

RESEARCH ARTICLE

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Diagnostic Value of Glypican-3 for Hepatocellular Carcinomas

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Abstract

Background: Hepatocellular carcinoma (HCC) is a common and dangerous malignancy in many parts of the world, and especially in Egypt. Early diagnosis is the most important step in successful HCC management. However most cases are detected at late stage making effective intervention impossible. **Aim:** The aim of this study was to evaluate the potential of Glypican-3 (GPC-3) to aid in diagnosis of HCC, especially in patients with low serum alpha-fetoprotein (AFP). **Subjects and methods:** Serum GPC-3 was assessed by flow-cytometry and serum AFP by enzyme-linked immunosorbent assay (ELISA) in 40 HCC patients with AFP < 400ug/l. (GI), 40 HCC patients with AFP > 400ug/l. (GII) and 20 healthy controls (GIII). **Results:** GPC-3 was found to be significantly elevated in HCC as compared to healthy subjects (GI 38.2±22.5, GII 50.2±22.6, and GIII 2.24±1.19), with sensitivities of 85% for GI and 84% for GII and specificities of 95% for GI and 92% for GII. AFP showed respective sensitivities of 50% and 79%, and specificities of 80% and 90%, for HCC diagnosis. The combination of GPC-3 with AFP achieved the highest sensitivity (98.5%) and specificity (97.8%). **Conclusion:** Serum GPC-3 has a better sensitivity than AFP for the diagnosis of HCC. Combination of two markers appears warranted for greatest accuracy

Keywords: Glypican-3- AFP- HCC- diagnostic sensitivity

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Introduction

Primary hepatocellular carcinoma (HCC) is one of the most dangerous malignant tumors and is the most common cause of cancer related death worldwide (Siegel et al., 2012). Egypt has a high prevalence of hepatocellular carcinoma (HCC). This high prevalence in Egypt is related to the high rate of viral hepatitis B and C and environmental risks (Su et al., 2013, El Azm et al., 2013; Ziada et al., 2016). A large portion of HCC patients are diagnosed in advanced stage making the medical intervention difficult or impossible and this makes the prognosis worse. Early diagnosis of HCC is very important to increase the chance of effective treatment and reduce the HCC related mortality (Yang et al., 2014). Abdominal ultrasound and checking of serum alpha-fetoprotein (AFP) level are recommended for patients with chronic liver diseases. However the accuracy of ultrasound depends greatly on the operator skill and can't differentiate between malignant and non malignant focal lesions (Yang et al., 2014) similarly, AFP has a limited sensitivity. Normal AFP values were reported in many HCC patients while AFP may be elevated in some benign hepatic focal lesions as well as non hepatic

malignancies (Abu El Makarem, 2012; Xu et al., 2013).

Many authors tried to evaluate the value of different biomarkers in HCC diagnosis aiming to improve the diagnostic accuracy as well as the prognosis. (Youns et al., 2013, El-Mashad et al., 2015, Ismail et al., 2015; Ismail et al., 2017). Glypican-3 (GPC-3) is one of these promising biomarkers. "GPC-3 is an oncofetal protein encoded on the X chromosome (Sung et al., 2003). GPC-3 is a member of the glypican family, a group of heparan sulfate proteoglycans joined to the cell surface through a glycosyl-phosphatidyl inositol-anchor. It has been found that glypicans interact with growth factors and modify their activities and perform an important role in cell growth, differentiation and migration" (Filmus and Selleck, 2001; Kandil and Cooper, 2009). GPC-3 is expressed abundantly in the fetal liver, minimally expressed in the normal adult liver. Currently, many studies have found that GPC-3 expression is increased in tissues and serum of HCC patients than in normal individuals and hepatitis patients (Zhang et al., 2010; Badr et al., 2014; Lee et al., 2014). The aim of this study is to evaluate the role of glypican-3 in the diagnosis of hepatocellular carcinoma especially in patients with serum alpha-fetoprotein (AFP) levels below

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the cutoff value of diagnosis.

