RESEARCH ARTICLE

Can Glypican3 be Diagnostic for Early Hepatocellular Carcinoma among Egyptian Patients?

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Abstract

Background: Because of the high prevalence of hepatocellular carcinoma (HCC) in Egypt, new markers with better diagnostic performance than alpha-feto protein (AFP) are needed to help in early diagnosis. The aim of this work was to compare the clinical utility of both serum and mRNA glypican3 (GPC3) as probable diagnostic markers for HCC among Egyptian patients. Materials and Methods: A total of 60 subjects, including 40 with HCC, 10 with cirrhosis and 10 normal controls were analyzed for serum GPC3 (sGPC3) by ELISA. GPC-3 mRNA from circulating peripheral blood mononuclear cells was amplified by RT-PCR. Both markers were compared to some prognostic factors of HCC, and sensitivity of both techniques was compared. Results: Serum glypican-3 and AFP were significantly higher in the HCC group compared to cirrhotic and normal controls ($p<0.001$). Sensitivity and specificity were (95% each) for sGlypican-3, (82.5% and 85%) for AFP, and (100% and 90%) for Glypican3 mRNA , and (80% and 95%) for double combination between sGPC3 and AFP respectively. Conclusion: Both serum GPC-3 and GPC-3mRNA are promising diagnostic markers for early detection of HCC in Egyptian patients. RT- PCR proved to be more sensitive (100%) than ELISA (95%) in detecting glypican3.

Keywords: Glypican3 - ELISA - RT/PCR - HCC - diagnosis - Egypt

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Introduction

Egypt has high prevalence of hepatocellular carcinoma (HCC). It is the 2nd most common cancer site among males and 7th among females (NCI Cancer Registry, 2002-2007).

The rising rates of HCC in Egypt are due to the high prevalence of hepatitis B virus (HBV) and hepatitis C virus infection (HCV) (25.9% and 78.5%) among Egyptian population. There is a shift in the relative influence of these viruses in HCC etiology in Egypt, as HBV infection significantly decreased while HCV did not (Lehman et al., 2009). The role of exposure to aflatoxin in Egypt may also contribute to the development of HCC (Anwar et al., 2008).

The identification of a biochemical marker with better sensitivity and/or specificity than alpha-feto protein (AFP) could be extremely helpful in improving early diagnosis of HCC (Trerotoli et al., 2009).

Glypican3 (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 linked to the cell surface through a glycosyl-phosphatidyl inositol-anchor. It has been found that glypicans interact with growth factors and modulate their activities; hence they play an important role in cell growth, differentiation and migration (Kandil et al., 2009). Glypican3 is expressed in the fetal livers but not in adult livers (Sung et al., 2003). In the adult, GPC-3 can only be detected in a limited number of tissues, including lung, ovaries, mammary epithelium, and mesothelium (Iglesias et al., 2008). Its expression tends to reappear with malignant transformation (Suriawinata et al., 2010). It was recently reported that GPC-3 is only detected in HCC cells but not in benign liver tissues and can thus be used as a potential biomarker for the screening and diagnosis of early HCC (Liu et al., 2010).

The aim of this study is to evaluate and compare the clinical utility of serum and mRNA Glypican-3 in differentiating HCC patients from cirrhotic patients and normal controls, and to compare them with AFP, the traditionally used marker for diagnosis and follow up of patients with HCC. Also to correlate the positivity of the studied tumor markers with different prognostic factors of HCC, and to compare the sensitivity of the 2 techniques (ELISA and RT-PCR).

