Diagnostic Role of Serum Glypican-3 as a Tumor Marker for Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is a major health problem. It has been increasing in Egypt with a doubling in the incidence rate in the past 10 years. It represents the most common primary malignant tumor of the liver and is one of the major causes of death among patients with cirrhosis. Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP). The reported sensitivity and specificity of AFP are not sufficient for early diagnosis, and so additional marker is needed. The development of effective marker for the diagnosis of HCC could have an impact on HCC-related cancer mortality and significant public health implications worldwide. In the adult, Glypican-3 (GPC3) can only be detected in a limited number of tissues, including the lung, ovaries, mammary epithelium, and mesothelium. It is expressed in fetal livers but not in adult livers. The soluble form of GPC3 was identified in the serum of patients with hepatocellular carcinomas, and can be used as a serological test for the diagnosis of hepatocellular carcinoma. It was reported that the frequency of GPC3 expression in AFP-negative HCC patients is as high as 90%, suggesting that it can be used in diagnostic of HCC. **The aim of the current study** was to detect the value of serum GPC3 in HCC Egyptian patients as a more specific, sensitive and accurate biomarker by comparing it with an established biomarker as AFP. **Subjects and methods**: The patients were selected from the Department of Hepatology, National Liver Institute, Minoufiya University. There were three groups (HCC group, Liver cirrhosis group and control group). The serum estimation of AFP and GPC-3 were done to all subjects. **Results:** When analysis of variance was done between the three groups, a highly statistical significant difference was found between these groups regarding the mean serum levels of both AFP and GPC-3 where the highest increase of both markers were found in the HCC group. Results of the ROC curves analysis showed that the optimal cut-off of GPC-3 to differentiate between cirrhotic patients from healthy subjects is 0.5 ng/ml with 90% sensitivity & 80% specificity and 19 ng/ml with sensitivity and the specificity 63.5% and 70% respectively to differentiate HCC patients from liver cirrhotic patients. **Conclusion:** GPC-3 could be a sensitive, specific and accurate serum marker for early diagnosis of HCC. Further studies in larger groups of patients are needed to confirm this finding.

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1. Introduction:

Hepatocellular carcinoma (HCC) is a major health problem, with more than 500.000 cases diagnosed annually [(1)]. The burden of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the incidence rate in the past 10 years [(2)]. Estimates of the burden of cancer caused by these factors provide an opportunity for prevention. Previously, there was strong evidence that hepatitis B virus (HBV) was the major cause of HCC in Egypt, but more recently hepatitis C virus (HCV) has become the predominant factor associated with the more recent epidemic of HCC. It has been well documented that Egypt has one of the highest prevalence rates of HCV infection in the world [(3)]. Other factors such as cigarette smoking, occupational exposure to chemicals such as pesticides, and endemic infections in the community, such as schistosomiasis, may have additional roles in the etiology or progression of the disease [(4)]. Early recognition of the onset of HCC would help to select more effective therapies for patients leading to a better prognosis and life span. Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP) [(5)].

Serum alpha-fetoprotein (AFP) was first described as a marker for HCC by **Abelev *et al.*, in the 1960** and used as a serum marker for HCC in humans for many years [(6)]. The first quantitative serum assays for AFP were established by **Ruoshlati and Seppala** [(7)]. It has a sensitivity of 39%–65%, a specificity of 76%–94%, and a positive predictive value of 9%–50% [(8)]. HCC patients with a high AFP concentration (≥ 400 IU/mL) tend to have greater

tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis and a lower median survival rate [9]. Though the measurement of AFP serves as an important tool in screening of HCC, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for: its high false-positive and false-negative rates, elevated levels in patients with acute exacerbation of viral hepatitis and that tumors other than HCC may also have markedly increased AFP levels like testicular tumors [9]. AFP with its reported sensitivity and specificity are not sufficient for early diagnosis as AFP concentrations are directly correlated with tumor size. So, the development of effective marker for the diagnosis of HCC could have an impact on HCC-related cancer mortality and significant public health implications worldwide [10]. Although ultrasonography has been widely used in clinical screening of HCC, it is highly dependent on the experience of its operator. Therefore, as AFP and ultrasonography only play a limited role in screening of HCC, some candidate biomarkers can be used in diagnosis of HCC including glypican-3 (GPC3) [11].

