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Research Aritlce

GOLGI PROTEIN 73 (GP73) mRNA EXPRESSION AS A SERUM MARKER FOR DETECTION OF HEPATOCELLULAR CARCINOMA AMONG EGYPTIAN PATIENTS

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A B S T R A C T

In the search for serum markers of hepatocellular cancer, several studies try to find a non-invasive marker for diagnosis of hepatocellular carcinoma (HCC) to facilitate the diagnosis and avoid the harmful complications of liver biopsy. Investigators have focused on Golgi protein 73 (GP73); also known as Golgi membrane protein 1 (Golm1) or Golgi Phosphoprotein 2 (Golph2) as non-invasive, available and inexpensive marker for the diagnosis of HCC. The present study aimed to evaluate the serum GP-73 mRNA expression as a marker for HCC diagnosis among Egyptian patients versus alpha fetoprotein. **Subjects and Methods:** The patients selected from the Hepatology, Gastroenterology and Infectious Diseases Department and from Gastroenterology and Hepatology Unit of Internal Medicine Department, Benha University Hospital. Subjects were classified into three groups: Group I : Included 20 apparently healthy subjects. Group II: Included 20 cirrhotic patients and not complicated by HCC, Group III: Included 40 patients diagnosed as liver cirrhosis complicated by HCC. The serum Golgi Protein 73 (GP73) mRNA was determined by semiquantitative RT-PCR. **Results:** GP-73mRNA was highly significant higher in HCC patients in comparison to cirrhotic and the control group ($P < 0.001$). ROC for AFP revealed that the best cut-off value is (>48ng/ml), at this point the sensitivity was 90 %, specificity was 90% and AUC was 0.96. However the ROC for GP-73mRNA expression revealed that the best cut-off value is (>5.11) at this point the sensitivity was 97.5%, specificity was 100% and AUC was 0.99. In conclusion, increased GP-73 mRNA expression could be associated with the presence of HCC and the serum Gp73 mRNA expression was more sensitive and more specific than AFP.

INTRODUCTION

In Egypt, HCC is the second most common cancer in men and the 6th most common cancer in women.[1] Over the last decade, a remarkable growth, from 4.0% to 7.2%, was observed in the proportion of chronic liver disease patients diagnosed with HCC.[2] The progression of liver disease into liver cancer is primarily monitored by serum levels of the oncofetal glycoprotein, α-fetoprotein (AFP).[3]

Alpha fetoprotein is the most widely used tumor biomarker currently available for the early detection of HCC. Findings of a previous clinical study demonstrated that serum AFP had a sensitivity of 41-65% and specificity of 80-94% when the cut-off value is 20ng/ml. Therefore, the use of AFP as a primary screen for HCC has been questioned and more sensitive serum biomarkers for HCC are desired.[4]

Investigators have focused on Golgi protein 73 (GP73); also known as Golgi membrane protein 1 (Golm1) or Golgi Phosphoprotein 2 (Golph2) as non-invasive, available and inexpensive marker for the diagnosis of HCC.[5] Golgi protein 73 has an N-terminal transmembrane domain a C-terminal coiled- coil domain located on the luminal surface of golgi apparatus.[6] Changes in the expression levels of Gp73 involves in the pathogenesis of many diseases. Over expression of Gp73 is first reported in adult gaint cell hepatits (GCH), some studies have identified Gp73 as a potential marker of cancer, including hepatocellular carcinoma and as an independent prognostic factor for tumor recurrence and poor over survival.[7] In a study aimed to investigate the expression of GP73 and its correlation with clinical parameters, significant overexpression of GOLPH2 at either protein or mRNA-levels or both were found to be associated with aggressive behavior of HCC, but not overall patient survival.[8] In addition to hepatocytes, GP73 was consistently expressed by normal biliary epithelial cells as well as hepatic stellate cells in injured livers.[9] Further studies demonstrated constitutive expression in cells of the epithelial lineage, especially in the prostate, gut, breast, and thyroid, and within the central nervous system.[5]

In the current study, we aimed to evaluate the expression of GP 73 mRNA in HCC patients to be used as tumor marker.

SUBJECTS AND METHODS

Sixty patients selected from the Hepatology, Gastroenterology and Infectious Diseases Department and from Gastroenterology and Hepatology Unit of Internal Medicine Department, Benha University Hospital. Forty of patients were diagnosed as HCC according to clinical examination, radiological investigations including abdominal ultrasonography, triphasic C.T, histopathological examination and laboratory investigations. The patients did not receive any chemotherapy. The remaining twenty patients were cirrhotic and not complicated with HCC. Furthermore, twenty apparently healthy subjects were included in this study.