Materials and Methods

This study was conducted on 40 newly diagnosed patients with hepatocellular carcinoma (HCC) who

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Table 1. Patients' Characteristics of the Hepatocellular Carcinoma Group

		N(%)
Sex	Males	32 (80)
	Females	8 (20)
Child's grade	Grade A	9 (22.5)
	Grade B	22 (55)
	Grade C	9 (22.5)
Stage	Stage I	2 (5)
	Stage II	30 (75)
	Stage III	8 (20)
Hepatomegaly	Absent	17 (42.5)
	Present	23 (57.5)
Splenomegaly	Absent	29 (72.5)
	Present	11 (27.5)
Ascites	Absent	20 (50)
	Present	20 (50)
Oedema	Absent	37 (92.5)
	Present	3 (7.5)
Portal vein thrombosis	Absent	32 (80)
	Present	8 (20)
Number of masses	1 mass	19 (47.5)
	2 masses	9 (22.5)
	3 masses	10 (25)
	4 masses	2 (5)
Hepatitis markers	Hepatitis B	7 (17.5)
	Hepatitis C	26 (65)
	Free of hepatitis	7 (17.5)
Size of mass	1-3 cm	17 (42.5)
	More than 3 cm	23 (57.5)

presented to the outpatients' clinics at the National Cancer Institute (NCI), Cairo University and the National Liver Institute from January to September 2012. They were 32 males and 8 females. Their age ranged from 44 to 77 years. Patient's characteristics are mentioned in (Table 1).The study also included 10 patients with cirrhosis and 10 apparently healthy volunteers as normal controls.

The study was conducted after approval from the ethics committee of the National Cancer Institute (NCI), Cairo University as well as the National Liver Institute. Written consent was obtained from all patients.

Measurement of the tumor markers:

AFP was measured using Axsym based on the microparticle enzyme immunoassay (MEIA) technology.

Serum glypican-3was measured using ELISA kit ,Uscn LifeScience Inc. Wuhan, China.

Detection of Glypican-3 mRNA by RT-PCR

The detection of specific mRNA expressed in cancer cells by RT-PCR in the peripheral blood samples of HCC patients indirectly suggests the presence of CTCs. So we tried to detect GPC3 mRNA in the peripheral blood of our patients.

Isolation of mononuclear cells was performed using Ficoll-Hypaque. RNA extraction was done from mononuclear cells using InviTrap Spin Blood RNA Mini Kit according to manufacturer's instructions. One μg RNA was reversely transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems). Reverse transcription was performed in 20 μl reaction containing 1x RT buffer, 0.2mM DNTP, 1x RT random primer, 50U multiscribe TM reverse transcriptase and nuclease free water. The reaction was performed at 25˚C for 10 min, followed by 25˚C for 120 min and 85˚C for 5 min then kept at 4˚C. PCR was performed using Dream Taq Green PCR Master Mix (ThermoScientific, Fermentas). The reaction for β actin was performed in 25 μl reaction containing 1 μl cDNA, 1x master mix, 25 pmole of each primer F: 5'-GTGGGGCGCCCCAGGCACCA-3'and R: 5'-GTCCTTAATGTCACGCACGATTTC -3' (Inoue et al., 1994). The cyclic condition consisted of initial denaturation at 94˚C for 5 min, followed by 34 cycles of denaturation at 94˚C for 1 min, annealing at 63˚C for 2 min and extension at 72˚C for 3 min. For Glypican-3 the primers were F:5'-GATACAGCCAAAAGGCAG-3'and R: 5'-ATCATTCCATCACCAGAG-3' (5). The cyclic condition consisted of initial denaturation at 94˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 58˚C for 45 sec, extension at 72˚C for 1 min and final elongation at 72˚C for 10 min. The PCR products were visualized on 2% agarose and were 540 bp for β actin and 250 bp for Glypican-3.

Statistical analysis

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as medians and ranges. Qualitative data were expressed as frequencies and percentages. For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" on rank of variables was used for pair-wise comparison. Spearman-rho method was used to test correlation between numerical variables. The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values. Sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were calculated for the different markers used. The p-value <0.05 was considered significant.

Results

Serum glypican-3 and AFP showed statistically significant results in HCC compared to cirrhotic and normal control groups (p<0.001).