GPC3 belongs to a family of glycosylphosphatidylinositol-anchored, cell-surface heparan sulfate proteoglycans. Six glypicans have been identified in mammals so far (GPC1 to GPC6) [12]. Although the homology of amino acids between glypican members is moderate, all glypicans are approximately 60 to 70 kd in size and share a characteristic pattern of 14 conserved cysteine residues [13]. Intact glypicans are decorated with heparan sulfate (HS), which is located in the last 50 amino acids of the C terminus, placing the HS chains close to the cell membrane [14].

It is an oncofetal protein that is located on the X chromosome, and is highly expressed in the embryo and involved in morphogenesis and growth control during development [15]. It is reported that a loss-of -function mutation in the GPC3 gene causes Simpson-Golabi-Behmel syndrome, a rare X-linked disorder characterized by pre- and postnatal overgrowth, increased risk of embryonic tumors during early childhood, and numerous visceral and skeletal anomalies [16].

In the adult, GPC3 can only be detected in a limited number of tissues, including the lung, ovaries, mammary epithelium, and mesothelium [17]. So, down regulation of GPC3 has been observed in several human malignancies, including mesothelioma and ovarian, breast and lung cancers [18-21]. These observations indicate that GPC3 is an inhibitor of cell proliferation and a tumor suppressor in a tissue-specific manner [22].

GPC3 is expressed in fetal livers but not in adult livers [23]. There have been a number of studies showing that GPC3 expression is frequently up-regulated in HCCs at the messenger RNA and protein levels when compared with normal livers and benign hepatic lesions [24]. The soluble form of GPC3 was identified in the serum of patients with hepatocellular carcinomas, and can be used as a serological test for the diagnosis of hepatocellular carcinoma [25]. The results of immunohistochemical studies have convincingly shown that GPC3 is a novel diagnostic marker for HCC [26]. It was reported that the frequency of GPC3 expression in AFP-negative HCC patients is as high as 90%, suggesting that it can be used in diagnostic of HCC [27].

So, the aim of the current study was to detect the value of serum GPC3 in HCC Egyptian patients as a more specific, sensitive and accurate biomarker by comparing it with an established biomarker as AFP.

2. Subjects and Methods:

Patients:

In the current study, the patients were selected from the Department of Hepatology ,National Liver Institute ,Minoufiya University .There were three groups.

First group (HCC group): It included 85 patients (67 males and 18 females). Their mean age was 55.74 ± 5.2 years. These patients were diagnosed as HCC by the presence of characteristic hepatic masses on liver CT, MRI and hepatic angiography (i.e., enlarged tumors and/or tumors with typical arterial vascularization. Tumor staging was determined according to the Cancer of liver Italian Program (CLIP) classification [28].

Second group (LC group): It included 50 liver cirrhotic (LC) patients (39 males and 11 females). Their mean age was 54.84 ± 3.68 years with no radiological evidence of HCC.

Third group (Control group): It included 35 apparently healthy subjects as a control group with no evidence of liver disease and/or neoplasm. They were 29 males and 6 females, with mean age 56.82 ± 6.17 years.