Materials and Methods

This study was conducted on 80 HCC patients presented to the tropical medicine and Clinical Oncology Departments. 20 healthy individuals were included as a control group. A written consent was taken from all patients and controls and the study was approved by the ethical committee.

The subjects of this study were classified into 3 groups:

Group (I): Included 40 patients with hepatocellular carcinoma with alpha-fetoprotein Less than 400ug/l.

Group (II): Included 40 patients with hepatocellular carcinoma with elevated alpha-fetoprotein level (More than 400ug/l).

Group (III): Included 20 healthy individuals as a control group.

Patients with HCC were diagnosed by triphasic CT with or without elevated AFP levels (Murakami et al., 2002).

All patients and control groups subjected to thorough history taking and full clinical examination, Laboratory investigations in the form of complete liver function tests (serum total and direct bilirubin, ALT, AST, total serum protein and serum albumin, prothrombin time and activity), Erythrocyte sedimentation rate (ESR), complete blood count (CBC) with platelet count, blood urea and serum creatinine. Modified Child Score was calculated in GI and GII (Kamath et al., 2007). Abdominal Ultrasonography for detection of signs of liver cirrhosis and HCC (Yu and Keeffe, 2003). Abdominal triphasic spiral CT: HCC appears enhanced during the arterial phase followed by a rapid wash-out in the portal phase (Murakami et al., 2002). Measurement of serum alpha-fetoprotein (AFP) by Enzyme-linked immunosorbent assay (ELISA) (Macias-Rodriguez et al., 2000).

Detection of serum Glypican-3 expression by flow-cytometry

Glypican-3 was assayed by (FAB2119P).

Sample preparation

Peripheral blood cells: Whole blood was collected in evacuated tubes containing EDTA or heparin as the anticoagulant. Contaminating serum components were removed by washing the cells three times in an isotonic phosphate buffer (supplemented with 0.5% BSA) by centrifugation at 500 x g for 5 minutes. 50 µL of packed cells transferred to a 5 mL tube for staining with the monoclonal antibody (Hutter and Stoher, 1982).

Cell Cultures:

Continuous cell lines or activated cell cultures centrifuged at 500 x g for 5 minutes and washed three times in an isotonic PBS buffer (supplemented with 0.5% BSA), as described above, to remove any residual growth factors that may be present in the culture medium. Then Cells re-suspended in the same buffer to a final concentration of 4 x 10⁶ cells/mL and 25 µL of cells (1 x 10⁵) transferred to a 5 mL tube for staining (Hayashi et al., 1992).

Sample Staining

1) Cells Fc-blocked by treatment with 1 µg of human IgG/105 cells for 15 minutes at room temperature prior to staining.

2) 25 µL of the Fc-blocked cells (1 x 10⁵ cells) or 50 µL of packed whole blood transferred to a 5 mL tube.

3) 10 µL of phycoerythrin (PE)-conjugated Glypican-3 reagent added.

4) Incubated for 30-45 minutes at 2°-8°C.

5) Following this incubation, we removed un-reacted Glypican-3 reagent by washing the cells twice in 4 mL of the same PBS buffer.

6) Finally, the cells resuspended in 200-400 µL of PBS buffer for final flow cytometric analysis.

7) As a control for analysis, cells in a separate tube treated with phycoerythrin (PE)-labeled mouse IgG2A antibody.

Principle of the test

Washed cells were incubated with the phycoerythrin-labeled monoclonal antibody, which binds to cells expressing Glypican-3. Unbound phycoerythrin - conjugated antibodies were then washed from the cells. Cells expressing Glypican-3 were fluorescently stained, with the intensity of staining directly proportional to the density of expression of Glypican-3. Cell surface expression of Glypican-3 was determined by flow cytometric analysis using 488 nm wave length laser excitation and monitoring emitted fluorescence with a detector optimized to collect peak emissions at 565 - 605 nm (Tometsko et al., 1993).

Reagent

Phycoerythrin-conjugated mouse anti-human Glypican-3 used as it is (no preparation necessary).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Comparison was performed using statistical package for social studies (SPSS). Comparison between groups was performed using one way ANOVA on rank test (Tukey's test) and P value less than 0.05 was considered statistically significant. The receiver operator characteristic (ROC) curve analysis, reporting area under the curve (AUC) and its 95% confidence interval. The sensitivity and specificity were determined.