Measuring sGlypican-3 by ELISA revealed 95% positivity in the HCC group, 10% positivity in the cirrhosis group, and was negative in the normal control group (p<0.001). Glypican-3mRNA measured by RT-PCR was positive in 100% of cases in the HCC group (Figure 3), 20% in the cirrhotic group, and was not detected in the normal control group (p<0.001).While AFP measurement revealed 82.5% positivity in the HCC group, 30% positivity in the cirrhosis group, and was negative in the normal control group (p<0.001).

Median levels of sGlypican-3 and AFP were (7.7, 2.74, 0.99 ng/ml) and (146.5, 15, 3.4 ng/ml) in the HCC group, cirrhosis group, and normal control group, respectively. Their comparisons revealed significant results (p<0.001 both).

Glypican-3 and AFP didn't give any significant results

with any of the prognostic factors of HCC (Table 2).

Twenty seven out of forty of the HCC patients (67.5%) were HCV positive and 7/40 (17.5%) were HBV positive. All hepatitis positive HCC patients were cirrhotic.

In HCC patients, the highest results of sGlypican-3 were detected in HBV positive compared to HBV negative patients and in HCV negative compared to HCV positive patients. While for AFP, the highest results were detected in HBV and HCV positive patients. The comparisons, however, didn't reach statistical significance.

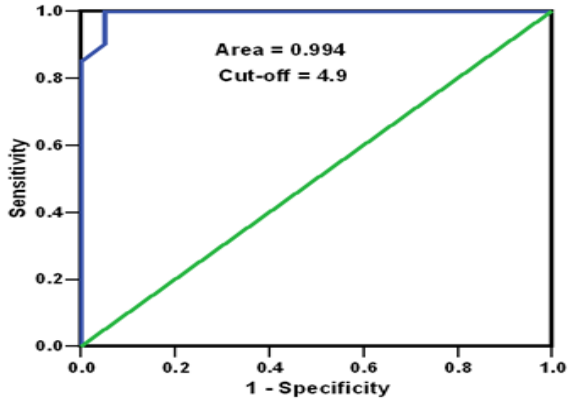

Figure 1. The Roc Curve for sGlypican-3

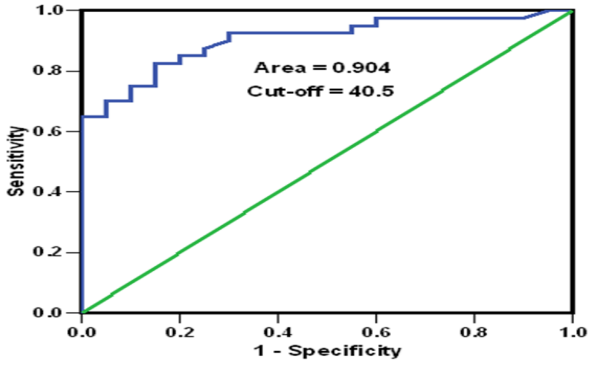

Figure 2. The Roc Curve of AFP

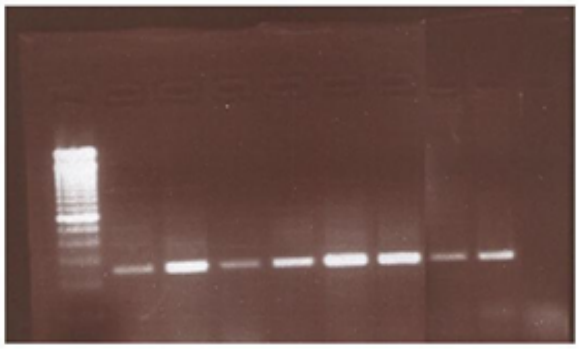

Figure 3. RT-PCR of Glypican3. Lane 1 Shows 100 bp Molecular Weight Marker, Lanes 2-9 Show Glypican3 Positive Cases (250bp) and Lane 10; Negative Control

Correlation analysis between sGPC3 and various biochemical parameters and prognostic factors in the HCC and cirrhotic groups showed no significant results. Also correlation between sGPC3 and AFP was non-significant (p=0.824).

