HBc antibodies were detected by Electro-Chemi-Luminescence Immunoassay (ECLIA).[17]

Serum AFP levels were measured by ELISA (MONBIND, Inc. Costa Mesa, CA92627 USA).

HCV-RNA levels were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using a commercial kit (Roche Diagnostic, Branchburg, NJ) according to the manufacturer's instructions.

Serum GLP-3 was determined by ELISA Kit provided by Uscn, (Inc-USA). This assay employs the quantitative sandwich enzyme immunoassay technique.[18]

2.3. Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) software version 10. Results are presented as mean ± standard deviation (SD) unless otherwise stated. For comparison of two means, the non-parametric Mann–Whitney test was used. The ANOVA test was used to compare among HCV-cirrhosis with HCC and controls. Fisher exact analysis was also applied to compare proportions between groups. Spearman correlation analysis was used to study correlations between different parameters. A *P*-value of $\leqslant 0.05$ was considered statistically significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy, were calculated from 2×2 tables. Cut-off point values were calculated from ROC curves.

3. Results

Thirty patients were diagnosed as HCC. They were 25 males and 5 females, with their mean age ± SD of 56.97 ± 10.94 years. Another thirty patients had HCV liver cirrhosis. They were 23 males and 7 females, their mean age ± SD of 53.26 ± 5.34 years. In addition, twenty healthy subjects were included and served as controls (16 males; 4 females; age 57.1 ± 6.9 years). Age and gender were not significantly different among the three studied groups ($p > 0.05$). Regarding Child Pugh classification, 7 (23.3%), 14(46.7%), and 9 (30%), patients with cirrhosis were in class A, B, and C, respectively. In contrast, 11 (36.7%), 10 (33.3%), and 9 (30%), patients with HCC were in class A, B, and C, respectively. There was no significant difference between the two groups ($p = 0.46$). Considering tumor size, 12 (40%) and 18 (60%) patients had small and large size HCC, respectively. Out of thirty HCC patients, twenty-three (76.7%) had unicentric tumor, and the rest (23.3%) had multicentric tumor. Twenty-three patients (76.6%) with HCC had the tumor located in the right lobe of the liver, and the rest (23.3%) in the left lobe.

Table 1, shows the laboratory data of the studied patients. There was a significant increase in serum creatinine, total and direct bilirubin, AFP and GLP-3 in HCC versus HCV cirrhosis group ($p < 0.05$). Also, serum AFP, and GLP-3 levels were significantly higher in these two groups, compared with the control group, with higher levels in HCC versus HCV cirrhosis group ($p < 0.05$). In contrast, platelet count, and serum albumin level, were significantly decreased in HCC versus HCV cirrhosis group ($p < 0.05$).

Correlation between serum GLP-3 levels and other laboratory parameters in HCC patients, is shown in Table 2. A significant positive correlation was found between serum GLP-3 level and blood urea, serum creatinine, AST, ALT, total and direct bilirubin, and AFP ($p < 0.05$). In contrast, a significant negative correlation was found between serum GLP-3 and hemoglobin concentration, platelet count, and prothrombin time ($p < 0.05$).

Also, on comparing serum GLP-3 levels with tumor characteristics of HCC patients, a significant difference was detected between serum GLP-3 level and some tumor characteristics, namely, unicentric tumor, portal vein thrombosis (tumor embolization), and tumor location in right hepatic lobe ($p = 0.002$, > 0.001, and > 0.001, respectively). However, there was no significant difference with Child Pugh class ($p = 0.14$).

Regarding HCC diagnosis, (Table 3), AFP at a cut-off value $\geqslant 200$ ng/ml had a sensitivity, specificity, PPV, and NPV of 83.3%, 93.3%, 92.6%, and 84.8% for HCC diagnosis, respectively. In contrast, GLP-3, at a cut-off value $\geqslant 240$ ug/L had a sensitivity, specificity, PPV, and NPV of 100%, 93.3%, 93.8%, and 100% for HCC diagnosis, respectively. Combined use of both markers (i.e. when either one of the two markers positive), at same cut-off values, increased sensitivity, specificity, PPV, and NPV to 100%, for HCC diagnosis.