Results

Table 1 shows the demographic and clinical data of the studied groups. Table 2 shows comparison of laboratory data (ALT, AST, prothrombin activity, Hemoglobin, White blood cell and platelet count, alpha-fetoprotein (AFP), as well as Glypican-3 expression in the studied groups. Significant differences were observed between the diseased groups (I and II) and the control group (GIII) as regards liver function tests (p<0.05) as well as blood picture except for white cell count which showed a non-significant difference between the three studied groups (p>0.05).

Serum alpha-fetoprotein levels showed a significant

Table 1. Shows the Demographic and Clinical Data of the Studied Groups

	Group I (40)	Group II (40)	Group III (20)
Male	15 (37.50%)	23 (57.5%)	10 (50%)
Females	25 (62.5%)	17 (42.50%)	10 (50%)
Age	57.93±11.14	57.38±8.58	44.28±12.01
Jaundice	29 (72.5%)	32 (80%)	-
Splenomegaly	32 (80%)	35(87.5%)	-
Ascites	25 (62.5%)	30 (75%)	-
Modified Child score	9.43±1.97	9.83±2.3	-
Tumor size (Cm)	3.183±1.928	4.063±2.067	

difference between all studied groups. The P value was <0.001 for comparison between Group I and II, and between group II and III and < 0.05 for comparison between I and III Table 2. The percentage of Glypican-3 expression showed a non-significant difference between the 2 HCC groups I and II (P. value >0.05), and significant difference between both HCC groups (I and

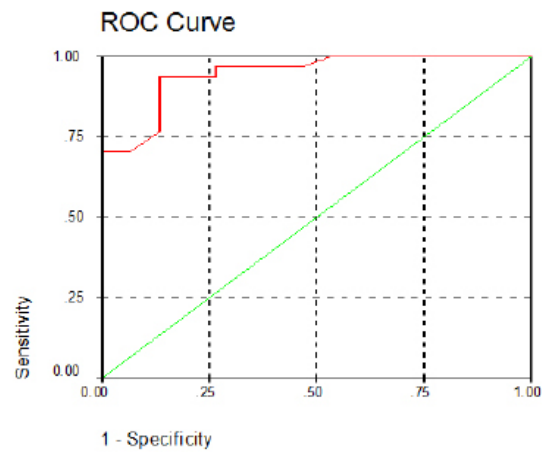


Figure 1. The Sensitivity and Specificity of Glypican-3% in Group I

II) in relation to the control group (P. value < 0.001) (Table 2). Receiver Operator Characterizing (ROC) curve analysis of our results showed that, Glypican 3 had 85%

Table 2. Comparison of the Laboratory Data of the Studied Group

	Group I	Group II	Group III	P
Serum bilirubin (mg\dl)	4.021±2.57	4.45±3.45	0.39±0.25	P1=0.764 P2 <0.001* P3 <0.001*
SGOT (IU/L)	36.88±31.69	37.32±19.19	16.95±4.322	P1 =0.996 P2 = 0.007* P3 =0.006*
SGPT (IU/L)	30.6±19.54	33.25±19.85	15.65±4.57	P1 = 0.783 P2 = 0.008* P3 = 0.001*
Serum albumin (gm\dl)	2.76±0.43	2.78±0.42	3.74±0.48	P1 = 0.957 P2 <0.001* P3 <0.001*
Prothrombin %	42.94±18.75	42.43±17.76	82.8±6.86	P1-0.990 P2 <0.001* P3 <0.001*
Hemoglobin (gm\dl)	8.9±1.27	9.45±1.466	12.27±1.42	P1= 0.179 P2 <0.001* P3 <0.001*
Total leukocytic count (x10 ³ cell/cc)	6.55±2.01	6.15±1.99	6.06±1.55	P1= 0.45 P2 = 0.09 P3 = 0.841
Platelets (x10 ³ /cc)	75.45±20.44	74.57±16.66	160.4±35.44	P1 = 0.887 P2 <0.001* P3 <0.001*
AFP(ug/l)	117.14±110.89	1,572.39±1496.37	3.36±2.22	P1<0.001* P2 < 0.05* P3<0.001*
GP-3 expression %	38.15±22.45	50.15±22.58	2.24±1.19	P1 >0.05 P2 <0.001* P3 <0.001*