All the procedures included in this study were approved by the Research Ethics Committee of National Liver Institute, Minoufiya University, Egypt. Venous blood sample were withdrawn by venipuncture from all individuals included in this study and subjected to the following parameters: prothrombin time (PT) & concentration (using Behring Fibrin timer II, Germany), total & direct bilirubin (T. & D.Bil) and albumin (using the Beckman Coulter, Synchron C9 ALX, Clinical Autoanalyzar, USA), AFP (using VIDAS instrument, BioMerieux, France by the Enzyme Linked Fluorescent Assay). 1ml of serum of each subject

involved in this study was frozen and stored until GPC3 assay. Serum level of GPC3 was determined by using Uscn Life Science Inc. Wuhan, Germany, by the enzyme linked Immunosorbent assay. By following the manufacturer's protocol, the concentration of GPC3 in the samples is determined by comparing the optical density of the samples to the standard curve. The minimum detectable dose of human GPC3 in the kit is 0.036 ng/ml.

Statistical Analysis:

Data were collected and entered to the computer using SPSS (Statistical Package for Social Science) program for statistical analysis. For comparing 2 groups, continuous normally distributed variables were tested for association by student's t-test. On the other hand, Mann–Whitney test is a non-parametric test for assessing whether two independent samples of observations have equally large values. For more than 2 groups, normally distributed variables were tested with ANOVA test. While, Kruskal Wallis test was done for variables that wasn't normally distributed.

The Pearson`s correlation coefficients were calculated for the normally distributed values. However, Spearman`s correlation coefficients were done for the not normally distributed values. Receiver Operating Characteristic (ROC) curves was produced for the measured parameters to investigate the sensitivity, specificity and the cut-off values of each AFP and GPC3. *P* value <0.05 and <0.01 were considered statistically significant.

3. Results:

Table (1) showed the statistical comparison between HCC group, LC group and the control group regarding the mean serum albumin & total bilirubin, INR and degree of ascites & encephalitis. When analysis of the results of the mean serum level of each AFP and GPC3 in the three studied groups, a statistical significant difference (p<0.001) was detected between them where the maximum increase of both parameters was observed in the HCC group as in table (2).

Table (1): Statistical comparison of different studied parameters in the three studied groups

	Studied groups						Test of sign.	*P* value
	HCC N = 85		LC N = 50		Control N = 35			
	N	%	N	%	N	%		
Albumin (g/dl):								
< 2.8	44	51.8	19	38.0	0	0.0	113.0	<0.001
2.8 – 3.5	29	34.1	22	44.0	0	0.0		
> 3.5	12	14.1	9	18.0	35	100		
Bilirubin (mg/dl):								
< 2	42	49.4	32	64.0	35	100 0.0	37.1	
2 – 3	23	27.1 23.5	11	22.0	0	0.0		<0.001
> 3	20		7	14.0	0			
INR:								
< 1.7	24	28.2	10	20.0	35	100	90.75	<0.001
1.71 – 2.1	41	48.2	35	70.0	0	0.0		
> 2.1	20	23.5	5	10.0	0	0.0		
Ascites:								
Non	38	44.7	26	52.0	35	100	43.84	<0.001
Mild	30	35.3	17	34.0	0	0.0		
Mod. / sever	17	20.0	7	14.0	0	0.0		
Encephalitis:								
Non	64	75.3	49	98.0	35	100	24.79	<0.001
Mild	17	20.0 4.7	1	2.0 0.0	0	0.0		
Mod. / sever	4		0		0	0.0		

P-value is highly significant at <0.001

Table (2): Statistical comparison of different studied parameters in the three studied groups

	Studied groups			Test of sign.	*P* value
	HCC N = 85 Mean ± SD	LC N = 50 Mean ± SD	Control N = 35 Mean ± SD		
Child score:	9.10 ± 2.87	8.24 ± 2.30	5.0 ± 0.0	52.05	<0.001
AFP (IU/ml):	5529.0 ± 7008.6	80.4 ± 159.3	6.6 ± 3.04	72.10	<0.001
GPC3 (ng/ml):	1646.3 ± 3980.2	12.7 ± 10.4	1.3 ± 2.9	102.05	<0.001

P-value is highly significant at <0.001

Table (3) showed the descriptive statistics of CLIP score items in the HCC patients where the mean CLIP score in these patients was 2.97 ± 1.68.