Table 1 Laboratory data of the studied patients.

	Cirrhosis $N = 30$ X ± SD	HCC $N = 30$ X ± SD	Mann–Whitney Test
Hemoglobin (g/dl)	10.46 ± 1.61	9.76 ± 1.57	$P > 0.05$
Leukocyte count ($\times 10^3$/L)	7.13 ± 5.72	7.03 ± 5.63	$P > 0.05$
Platelets ($\times 10^3$/L)	105.23 ± 68.42	72.57 ± 24.11	$P < 0.01$*
Urea mg/dl	43.87 ± 18.74	53.83 ± 26.73	$P > 0.05$
Creatinine (mg/dl)	1.37 ± 0.49	2.08 ± 1.01	$P < 0.01$*
AST (IU/ml)	42.23 ± 13.78	58.13 ± 57.48	$P > 0.05$
ALT (IU/ml)	33.57 ± 18.44	39.57 ± 20.33	$P > 0.05$
Total bilirubin (mg/dl)	2.62 ± 1.54	4.80 ± 2.69	$P < 0.01$*
Direct bilirubin (mg/dl)	1.11 ± 0.89	1.85 ± 1.31	$P < 0.05$*
Serum albumin (mg/dl)	2.36 ± 0.47	2.82 ± 0.76	$P < 0.01$*
Prothrombin time (%)	55.30 ± 13.60	54.63 ± 14.07	$P > 0.05$
Serum AFP (ng/ml)	80.04 ± 100.92	703.43 ± 744.69	$P < 0.01$*
Serum glypican-3 (ug/L)	98.23 ± 73.54	551.47 ± 185.25	$P < 0.01$*

HCC = Hepatocellular carcinoma AFP = Alpha-fetoprotein.
AST = Aspartate transaminase ALT = Alanine transaminase.
* Significant.

Table 2 Correlation between serum glypican-3 levels and other laboratory parameters in HCC group.

	Serum glypican-3 Spearman correlation analysis	*P* value
Hemoglobin	−0.45	<0.001*
Leukocyte count	−0.04	0.75
Platelets	−0.52	<0.001*
Urea	+0.37	0.001*
Creatinine	+0.53	<0.001*
AST	+0.27	0.01*
ALT	+0.24	0.03*
Total bilirubin	+0.55	<0.001*
Direct bilirubin	+0.45	<0.001*
Serum albumin	−0.21	0.06
Prothrombin time	−0.41	<0.001*
Serum AFP	+0.61	<0.001*

HCC = Hepatocellular carcinoma.
AST = Aspartate transaminase.
ALT = Alanine transaminase.
AFP = Alpha-fetoprorein.
* Significant.

Considering tumor size (i.e. small size of 3 cm or less; and large size of more than 3 cm in diameter), (Table 4), AFP at a cut-off value ⩽300 ng/ml had a sensitivity, specificity, PPV, and NPV of 41.7%, 83.3%, 62.5%, and 68.2%, for diagnosis of small HCC, respectively. In contrast, GLP-3, at a cut-off value ⩽560 ug/L had sensitivity, specificity, PPV, NPV of 50%, 50%, 40%, and 60% for diagnosis of small HCC, respectively. Combined use of both markers (AFP or GLP-3), at same cut-off values, increased specificity, PPV, and NPV to 88.9%, 75%, and 72.7%, respectively; whereas, sensitivity was not changed for diagnosis of small HCC.

In respect to unicentric and multicentric HCC, (Table 5), AFP at a cut-off value ⩽765 ng/ml had a sensitivity, specificity, PPV, and NPV of 73.9%, 42.9%, 81%, and 33.3% for diagnosis of unicentric HCC, respectively. In contrast, GLP-3, at a cut-off value ⩽580 ug/L had sensitivity, specificity, PPV, and NPV of 65.2%, 57.1%, 83.3%, and 33.3% for diagnosis of unicentric HCC, respectively. Combined use of both markers (AFP or GLP-3), at same cut-off values, increased sensitivity, specificity, PPV, and NPV to 82.6%, 71.4%, 90.5%, and 55.6%, respectively, for diagnosis of unicentric HCC.