GPC-3 %, Glypican-3 expression ; AFP, Alpha-fetoprotein; P1, GI versus GII; P2, GI versus GIII; P3, GII versus GIII; *, significant

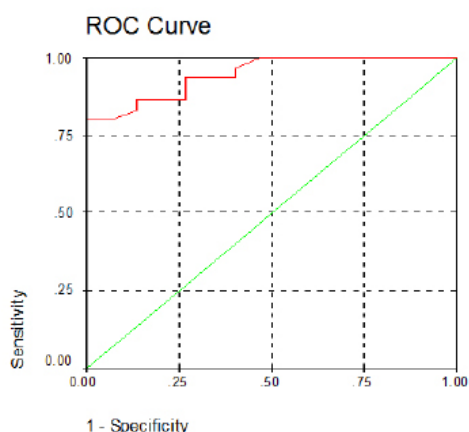


Figure 2. The Sensitivity and Specificity of Glypican-3% in Group II.

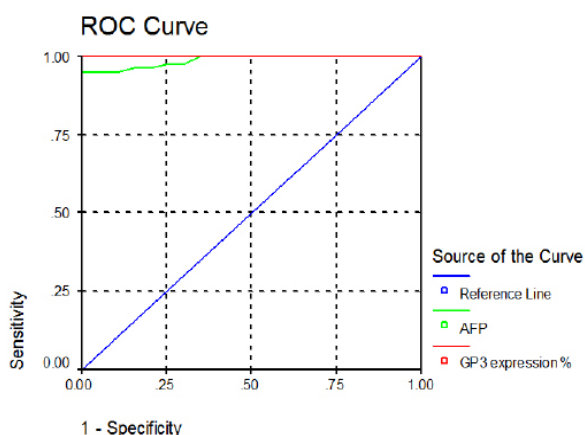


Figure 3. The Sensitivity and the Specificity of Combined Glypican-3% and Serum Alpha-fetoprotein (ug/l).

Table 3. Sensitivity, Specificity and the Accuracy of Alpha-fetoprotein and Glypican-3 in Patient Groups

	Sensitivity%	Specificity %	Accuracy%
AFP GI	50	80	60
AFP GII	79	90	80
GPC-3 GI	85	95	89
GPC-3 GII	84	92	85
AFP& GPC-3	98/5	97/8	93

GPC-3 %, Glypican-3 expression; AFP, Alpha-fetoprotein

sensitivity, 95% specificity and 89% accuracy in group I and 84% sensitivity, 92% specificity and 85% accuracy in group II Table 3, Figure 1 and 2. On the other hand, Alpha Fetoprotein had 50% sensitivity, 80% specificity

and 60% accuracy in group I (GI) and 79% sensitivity, 90% specificity and 80% accuracy in group II Table 3. Sensitivity and specificity of combined glypican-3% and serum alpha Fetoprotein ug/l: glypican-3% and serum alpha Fetoprotein together had 98.5% sensitivity, 97.8% specificity and 93% accuracy Table 3, Figure 3 and Table 4 shows a non-significant correlation between Glypican-3 (GPC-3) expression and the size of the tumor or child score while there was a significant correlation between alpha-fetoprotein (AFP) and the Tumor size. Also, non-significant correlation was found between Glypican-3 expression and alpha-fetoprotein (AFP).