As regards the diagnostic performance of the two markers, the specificity, sensitivity, positive predictive value, negative predictive value, and diagnostic accuracy for sGlypican3 were (95%, 95%, 97.5%, 90.5% and 95%) respectively at a cut off 4.9ng/ml (Figure 1), and for AFP were (85%, 82.5%, 91.7%, 70.8% and 83.3%) respectively at a cut off 40.5ng/ml (Figure 2). For Glypican3 mRNA, they were (90%, 100%, 95%, 100%, and 96%) respectively. While they were (95%, 80%, 97%, 70% and 85%) respectively for the double combination between sGPC3 at a cut off of 4.9ng/ml, and AFP at a cut off 40.5ng/ml (Table 3).

Discussion

It was recently reported that GPC-3 is only detected in

Table 2. Comparison of sGlypican-3, and Serum Alpha-Feto Protein with Different Prognostic Factors in the Hepatocellular Carcinoma Group

	Serum glypican-3 (ng/ml)	p value	Serum alpha-feto protein (ng/ml)	p value
Age		0.405		0.968
Up to 60 years	7.41 (4.90-11)		134 (47.63-505.25)	
>60 years	8.47 (5.23-11.02)		159 (66-385)	
Sex		0.342		0.532
Male	7.43 (4.90-11.02)		184 (64-442)	
Female	8.38 (6.23-11)		90 (43-15049)	
Hepatomegaly		0.448		0.692
Absent	8.04 (4.90-11.02)		159 (59-708)	
Present	7.32 (4.90-11.00)		121 (53-385)	
Splenomegaly		0.929		0.832
Absent	8.04 (4.90-10.91)		183 (65 -421)	
Present	7.32 (5.23-11.02)		97 (43-940)	
Ascites		0.904		0.626
Absent	7.43 (4.90-11.02)		184 (67-382)	
Present	8.02 (4.90-11)		94 (44-584)	
Portal vein thrombosis		0.278		0.612
Absent	8.07 (4.90-11.02)		128 (64-382)	
Present	6.51 (4.90-11)		280 (45-870)	
Masses in the liver		0.738		0.647
1-2 masses	7.73 (4.90-11.02)		171 (69-382)	
3-4 masses	7.78 (5.41-11)		80 (40-855)	
Tumor size		0.075		0.557
Up to 3 cm	7.01 (4.90-10.91)		201 (59-505)	
More than 3 cm	9.01 (4.90-11.02)		121 (35 -346)	
Child's grade		0.459		0.544
A	6.47 (5.93-10.02)		260(131-7800)	
B	8.41 (4.90-11)		106 (63-484)	
C	7.32 (4.90-11.02)		97 (45-404)	
Stage		0.6		0.264
Stage I & II	7.745 (6.46-9.31)		381 (55-109)	
Stage III	7.29 (5.67-10.165)		870 (91-280)	

Table 3. Diagnostic Performance of sGPC-3, GPC3 mRNA and sGPC-3 and AFP in Double Combination

	Serum glypican3 (cutoff 4.9 ng/ml)	Serum alpha-feto protein (cutoff 40.5 ng/ml)	Glypican3 mRNA	Combined sGlypican3 and AFP (cutoff 4.9ng/ml& 40.5 ng/ml)
Sensitivity% (95% CI)	95 (85.5-98.7)	82.5 (70.1-90.7)	100 (91.0-100.0)	80 (64.0-90.0)
Specificity% (95% CI)	95 (85.2-98.7)	85 (72.9-92.5)	90 (68.0-98.0)	95 (75.0-99.0)
Positive Predictive value % (95% CI)	97.5 (88.5-99.7)	91.7 (80.9-96.9)	95 (83.0-99.0)	97 (84.0-99.0)
Negative Predictive value % (95% CI)	90.5 (79.4-96.2)	70.8 (57.5-81.5)	100 (81.0-100.0)	70 (49.0-86.0)
Diagnostic Accuracy % (95% CI)	95	83.3	96	85

*95%CI: Confidence Interval

HCC cells, not in benign liver tissues and can thus be used as a potential biomarker for the screening and diagnosis of early HCC (Liu et al., 2010).