Figure (1) represented the ROC curve of GPC3 and AFP to differentiate HCC patients from liver cirrhotic patients. When analysis of the results of the ROC curve, this study found the cut-off point is 19 ng/ml for GPC3 and 78 IU/ml for AFP. The sensitivity and the specificity for GPC3 is 63.5% and 70% respectively and for AFP is 76.5% and 82% respectively as described in table (4).

Figure (2) showed the ROC curve of GPC3 and AFP to differentiate liver cirrhotic patients from healthy subjects. By analysis of the data in the ROC curve as in table (5), the cut-off to differentiate between cirrhotic patients from healthy subjects is 0.5 ng/ml for GPC3 with 90% sensitivity & 80% specificity and 8.5 IU/ml for AFP with 76% sensitivity & 80% specificity.

Table (6) showed a statistically significant positive correlation between GPC3 and each of AFP (r=0.593, p=<0.001), tumor size (r=0.277, p=<0.05) and CLIP score (r=0.505, p=<0.001). Also, a statistically significant positive correlation was found between AFP and each of Child score (r=0.302, p=<0.05), CLIP score (r=0.640, p=<0.001 and the tumor size (r=0.469, p=<0.001).

Regarding the mean serum level of GPC3 in HCC patients as in table (7), it showed a statistically significant difference (p<0.001) with tumor size and presence of portal vein invasion. Considering the mean serum level of AFP in the same patients, a statistically significant difference (p<0.001) was found with the same parameters. While no statistically significant difference was detected between each of GPC3 and AFP with Child score.

Table (3): Descriptive statistics of CLIP score items in HCC group:

	N=85	%
Child score:		
5 – 6	19	22.4
7 – 9	27	31.8
10 – 15	39	45.9
Tumor:		
Uninodular	25	29.4
Multi nodular < 50	42	49.4
Multi nodular > 50	18	21.2
AFP (ng/ml):		
< 400	50	58.8
> 400	35	41.2
Portal vein invasion:		
Yes	24	28.2
No	61	71.8
CLIP score: (Mean ± SD)	2.97 ± 1.68	

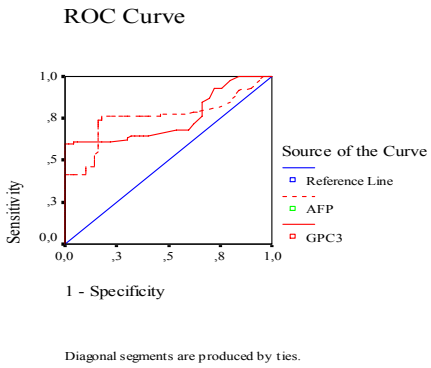

Figure (1): ROC curve of GPC3 and AFP to differentiate HCC patients from liver cirrhotic patients

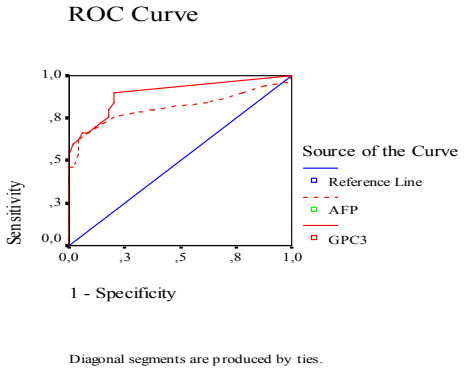

Figure (2): ROC curve of GPC3 and AFP to differentiate liver cirrhotic patients from control subjects

Table (4): ROC curve analysis of the studied parameters (GPC3 and AFP) to differentiate HCC patients from liver cirrhotic patients

