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Glypican-3 in hepatocellular carcinoma

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ABSTRACT

HCV infection patients without any complications, group III: 100 HCV infected patients complicated with cirrhosis and group IV: 150 HCV infected patients complicated with (90 localized and 60 metastatic) HCC. Detection of serum GPC3 and alpha-fetoprotein using an enzyme-linked immunosorbent assay (ELISA) were done to all subjects. Results: When analysis of variance was done between the four groups, a highly statistical significant difference was found between these groups regarding the mean serum levels of AFP and GPC-3 where the highest increase of three markers were found in the HCC group. The combined AFP and Glypican-3 improve the sensitivity and specificity of AFP alone. Conclusions: GPC-3 could be a sensitive, specific and accurate marker for early detection of HCC diagnosis.

KEYWORDS

Hepatocellular carcinoma;
Glypican-3;
Alpha-fetoprotein.

INTRODUCTION

Hepatocellular carcinoma (HCC), a common solid malignancy, is the third most common cause of cancer-related mortality worldwide[1] However, the prognosis of HCC patients remains poor, with its 5 year survival rate after surgery as low as 25–39%[2]. Surgical resection remains to be the standard choice of treatment for patients in the early stage of HCC. However, even with radical resection, 60–70% of patients develop metastasis and recurrence within 5 years after surgery[3,4].

Glypican-3 (GPC3) is a member of the glypican family of heparan-sulfate proteoglycans (HSPGs), which is bound to the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor. GPC3 is highly expressed in HCC, and it has been discovered as a potential serological and histochemical marker specific for the differentiation between the early stage of HCC formation and its precancerous state[5]. 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)[6]. Although the homology of amino acids between glypican members is moderate, all glypicans are approximately 60 to 70 kd in size and share acharacteristic pattern of 14 conserved cysteine residues[7]. 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[8]. 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

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control during development[9]. 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[10]. In the adult, GPC3 can only be detected in alimited number of tissues, including the lung, ovaries, mammary epithelium, and mesothelium[11] So, down regulation of GPC3 has been observed in several human malignancies, including mesothelioma and ovarian, breast and lung cancers[12-15] These observations indicate that GPC3 is an inhibitor of cell proliferation and a tumor suppressor in a tissue-specific manner[16]. 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[17]. The results of immunohistochemical studies have convincingly shown that GPC3 is a novel diagnostic marker for HCC[18]. 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[19].For example, a dramatic elevation of GPC3 and protein levels has been reported in large amounts of HCC, whereas its expression was less frequent in preneoplastic or entirely absent in non-neoplastic liver[20–23]. We have hypothesized that GPC3 over-expression might be closely related with prognosis and post-operative metastasis/recurrence in HCC patients. However, until now, the clinical significance of GPC3 over-expression in HCC remains to be vague, which includes the relationship between GPC3 over-expression and post-operative metastasis/recurrence. In this study, the relationship of GPC3 expression with its corresponding clinicopathological features, especially that between GPC3 expression and the prognosis of HCC patients, was examined using immunochemical analysis.

MATERIALS AND METHODS

Samples

The present study was carried out on 400 individuals (185 females and 215 males) with age ranged from (19 to 69 years). They were selected from Clinical Oncology and Internal Medicine Outpatient clinics, Faculty of Medicine, Zagazig University Hospital. They were divided into the following groups: Group I: consists of 50 healthy individuals (26 males and 24 females) aged from 19-49 years served as control. Group II: consists of 100 HCV infected patients without complications (66 males and 34 females) aged from 22 to 61 years. Group III: consists of 100 HCV infected patients with Cirrhosis (64 males and 36 females) aged from 42-68 years. Group IV: consists of 150 HCV infected Patients complicated with cirrhosis and HCC (90 localized and 60 metastatic) (74 males and 76 females) aged from 43-69 years. Each patient was subjected to the following:

Alpha-fetoprotein detection

ELISA for the detection of Alpha-fetoprotein in serum according to Herberman[24].