In the HCC group, GPC-3 mRNA was the most sensitive among the studied markers for diagnosing HCC among Egyptian patients with 100% positivity rate, followed by sGPC3 (95%), then AFP (82.5%). While in the cirrhosis group, AFP showed the highest positivity (30%), followed by GPC-3 mRNA (20%), then sGPC-3 (10%). The three markers were negative in the normal control group.

Comparison of sGPC-3, and AFP between the three studied groups showed significant difference between HCC and both liver cirrhosis and normal control groups. This is in accordance with other researchers (Nakatsura et al., 2003 ; Youssef et al., 2010; El-Shenawy et al., 2012; Gomaa et al., 2012). Serum levels of GPC-3 and AFP levels were increased in the HCC patients and absent in other benign liver conditions (Liu et al., 2010). The absence of serum GPC-3 in the healthy individuals was also reported by some researchers (Filmus et al., 2004; Hippo et al., 2004; Nakatsura et al., 2005).

As regards the diagnostic performance, Sensitivities of GPC-3 mRNA, sGPC-3 and AFP were (100%, 95%, and 82.5%), and specificities were (90%, 95% and 85%) respectively. GPC-3 mRNA is slightly more sensitive but less specific than sGPC-3 and both are more sensitive and specific for diagnosing HCC and differentiating HCC from benign liver conditions and normal controls than does AFP.

Double combination of sGPC-3 and AFP revealed sensitivity of 80% which is lower than the sensitivity of each marker alone, and specificity of 95% which is the same for sGPC3, and higher than that of AFP. So using sGPC-3 alone is more satisfactory and informative than using it in double combination with AFP. Gomaa et al. (2012), reported a sensitivity and a specificity of (90.3% and 98%) respectively at a cut-off value of 5.41ng/ml for sGPC-3, and (77.4% and 60%) respectively for AFP at a cut-off value of 42.32ng/ml. Another study done by El-Shenawy et al. (2012), reported a sensitivity and a specificity of (63.5% and 70%) for serum GPC-3 and (76.5% and 82%) for AFP respectively, at cut-off values of (19ng/ml and 78 ng/ml) respectively. Youssef et al. (2010) calculated the sensitivity of sGPC-3 and AFP as (82.5% and 80%), their specificities as (95% and 90%) at cut off values of (4.6ng/ml and 66ng/ml) respectively. Several other studies by many researchers were done for GPC-3, Liu et al. (2010), and Qiao et al. (2011) reported sensitivities of (69.3% and 46.7%) and specificities of (88.7% and 93.5%) at cut off values of (20.68ng/ml and 30ng/ml) respectively. Shafizadeh et al. (2008) stated that serum GPC-3 level was increased in early HCC patients with their serum level 400ng/ml. So, they concluded that GPC-3 is a sensitive serum and tissue marker for the diagnosis of early HCC. Several studies have shown that Glypican-3 is superior to AFP in early detection of HCC, being highly sensitive and specific (Suriawinata et al., 2010; Zakhary et al., 2012). Studies performing double combination of Glypican-3 and AFP found sensitivities between (84%-92%) and specificities between (90%-95%). (Tangkijvanich et al., 2010; Youssef et al., 2010; Gomaa et al., 2012).