All cases were subjected to the following: Complete history taking and full clinical evaluation with special emphasis on previous history of encephalopathy, liver size, splenomegaly, presence of ascites and jaundice. ALT, AST, albumin, total bilirubin level were done using BS 350 (Biosystem company). Prothrombin concentration, INR was done. Complete blood cell count was done using **Sysmex xs 800**. Serum alpha fetoprotein (AFP) was measured by ELISA. Hepatitis markers (HBsAg, and HCV antibody) were done by EIA. HCV-RNA levels were analyzed by polymerase chain reaction (PCR). Determination of Golgi Protein 73 (GP73): It was be determined by (RT-PCR).

METHODS

Gp73

Three steps were done to reach semiquantitation:

RNA purification

Venous blood samples (3 ml) were obtained by sterile venipuncture and the serum was separated by centrifugation at 3500 rpm. RNA Extraction was done from 200µl of serum using **RNA purification Step Direct-zol™ RNA MiniPrep (Zymo research) Catalog Nos R2051.**

Synthesis of cDNA from mRNA by using: HisenscriptRH [-] cDNA Synthesis kit (Intron Biotechnology). The reaction was carried out according to instruction of manufacturer by using 2 µl of the isolated RNA.

Semiquantitative RT-PCR

Using qPCR Green Master–clear (Jena Bioscience) Master mix for real time qPCR with green-fluoresent DNA stain.

Preparation of the qPCR master mix **Table1**

component	20 µl assay
qPCR Green-Master	10 µl
primer forward (10 µM)[1]	0.6 µl
primer reverse (10 µM)[1]	0.6 µl
UNG (PCR 353)[2]	0.2 µl
template DNA	2 µl
PCR-grade water	fill up to 20 µl

qPCRGreenMaster is designed for the quantative real-time analysis of DNA samples using the fluorescent DNA stain EvaGreen. The fluorescent dye in the master mix intercalates into amplification product during the PCR process and enables the rapid analysis of target DNA without the need to synthesize sequence-specific labeled probes. Real time PCR was carried in a total volume 20µl with 2µl of cDNA, 10µl of qPCR Green Master containing qPCR Polymerase, dATP, dCTP, dGTP, dUTP, Eva Green, reaction buffer with KCl,(NH4)2SO4, MgCl2 and stabilizers, 6.6µl of PCR-grade water, 0.6µl of primer forward and 0.6µl of primer reverse. Upon binding to DNA, the non-fluorescent dye becomes highly fluorescent while showing no detectable inhibition to the PCR process. The dye is extremely stable both thermally and hydrolytically, providing convenience during routine handling.

Cycling protocol **Table 2**

PCR was performed in a thermal cycler with heated lid.

Recommended cycling conditions:

UNG treatment[3]	50 °C	2 min	1x
initial denaturation and polymerase activation	95 °C	2 min	1x
Denaturation	95 °C	15 sec	35-45x
Annealing and elongation	54 °C[4]	1 min[5]	40x

Statistical analysis: The collected data were tabulated and analyzed using SPSS version 16 software (SpssInc, Chicago, ILL Company). Categorical data were presented as number and percentages, using Chi square test (X^2) or Fisher's exact test (FET) to analyze them. Quantitative data were tested for normality using Shapiro-Wilks test assuming normality at P>0.05. Quantitative data were expressed as mean ± standard deviation, medianand range. ANOVA test was used to analyze normally distributed variables among 3 independent groups. While non-parametric variables were analyzed using Krauskal Wallis test (KWT). Significant ANOVA and KrauskallWallis test were followed by post hoc multiple comparisons using Bonferroni and Bonferroni adjusted Mann-Whitney U test respectively to detect the significant pairs. Spearman's correlation coefficient (rho) was used to assess correlation between non parametric variables. ROC curve was used to detect cut-off values of AFP and GP-73 mRNA expression with optimum sensitivity and specificity in detection of HCC and tumor size. The accepted level of significance in this work was stated at 0.05 (*P <0.05* was considered significant). *P value >0.05 is non-significant (NS). P<0.05 is significant (S). P≤0.001 is highly significant (HS).*

RESULTS

Gp73 mRNA expression was evaluated and compared between HCC patients, cirrhotic patients and healthy control. The housekeeping gene *B*-actin were detected in all patients. The serum Gp73 mRNA expression was highly statistically significant increase (*P value <0.001*) in cirrhotic (Mean 2.11±0.59) and HCC group (Mean 18.15±7.87) in comparison to the control group **(Table 3)**.