4. Discussion

Hepatocellular carcinoma most frequently develops in patients with cirrhosis related to chronic viral hepatitis.[19] The use of biomarkers in predicting disease holds considerable promise and has played an important role in early diagnosis.[20] In HCC, GLP-3 fosters HCC growth by altering Wnt signaling,[21] modulating growth factors such as insulin-like growth factor-2 (IGF-2), bone morphogenetic protein-9 (BMP-9), and fibroblast growth factor-2 (FGF-2) and possibly by playing a role in M2 macrophage recruitment.[22] GLP3 may be cleaved from the surface of expressing hepatocytes, thereby entering the circulation.[23]

In the present study, we found that serum levels of GLP-3 were significantly higher in patients with HCC compared with HCV cirrhosis group. Also, these levels were significantly higher in these two patients' groups versus controls. These results are in agreement with many studies.[5,24–26]

Several studies have demonstrated the efficacy of GLP-3 as a diagnostic tool in HCC. It was reported that the sensitivity and specificity ranged from 47–93.3%, and 41.8–100%, respectively.[7,12,24,27,28] This wide range of difference may be due to different patients' characteristics, the presence of HCV as an etiological factor for HCC,[24] or using different cut-off values for GLP-3. Our results are similar to the literature, with 100% sensitivity and specificity, for HCC diagnosis.

Consistent with our and these previous studies, tumors arising in cirrhotic liver are more likely to express GLP-3.[5,29] Also, in line with our work, recently the American Association for the Study of Liver Disease has stated in management of HCC that 'Expert pathology diagnosis is reinforced by staining for GLP-3, heat shock protein 70 and glutamine synthetase, because positivity for two of these three stains confirms HCC'.[30] Likewise, the Clinical Practice Guidelines of the European Association for the Study of the Liver recommend the use of these three markers to confirm HCC diagnosis.[31] In contrast, Yasuda et al.[32] and Ozkan et al.[33] reported that there was no increase in serum GLP-3 level in patients with HCC, and serum GLP-3 level was not a useful diagnostic marker for HCC. This may be due to the measuring procedure used in these studies, as suggested by the former.

Regarding AFP, it was reported that the sensitivity and specificity ranges from 36.3–83.3%, and 60–100%, respectively.[24,27,28,33] Our results were in accordance with these

Table 3 Predictive power of serum AFP, GLP-3, and combined (AFP or GLP-3) in the diagnosis of HCC.

	AFP: cut off point ⩾ 200 ng/ml		GLP-3: cut off point ⩾ 240 ug/L		Combined GLP-3: cut off point ⩾ 200 ug/L OR AFP: cut off point ⩾ 240 ng/ml	
	HCC + ve (*n* = 30)	HCC −ve (*n* = 30)	HCC + ve (*n* = 30)	HCC −ve (*n* = 30)	HCC + ve (*n* = 30)	HCC −ve (*n* = 30)
Tumor marker (+ve)	25	2	30	2	30	0
Tumor marker (−ve)	5	28	0	28	0	30
Sensitivity	83.3%		100%		100%	
Specificity	93.3%		93.3%		100%	
PPV	92.6%		93.8%		100%	
NPV	84.8%		100%		100%	
Accuracy of the test	88.3%		96.7%		100%	

AFP = Alpha-fetoprotein; GLP-3 = Glypican-3; HCC = Hepatocellular carcinoma; PPV = Positive predictive value; NPV = Negative predictive value.

Table 4 Predictive power of serum AFP, GLP-3, and combined (AFP or GLP-3) in the diagnosis of small HCC (diameter ⩽ 3 cm).