	GPC3 (ng/ml)	**AFP** (IU/ml)
Area under the curve	0.753 (<0.001)	0.758 (<0.001)
95 % CI	0.672 – 0.834	0.677 – 0.840
Cut-off point	19.0	78.0
Sensitivity	63.5%	76.5%
Specificity	70.0%	82.0%
Positive predictive value	78.3%	87.8%
Negative predictive value	53.0%	67.2%
Accuracy of the test	65.9%	78.5%

Table (5): ROC curve analysis of GPC3 and AFP differentiate liver cirrhotic patients from control subjects

	GPC3 (ng/ml)	**AFP** (IU/ml)
Area under the curve	0.897 (<0.001)	0.813 (<0.001)
95 % CI	0.834 – 0.960	0.723 – 0.904
Cut-off point	0.50	8.50
Sensitivity	90.0%	76.0%
Specificity	80.0%	80.0%
Positive predictive value	81.8%	79.2%
Negative predictive value	88.9%	76.9%
Accuracy of the test	85%	78%

Table (6): Correlation analysis between each of GPC3 and AFP with different parameters in HCC patients (N=85)

	GPC3 (ng/ml)		**AFP** (IU/ml)	
	(r)	***P* value**	**(r)**	***P* value**
Child score	+ 0.161	>0.05	+ 0.302	<0.05
Tumor size	+ 0.277	<0.05	+ 0.469	<0.001
AFP (IU/ml)	+ 0.593	<0.001		
Portal vein invasion	+ 0.431	<0.001	+ 0.455	<0.05
CLIP score	+ 0.505	<0.001	+ 0.640	<0.001

P-value >0.05 isn't significant *P*-value is significant at <0.05 & <0.001
Spearman's correlation was used for all the variables except for AFP and CLIP score as Pearson's correlation was done.

Table (7): Statistical comparison between each of GPC3 and AFP with different parameters in HCC patients (N=85)

	GPC3 (ng/ml)			**AFP** (IU/ml)		
	GPC3 (ng/ml) **Mean ± SD**	**Test of sign.**	***P* value**	**AFP** (IU/ml) **Mean ± SD**	**Test of sign.**	***P* value**
Child score						
5 – 6	225.94 ± 312.05	0.18	>0.05	2294.0 ± 3860.11	2.72	>0.05
7 – 9	1464.59±2702.38			5462.33±6082.92		
10 – 15	2464.17±5315.91			7151.17 ±8262.65		
Tumor						
Uni nodular	316.48 ±324.96	11.17	<0.001	3572.64±4437.38	22.36	<0.001
Multi nodular<50	935.47±2265.11			3536.23±6108.05		
Multi nodular>50	5152.11±6992.50			12895.94±7213.51		
AFP (IU/ml)						
< 400	76.92± 153.39	7.66	<0.001	71.06 ± 57.83	7.81	<0.001
> 400	3888.40±5505.43			13326.06 ±3868.23		
Portal vein invasion						
Yes	531.16 ±1320.54	3.95	<0.001	3650.29±5974.74	4.17	<0.001
No	4480.79±6452.06			10304.04±7288.42		

P-value >0.05 isn't significant *P*-value is significant at <0.05 & <0.001

4. Discussion:

Hepatocellular carcinoma (HCC) is characterized by a multi-cause, multi-stage and multi-focus process of tumor progression. Its prognosis is poor due to both its late detection and the lack of effective therapies for advanced stage disease[29-30]. Up to 80% of HCCs develop against a background of cirrhosis of the liver and the surveillance of the at risk cirrhotic population could aid earlier detection of the disease and decrease the cancer related mortality rate [31]. Currently, standard surveillance includes a combination of 6 monthly abdominal ultrasound scan and serum alphafetoprotein measurement, but this strategy does not reliably detect early disease [32]. The aim of this study was to evaluate the value of serum GPC[3] in HCC Egyptian patients .