As regards GPC-3 expression at mRNA level by RT-PCR, our results revealed that GPC-3mRNA was detected in all cases of HCC (100%), two cases out of 10 in liver cirrhosis patients (20%), and was not detected in any of the normal healthy controls. Our results were in agreement with Young et al. (2003), Li et al. (2006), Youssef et al. (2010), Yan et al. (2011), Gomaa et al., (2012) who found that the percentages of mRNA expression in HCC patients were (100%, 90%, 76%, 80.5%, and 85%) respectively. Several other studies reported similar results, Jackbovic et al. (2007); Nishimura et al. (2008); Yasuda et al. (2010) concluded that GPC-3 mRNA is significantly up regulated in HCC compared to normal and benign liver samples, and hence GPC-3 could serve as a molecular marker for early detection of HCC.

Regarding the ten cirrhotic patients, involved in the current study, two of them were positive for GPC-3 mRNA. These two cases were followed up for 12 months. The first one who was also having mildly elevated sGPC3 and AFP levels has been diagnosed as HCC after 9 months of follow up, while the other died during the study. These results could predict that GPC-3 can be used for screening and early detection of HCC among cirrhotic patients. This was in accordance with Hippo et al. (2004) who demonstrated that during the follow-up of their patients with liver cirrhosis having detectable sGPC-3 levels, HCC developed within 6 months among considerable number of patients with neither significant change of serum AFP levels nor in abdominal ultrasonography.

In this study, neither GPC-3 nor AFP showed any significant results when compared to tumor size and TNM staging. Contrary to our results, a positive correlation was found between serum levels of each of AFP and GPC-3 with both tumor size and portal vein invasion by El-Shenawy et al. (2012), while Youssef et al. (2010) reported statistically significant results between GPC-3 and the staging of HCC. This discrepancy of results may be due to the different sample size, or the different underlying etiology of HCC.

The lack of correlation between GPC-3 and positivity of HBV and HCV infections in this study proves the high specificity of GPC-3 in HCC versus non HCC hepatitis cases, as positivity of HBV or HCV infection will not give false positive results especially in a country like Egypt, where there is a high prevalence of hepatitis viral infection. Capurro et al. (2003) and Nakastura et al. (2003) reported that GPC-3 was present in the serum of HCC patients, but was undetectable in all patients with hepatitis as well as healthy individuals.

In this study no significant correlation was detected between AFP and Glypican-3 in the HCC group. Our results are in agreement with many other researchers (Nakatsura et al., 2003; Hippo et al., 2004; Nakatsura et al., 2005; Jackbovic et al., 2007). Thus, the simultaneous use of both markers significantly increases the sensitivity without compromising the specificity of any.

Comparing the sensitivity of the two techniques used, GPC-3 by PCR proved to be more sensitive (100%) than ELISA (95%).

Oncofetal proteins do not seem to play a critical role in

tumor progression, but have been used as tumor markers or as targets for immunotherapy (Nishimura et al., 2008). Overall, up regulation of GPC-3 protein in HCC, together with the nature of shedding and oncofetal behavior, strongly suggests that GPC-3 is a good molecular marker for HCC. Evaluation of GPC-3 as a diagnostic and immunotherapeutic target may be worthwhile for the prevention and treatment of liver cancer (Sung et al., 2003).

In conclusion, GPC-3 is a promising diagnostic marker with high sensitivity and specificity for HCC which can substitute AFP in early diagnosis of HCC and in screening and follow up of patients with cirrhosis among the Egyptian population. There is no impact for the presence of hepatitis viral infection on the diagnostic accuracy of glypican3 in diagnosing HCC among Egyptian patients. Measuring GPC-3 by RT-PCR proved to be more sensitive (100%) than ELISA (95%), hence it is more suitable for follow up of cirrhotic patients. Reverse transcription PCR is a sensitive technique for early detection of HCC, but it's more time consuming and more tedious than ELISA. Further studies with larger sample sizes and long follow up of HCC and cirrhosis patients are needed to clarify the role of Glypican3 in the early diagnosis of HCC.

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