	AFP: cut off point ⩽ 300 ng/ml		GLP-3: cut off point ⩽ 560 ug/L		Combined GLP-3: cut off point ⩽ 560 ug/L OR AFP: cut off point ⩽ 300 ng/ml	
	HCC ⩽ 3 cm +ve (n = 12)	HCC ⩽ 3 cm –ve (n = 18)	HCC ⩽ 3 cm + ve (n = 12)	HCC ⩽ 3 cm –ve (n = 18)	HCC ⩽ 3 cm + ve (n = 12)	HCC ⩽ 3 cm –ve (n = 18)
Tumor marker (+ve)	5	3	6	9	6	2
Tumor marker (−ve)	7	15	6	9	6	16
Sensitivity	41.7%		50.0%		50.0%	
Specificity	83.3%		50.0%		88.9%	
PPV	62.5%		40.0%		75.0%	
NPV	68.2%		60.0%		72.7%	
Accuracy of the test	66.7%		50.0%		73.3%	

AFP = Alpha-fetoprotein; GLP-3 = Glypican-3; HCC = Hepatocellular carcinoma; PPV = Positive predictive value; NPV = Negative predictive value.

Table 5 Predictive power of serum AFP, GLP-3, and combined (AFP or GLP-3) in the diagnosis of unicentric HCC.

	AFP: cut off point ⩽ 765 ng/ml		GLP-3: cut off point ⩽ 580 ug/L		Combined GLP-3: cut off point ⩽ 580 ug/L OR AFP: cut off point ⩽ 765 ng/ml	
	HCC (unicentric) +ve (n = 23)	HCC (unicentric) –ve (n = 7)	HCC (unicentric) +ve (n = 23)	HCC (unicentric) –ve (n = 7)	HCC (unicentric) +ve (n = 23)	HCC (unicentric) –ve (n = 7)
Tumor marker(+ve)	17	4	15	3	19	2
Tumor marker (−ve)	6	3	8	4	4	5
Sensitivity	73.9%		65.2%		82.6%	
Specificity	42.9%		57.1%		71.4%	
PPV	81.0%		83.3%		90.5%	
NPV	33.3%		33.3%		55.6%	
Accuracy of the test	66.7%		63.3%		80.0%	

AFP = Alpha-fetoprotein; GLP-3 = Glypican-3; HCC = Hepatocellular carcinoma; PPV = Positive predictive value; NPV = Negative predictive value.

results (83.3% and 93.3%, sensitivity and specificity, respectively). In agreement with previous studies, our data also showed that the simultaneous measurement of AFP and GLP-3 will improve the specificity for HCC diagnosis.[24,25,34]

We found that serum GLP-3 levels were not correlated with tumor size and Child-Pugh class. However, for HCC with small tumor size (diameter equal or smaller than 3 cm), we found that GLP-3 at cut-off value of ⩽560 ug/l had superior sensitivity than AFP at cut-off values of ⩽300 ng/ml (50% and 41.7%, respectively). Also, the combined use of both markers improved the specificity to 88.9%. These findings were in agreement with previous studies.[18,24,25,34] In respect to the unicentric HCC, we found that the combined use of GLP-3 at cut-off value of ⩽580 ug/L, and AFP at cut-off values of ⩽765 ng/ml, improved both sensitivity and specificity for diagnosis. This is in agreement with previous study.[24]

5. Conclusion

Serum GLP-3 is highly sensitive and specific for detecting HCC, and differentiating HCC from liver cirrhosis. Also, GLP-3 is more sensitive than AFP for the detection of smaller HCC, with diameter of 3 cm or less. Moreover, a combination of both markers yielded an improved specificity and both sensitivity and specificity for the diagnosis of small and unicentric HCC, respectively.

6. Limitations of the study

This work included a relatively small number of patients, which may affect the final conclusions. In addition, the experience with GLP-3 is still limited and its expression in adenocarcinomas of various sites and other tumors that can mimic HCC such as renal cell carcinoma have not been widely studied. Also, some studies have reported negative results in cholangiocarcinomas and majority of metastatic adenocarcinomas,[26,35] but GLP-3 expression has been described in melanomas, ovarian carcinoma and rarely in metastatic colonic adenocarcinoma.[36,37] Moreover, it was reported that, 14% of the metastases from gastrointestinal, and pancreatic tumors, are positive for GLP-3, suggesting that this marker might be not very specific for distinguishing HCC from extra-hepatic metastases.[38]

Conflict of interest

None declared.

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