Table 3 Comparing the studied groups regarding AFP (ng/ml) and GP -73 mRNA expression

Variable	Controls (N=20)			Cirrhotic group (n=20)			HCC group (n=40)			KWT	P	Post hoc multiple comparison
	Mean	± SD	Median (Range)	Mean	± SD	Median (Range)	Mean	± SD	Median (Range)			
AFP (ng/ml)	6.0	2.61	6 (2-11)	29.4	26.09	16.5 (7-102)	982.8	1183.33	442 (4.6-4490)	57.2	<0.001 (HS)	HCC≠ Controls HCC≠ Cirrhotic
GP-73 mRNA	1.0	0.00	1-1	2.11	0.59	2.07 (1.06-2.99)	18.15	7.87	15.9 (7.23-36.2)	67.7	<0.001 (HS)	HCC≠ Controls HCC≠ Cirrhotic

In HCC patients serum AFP was highly significant higher (*P value <0.001*) (Mean 982.8±1183.33) in comparison to cirrhotic patients (Mean29.4±26.09) and the control group (Mean 6.0±2.61) **(Table 3)**. Additionally, there was positive significant correlation between GP-73 mRNA expression and serum level of AFP (*P<0.001*). The mean size of HCC is (4.16) cm and median size is (3.0) cm with standered deviation of 2.8 cm.

There was significant positive correlation between GP-73 and tumor size (*P<0.001*). The sensitivity and specificity of Gp73 mRNA expression and serum level of AFP for HCC patients were illustrated in **(Table 4)**.

Serum Gp73 expression sensitivity and specificity was high when compared to serum AFP (97.5 and 100 vs 90 and 90%).

(Golm1) or [Golph2]. GP73 is a 400 amino acid, 73 kDa transmembrane glycoprotein that normally resides within the cis-Golgi complex. Its mRNA was first identified in a search for upregulated hepatic genes in a patient with syncytial giant cell hepatitis.[5] Subsequent studies revealed minimal GP73 expression in normal hepatocytes but marked expression in patients with acute and chronic hepatitis and liver cirrhosis, regardless of the specific disease aetiology.[9]

In the present study we aimed at evaluating the diagnostic value of mRNA expression of Gp-73 in patients with HCC.

As regarding expression of mRNA of Gp73 and its significance in diagnosis of HCC, in the present study the expression of mRNA of GP73 was statistically significantly higher in HCC cases when compared with the cirrhotic group

Table 4 ROC curve for the performance of AFP ng/ml and GP-73 mRNA expression in detection of HCC.

Variable	Sens%	Spec%	PPV%	NPV%	Accuracy%	AUC	95% CI	P
AFP >48ng/ml	90%	90%	90%	90%	90%	0.96	0.91-1.0	<0.001 (HS)
GP-73 >5.11	97.5%	100%	100%	97.6%	98.7%	0.99	0.98-1.0	<0.001 (HS)
Combined AFP and GP73	100%	100%	100%	100%	100%	0.99-1.0	1.0	<0.001 (HS)

The ability of Gp73 mRNA and AFP to distinguish HCC patients was evaluated by area under the ROC curve **(Table 4)**, for AFP revealed that the best cut-off value is (>48ng/ml and AUC 0.96, for GP-73mRNA expression revealed that the best cuttof value is (>5.11) and AUC 0.99. Combined AFP and GP-73 mRNA expression shows sensitivity was 100 % , specificity was 100% and AUC 0.99-1.0 with (P< 0.001). Sensitivity of AFP in detection of tumor size > 3 cm among HCC patients showed that the best cut-off value was (>390 ng/ml), at this point the sensitivity was 89.5%, specificity was 81%, and AUC 0.91 with (P< 0.001). The sensitivity of Gp73 mRNA in detection of tumor size>3cm among HCC patients showed that the best cut-off value was (>15.53) at this point the sensitivity was >94.7%, specificity was 85.7%, and AUC (0.96).

and the control group. Furthermore expression of mRNA of GP73 was statistically significantly higher in cirrhotic patients when compared with control group.

Serum GP73 in HCC was higher than in LC and in all two groups were higher than those in healthy individuals. [11]Serum level of GP73 in patients with liver disease was significantly higher than in healthy individuals.[13] Similar results in a Chinese study on patients with predominantly hepatitis B virus–related liver cancer. In response to these encouraging reports, GP73 was added to a group of emerging candidate HCC serum markers.[13] There was a study found that the elevation of serum GP73 is mildest in virus carriers, moderate in patients with cirrhosis and dramatic in patients with HCC.

Table 5 ROC curve for the performance of sensitivity of AFP and GP-73 mRNA expression in detection of tumor size > 3 cm among HCC patients.