Regarding the mean serum level of AFP, a highly statistically significant difference was observed between the three groups (HCC, Liver cirrhosis and control groups). A marked increase showed in the HCC group, while a slight increase occurred in the cirrhotic group. In the current study by applying the ROC curves, analysis showed the best cut-off value for AFP to differentiate HCC patients from cirrhotic patients was 78 IU/ml. This gave 76.5% in sensitivity and 82% in specificity. While the best cut-off recorded in this study to diagnose liver cirrhosis was 8.5 IU/ml. It yielded a 76% sensitivity and 80% specificity. **Soresi *et al.*** [33]

showed that the best cut-off value of AFP has been reported to be 30 IU/ml (sensitivity of 65%, specificity of 89%) in Sicilian population compared with 200 IU/ml (sensitivity of 70%, specificity of 100%) in Burman population. However, **Zhou and his colleagues**, [34] reported that soma investigations have showed that the cut-off value is fluctuant in different ethnic groups and one of possible reasons for this difference is the diverse living circumstance which has a great influence on epidemiology. They also reported that AFP is more useful in detecting HCC patients with non-viral etiology. **Lau and Lai** [35] stated the specificity of AFP is very high when the levels are above 400 IU/ml in patients without testicular tumor. Also, **Goma *et al.*** [8] revealed an AFP value above 400-500 IU/ml has been considered to be diagnostic for HCC in patients with cirrhosis. Therefore, all these results indicate that serum AFP level plays a limited role in diagnosis of HCC, especially early HCC.

Regarding the mean of the serum level of GPC3, The present study showed a highly statistically significant difference was observed between the studied three groups with highest increased in the HCC group and a slight increase only occurred in the cirrhotic group. Moreover, the appropriate cut-off value for serum GPC3 that distinguishes between HCC patients from cirrhotic patients was >19 ng/ml; it yielded a 63.5% sensitivity and 70% specificity. While the best cut-off that differentiates cirrhotic patients from control subjects was >0.5 ng/ml, it gave a 90% sensitivity and 80% specificity. **Liu and his coworkers** [11] agreed with this study as they stated that GPC3 can be used as a potential biomarker for the diagnosis of early HCC and can be used in screening of HCC as they found that the serum GPC3 level was higher than 300 ng/l in 50% of early HCC patients, although their serum AFP level was below 100 µg/L in their study. They recorded that at cut-off 300 ng/L for GPC3, the sensitivity and specificity for the diagnosis of HCC was 46.7% and 93.5% respectively. **Shafizadeh *et al.*** [27] found GPC3 positive cells in 90% of patients with their serum AFP level <400 µg/L. They also found that serum GPC3 level was increased in early HCC patients with their serum AFP level <400 µg/L. So, they concluded that GPC3 is a sensitive, specific serum and tissue marker for the diagnosis of early HCC. Also, **Nakatsura *et al.*** [36] demonstrate that the expression of GPC3 (at both mRNA and protein levels) in the serum of HCC patients is significantly higher than that in serum of healthy adults (p<0.001) or patients with nonmalignant hepatopathy (p<0.01), and it can be detected in 40-53% of HCC patients and 33% of HCC patients with seronegative for AFP. **Yao *et al.*** [30] concluded that an oncofetal antigen GPC3 and GPC-3 mRNA expression in hepatocarcinogenesis is a promising molecular markers for early diagnosis of HCC, especially in poor-differentiated or small HCC.

However, **Beale and his colleagues** [37] revealed that GPC3 has no role at all in the surveillance of HCC in individuals with steatohepatitis related cirrhosis as they found both the sensitivity and specificity of GPC3 were poor in their patient set.

At the current study, a positive correlation was found between serum level of each of AFP and GPC3 with both tumor size and portal vein invasion. This comes in accordance with **Zhou *et al***. [34] who stated that HCC patients with a high AFP (≥400 ng/ml) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis, and a lower median survival rate.

In conclusion, GPC-3 could be a sensitive, specific and accurate serum marker for early diagnosis of HCC. Further studies in larger groups of patients are needed to confirm this finding.

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