Discussion

Hepatocellular carcinoma (HCC) is the seventh most common malignant tumor in the world (Yao et al., 2011). Egypt showed a doubling in the incidence of HCC rate in the past 10 years (EL-Shenawy et al., 2012). Usually HCC starts asymptomatic but it grows rapidly making the diagnosis occurs in a late stage with losing the opportunity of effective interventions and poor prognosis (Yao et al., 2007). So, the most important step in treatment is the early diagnosis of hepatocellular carcinoma (Bruix and Sherman, 2005). The clinical value of AFP is still unsatisfying due to its low sensitivity and specificity in diagnosis of HCC because many patients with HCC have no elevated level of AFP, while some patients with benign hepatic lesions, chronic hepatitis, liver cirrhosis, or other gastrointestinal cancer also may cause elevated serum levels of AFP (Spangenberg et al., 2006; Bruix and Sherman, 2011). Also, ultrasonography it is highly dependent on the experience of its operator (Poon et al., 2009). Specific and sensitive biomarkers are needed for accurate diagnose HCC especially in early stage (Yang et al., 2014). A novel biomarker is sensitive, specific, accurate, cheap and easy to perform to both the doctor and the patients (Mendy and Walton, 2009). Our study aimed to evaluate the role of glypican-3 in diagnosis of hepatocellular carcinoma patients in whom serum alpha-fetoprotein (AFP) levels are below the cutoff value of diagnosis. The results of our study revealed that HCC patients had significantly higher serum Alpha-Fetoprotein level when compared to healthy individuals. This agreed with El-Housseini et al., (2005), Hussein et al., (2008) and Ma et al., (2013) who found that AFP level was higher in HCC patients compared with healthy subjects. Also, there was a statistically significant difference between the 2 groups of HCC (GI and GII) as regard AFP. This could be explained by selecting patients in group I with

Table 4. Correlation Between Studied Parameters

	GPC-3 % GI	GPC-3 % GII	AFP GI (ug\l)	AFP GII (ug\l)
Tumor size	r= 0.03 P= 0.81	r= 0.24 P= 0.12	r=0. 46 P= 0.003*	r=0.397 P=0.011*
Child score	r=0.24 P= 0.13	r= 0.12 P= 0.42	r=0.086 P= 0.59	r=0.23 P= 0.13
AFP(ug\l)	r=0.09 P= 0.56	r= 0.15 P= 0.34		

GPC-3 %, Glypican-3 expression, AFP; Alpha-fetoprotein, *: significant

AFP < 400ug/l and in group II AFP > 400ug/l. So, this significant difference between HCC groups (I and II) in AFP suggests that serum AFP may be of little benefit in the diagnosis of HCC in patients with AFP less than 400u/l (Xu et al., 2013). The results of our study revealed that Glypican-3% was significantly higher in both HCC groups than in the control group. This was in accordance with Nakatsura et al., (2003); Tangkijvanich et al., (2010) and El-Shenawy et al., (2012) who found the GPC-3 expression in the serum of HCC patients to be significantly higher than that in the serum of healthy adults. Moreover, many authors found the level of serum GPC-3 in HCC to be higher than that in healthy subjects or patients with other liver diseases which may show elevated AFP (Zhang et al., 2010; Lee et al., 2014; Yu et al., 2015). Recently, Saber et al., (2017) found, GPC-3 mRNA to be expressed in the tissues all HCC cases, with overexpression in 81.9% of these cases. They considered GPC-3 to be a good marker for HCC diagnosis. Our study revealed that there was no significant difference between group I&II as regard GPC-3 expression as it is elevated in all our HCC cases. These data could suggest that GPC-3 may be a more effective diagnostic marker for HCC than AFP which may be lower than the diagnostic levels in some cases (Xu et al., 2013). The present study showed that there was no significant correlation between Glypican-3% and Child score as well as tumor size. This agreed with Chen et al., (2013) and Mohamed et al., (2013) who found no significant correlation between GPC3 expression and Child-Pugh score or tumor size similarly, Lee et al., (2014) reported a non-significant correlation between serum Glypican-3 levels and tumor size or tumor stage. This means that, Glypican-3 expression was not affected by the HCC size suggesting its role as a potential biomarker for the diagnosis of early stage and small sized HCC (Tangkijvanich et al., 2010; Tsuchiya et al., 2015). The present results reported that, Glypican-3 is more accurate than AFP (Glypican-3 accuracy was 89.11% in GI and 85.7% in GII versus 60% in GI and 80% in GII for AFP) in the diagnosis of HCC. The sensitivity of GPC3 was 85 in GI and 84% in GII while the sensitivity of AFP was 50% in GI and 79% in GII. The specificity of GPC-3 was 95.12% in GI and 92.36% in GII on the other hand; the specificity of AFP was 80% in GI and 90% in GII. The combined use of Glypican-3 and AFP results in 98.5% sensitivity, 97.8% specificity and 93.2% accuracy. This is in accordance with the study of Zhang et al., (2010) which showed that the diagnostic sensitivity of Glypican-3 (91.7%) was much higher than AFP (41.7%) and the diagnostic specificity of Glypican-3 (100%) was also much higher than AFP (80.4%). They considered GPC-3 alone to have a high sensitivity and specificity thus GPC-3 could be a valuable serum tumor marker in the diagnosis of HCC. Similarly, Tangkijvanich et al., (2010) found serum GPC-3 to be better than AFP in detecting small HCC (56.3% and 31.3%, respectively). Mohamed et al., (2013) found that Glypican-3 was more accurate than AFP (80% for GPC-3 versus 71% accuracy for AFP) the sensitivity of GPC-3 was 91% compared to that of AFP 80% in the diagnosis of HCC but with similar specificity (70%). The two Egyptian studies of Abd El Gawad et al.,