Glypican-3 detection

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. GPC-3 ELISA kit is intended for laboratory research use only and not for use in diagnostic or therapeutic procedures. The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm using a spectrophotometer. In order to measure the concentration of GPC-3 in the sample, this GPC-3 ELISA kit includes a set of calibrators. The calibrators are assayed at the same time as the samples and allow the operator to produce a calibration curve of optical density versus GPC-3 concentration. The concentration of GPC-3 in the samples is then determined by comparing the O.D. of the samples to the calibration curve. The coated well immunoenzymatic assay for the quantitative measurement of GPC-3 utilizes a polyclonal anti-GPC-3 antibody and a GPC-3-HRP conjugate. The assay sample and buffer are incubated together with GPC-3-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color

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is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the GPC-3 concentration since GPC-3 from samples and GPC-3-HRP conjugate compete for the anti-GPC-3 antibody binding site. Since the number of sites is limited, as more sites are occupied by GPC-3 from the sample, fewer sites are left to bind GPC-3-HRP conjugate. Calibrators of known GPC-3 concentrations are run concurrently with the samples being assayed and a calibration curve is plotted relating the intensity of the color (O.D.) to the concentration of GPC-3. The GPC-3 concentration is interpolated from this calibration curve. To determine the amount in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the calibration curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding concentration. The sensitivity in this assay is 0.1 ng/mL

RESULTS AND DISCUSSION

The serum level of AFP was significantly increased in HCC patients as compared to liver cirrhosis, hepatitis C without copmlications and controls (p<0.001) for each. Also GPC-3 was significantly higher in HCC compared to other groups (P<0.001) (TABLE 1, Figure 1).

As regard the obtained results of the mean value of GPC-3, a significant increase was detected in HCC group (1530.6 ± 2950.3) than that found in patients with cirrhosis (15.7 ± 11.5) or with chronic hepatitis (1.4 ± 1.9) and in control subjects (1.0 ± 2.8), (p < 0.001). When Spearman's correlation coefficient was done in the patients, a significant positive correlation (P value <0.001) of serum levels of AFP & GPC-3 was found (TABLE 2).

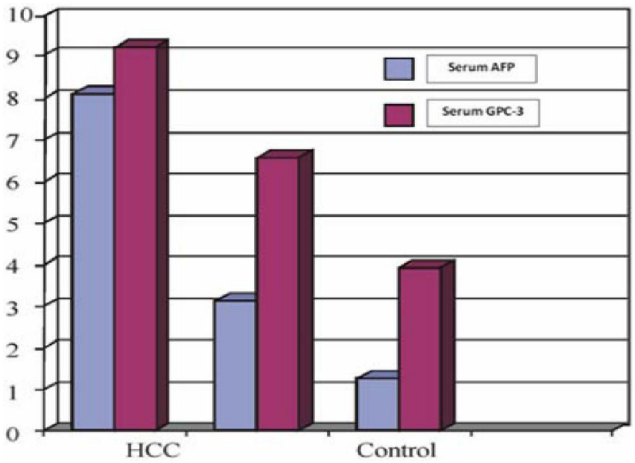

Figure 1 : Level of serum GPC-3 and AFP in the studied groups.

TABLE 1 : Mean and standard deviation of AFP and GPC-3 in study groups.

Parameter	HCC	Cirrhosis	Hepatitis	Control	*P value
AFP (ng/ml)	511.7±1195.1	22.4±24.3	14.2±8.6	3.9±2.0	< 0.001
GPC-3 (ng/ml)	1530.6 ± 2950.3	15.7 ± 11.5	1.4 ± 1.9	1.0 ± 2.8	<0.001

***p>0.05 is considered non-significant; p<0.05 is considered significant.**

TABLE 2 : Spearman,s correlation coefficient between AFP and GPC-3 in all patients (N=200).

	r	Sig (2-tailed)	P value
GPC-3&AFP	0.615	0.000	<0.001*

P value is highly significant correlation at <0.001*.

TABLE 3 : Descriptive statistics of the studied parameters (AFP & GPC-3) in HCC group and LC group.

	HCC group (N=150)				LC group (N=100)			
	+ve		-ve		+ve		-ve	
	N	%	N	%	N	%	N	%
AFP (ng/ml)	140	93%	10	7%	48	48%	52	52%
GPC-3 (ng/ml)	144	96%	6	4%	54	54%	46	46%

On analysis of the results of AFP in the patients with HCC (TABLE 3), 140 (93%) patients are + ve AFP level (high AFP than its normal range), while 144 HCC patients (96%) are + ve for GPC-3 level (high GPC-3 than its normal range). In the patients with liver cirrhosis, 48 (48%) patients have +ve AFP while 54 patients with cirrhosis are + ve for GPC-3 serum levels.

Concerning the obtained results of serum AFP, when localized and metastatic HCC group was compared with cirrhosis, hepatitis and healthy controls ones, a significant increase was observed (p < 0.000). Also, a non significant differences was detected between cirrhosis and hepatitis groups but when they were compared with healthy control one, a significant increase of serum AFP was detected (p< 0.001) (figure 2).

The combined AFP& GPC-3 gave a sensitivity of 92 % and specificity of 95%, which is higher than AFP alone and AUROC of 0.701 (Figure 3). In conclu-

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sion, detection of Glypican-3 and serum AFP has different significances; As regard GPC-3, it seems to be a parameter to detect HCC earlier than any other means, so detection of Glypican-3 may help to prefigure HCC metastasis and monitor the response to the therapy.

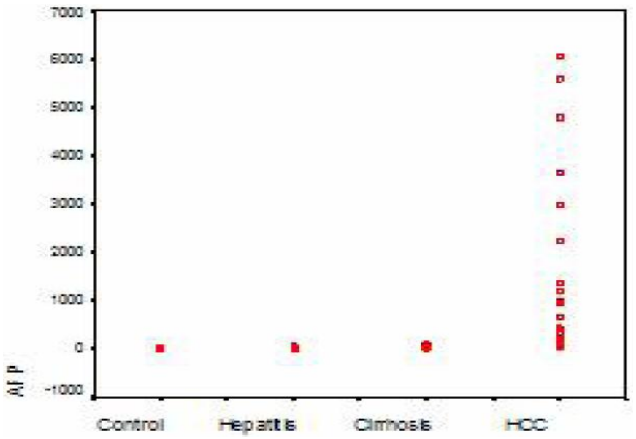

Figure 2 : A scattar diagram showing the individual values of AFP in patients and control subjects.

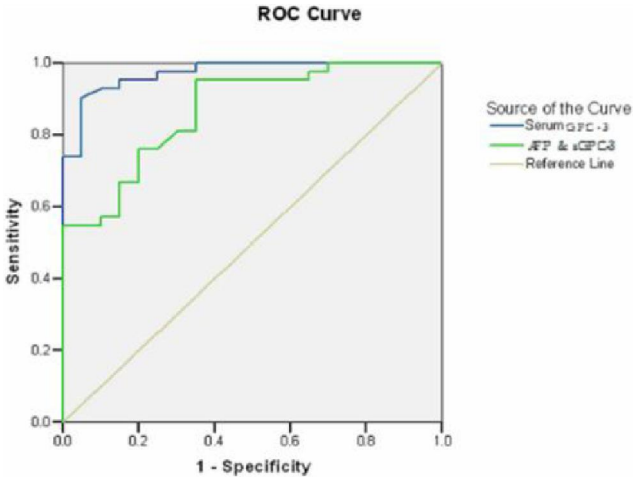

Figure 3 : ROC curve of serum GPC-3 and compined AFP&GPC-3 for HCC prediction.

TABLE 4 : General characteristics of patients in this study:

Characteristics	Group I	Group II	Group III	Group IV
Female	48 %	34 %	36 %	50.7 %
Male	52 %	66 %	64 %	49.3 %
Age	19 - 49	22 - 61	42 - 68	43 - 69

Molecular biology 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[26-27]. Up to 80% of HCCs develop against a background of cirrhosis of the liver and at risk cirrhotic population could aid earlier detection of the disease and decrease the cancer related mortality rate[28]. 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[29]. 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 four groups (HCC, Liver cirrhosis. Hepatitis without complications 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 45.4 ng/ml. This gave 86.5% in sensitivity and 92% in specificity. While the best cut-off recorded in this study to diagnose liver cirrhosis was 15.5 ng/ml. It yielded a 78% sensitivity and 80% specificity. Soresi *et al.*[30]. showed that the best cut-off value of AFP has been reported to be 20 ng/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[31] 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[32] 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.*[33] 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 ⍰21 ng/ml; it yielded a 92 % sensitivity and 89% 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[34] 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.*[35] 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*[27] 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.*[31] who stated that HCC patients with a high AFP (e"400 ng/ml) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis, and a lower median survival rate.

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. Therefore, combination of multiple markers may be more valuable in the diagnosis and prognosis of HCC.

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