in 2013 and 2014 reported that, serum Glypican-3 and AFP were significantly higher in HCC patients compared to cirrhotic and normal control groups. They found Glypican-3 to have higher sensitivity, specificity and diagnostic accuracy compared to AFP. In accordance with our study, the Egyptian study of Ibrahim and Abdel-Raouf (2015) who found the sensitivity and specificity of GPC-3 in HCC diagnosis to be 96.7% and 100% respectively and their findings were similar to results of Geramizadeh and Seirfar (2015) who reported 97.7%, sensitivity and 91.3% specificity for GPC-3 in the diagnosis of HCC. Moreover, Wasfy and Shams-Eldeen (2015) found Glypican-3 to have a high specificity and sensitivity in detecting HCC when compared to cirrhosis, dysplasia and metastatic cancers. Moreover, Badr et al., (2014) reported a high sensitivity and specificity for Glypican-3 in HCC diagnosis with a higher sensitivity than AFP in small HCC detection. Our result revealed that the combination between GPC-3 and AFP had a high sensitivity and specificity as diagnostic markers for HCC with 98.5% sensitivity, 97.8% specificity and 93.2% accuracy. This is in agreement with Liu et al., (2010), Tangkijvanich et al., (2010) and Xu et al., (2013) who reported high sensitivity of combined GPC-3 and serum AFP measurement for the diagnosis of HCC at all stages. Badr et al., (2014) reported improved sensitivity and specificity of HCC diagnosis by combination of both AFP and GPC-3. Recently, Sun et al., (2017) found overexpression of serum Glypican-3 in HCC patients. Also, they reported that, combined detection of serum AFP and GPC-3 can increase accuracy and efficacy of HCC diagnosis. In the last few years, the therapeutic value of Glypican-3 was studied by many investigators. They suggested that, Glypican-3 which is up-regulated in HCC (and is not expressed in normal liver cells) stimulates HCC development, transformation, proliferation and metastasis therefore; Glypican-3 may be a potential target for HCC treatment through tumor suppressive effect of some micro-RNAs targeting GPC-3 and reducing its expression and activity in the malignant liver cells. Another mechanism is bi-specific T cell engager targeting the Glypican-3 and CD3 which found to promote T cell destruction of GPC-3 positive human HCC cells in vitro and in vivo (Wang et al., 2015; Bi et al., 2017; Cartier et al., 2017). Finally, according to our results we can conclude that, serum Glypican-3 (GPC-3) is more accurate and has a better sensitivity than AFP in the diagnosis of HCC. It may be a promising marker in the diagnosis of HCC especially in cases without AFP elevation. Combination of GPC-3 and AFP improves the sensitivity and accuracy of HCC diagnosis.

Recommendations

We can recommend further evaluation of Glypican-3 therapeutic values in patients with HCC.

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