Variable	Sens%	Spec%	PPV%	NPV%	Accuracy %	AUC	95% CI	P
AFP >390ng/ml	89.5%	81%	81%	89.5%	85%	0.91	0.81-1.0	<0.001 (HS)
GP-73 >15.53	>94.7%	85.7%	85.7%	94.7%	90%	0.96	0.89-1.0	<0.001 (HS)

DISCUSSION

In the search for serum markers of hepatocellular cancer, several investigators have focused on Golgi protein 73 (GP73); also known as Golgi membrane protein 1

Therefore, serum GP73 can be used to monitor disease progression from HBV infection to cirrhosis to HCC. Moreover, they found that both liver benign tumors and non-HCC liver malignant lesions had elevated serum GP73,

although the magnitude is much smaller than that in HCC. Serum GP73 can therefore be a useful tool in determining the nature (benign vs. HCC) of hepatic tumors. Furthermore, in patients with non-liver cancers also had moderate elevation of serum GP73, none of which, however, reached the level identified for HCC cases. Serum levels of GP73 diagnostic for HCC thus seemed not to be a pan-cancer marker [14].

Additionally, a study demonstrated that surgical resection of the tumor results in diminished serum GP73 levels and that tumor recurrence correlates with the recurrence of elevated GP73 in the blood. Reappearance of serum GP73 indicates the existence of tumor lesions and thus may serve as an indicator for the recurrence of HCC [14]. The need for closer monitoring of patients with chronic hepatitis who have a high risk of developing HCC during the course of the disease has long been stated. In these patients, AFP has been a particularly unsatisfactory screening tool for early detection of HCC. [15] GP73 is not a general HCC serum tumor marker but could rather be a valuable complementary tool in the surveillance of at risk patients. The data presented in the study provides further evidence that GP73 protein is strongly expressed in HCC and bile duct carcinoma tissues and is secreted into the blood. Possibly, it is either involved in posttranslational protein modification, transport of secretory proteins, cell signalling regulation, or simply maintenance of Golgi apparatus function [13]. However the expression levels in benign liver lesions-focal nodular hypertrophy and hepatic adenoma were not significantly different from those of the surrounding areas. These findings provide evidence that the increased sGP73 in HCC patients originates from cancerous hepatocytes, an important requirement for the validation of tumor biomarkers. [16] Resolution of hepatitis is paralleled by a reduction and normalization of GP73 expression, indicating that GP73may be triggered by the hepatic injury response. [12]These data indicate that serum GP73 is a promising diagnostic serum marker for liver cancer. [17]
AFP has been used as a serum marker for HCC for many years, but it lack of high sensitivity and specificity.[18]
Another one finding is the marked up-regulation of GP73 expression in cancers of biliary origin.[19]

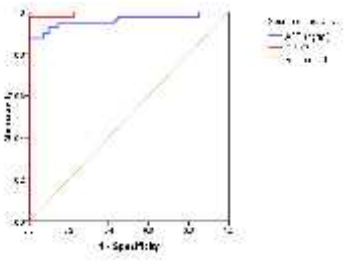

Figure 1ROC curve for the performance of AFP and GP-73 mRNA in detection of HCC.

In previous studies have shown a better sensitivity of GP73 than AFP in diagnosis of HCC.[17]

Accordingly, the role of AFP in the diagnosis of HCC is limited and controversial, AFP is not elevated in all patients with HCC, its sensitivity for detecting HCC ranges between 25%-60%, and its specificity is also low because serum AFP can also be detected in patients with cirrhosis and chronic hepatitis. [20] A mass in the liver with an elevated AFP does not automatically indicate HCC, intrahepatic cholangiocarcinoma (ICC) is also more common in cirrhosis than in noncirrhotics. The fact that both are more common in cirrhosis means that care must be taken to distinguish between them given the differences in treatment and outcomes. Since AFP can be elevated in either condition, it is recommended that it no longer be used.[21] There is a debate in defining the AFP cut-off level for the diagnosis of HCC, an AFP value above 400-500 ng/mL has been considered to be diagnostic for HCC in patients with cirrhosis, however, such a cut-off value is problematic in absolute diagnostic terms, since high levels of this magnitude are not as common in the presence of smaller tumors (< 5 cm) and furthermore, only 30% of HCC patients have levels higher than100 ng/ml. [22]

Also in this study expression of mRNA of GP73 had significant positive correlation with tumor size.

Significant overexpression of GP73 at both protein and mRNA levels along with overexpression of GP73 protein is associated with aggressive behavior of HCC. [8]

The degree of GP73 expression correlated with the tumor grade. [19]

In contrast, serum levels of GP73 in patients with HCC were not consistently affected by the tumor sizes and the status of tumor differentiation [13]

In the present study, there was positive significant correlation between mRNA expression of Gp73 and AFP in HCC patient and LC patient. In present study, whether GP73 is a better serum biomarker than AFP is controversial. The sensitivity and specificity of GP73 for HCC were superior to those ofAFP. However, when used in combination with AFP, they lead to an enhanced the sensitivity of detection of HCC up to 100% and sensitivity to100%.[17] GP73 is up-regulated in HCC and measurement of serum GP73 revealed a sensitivity and specificity of 69% and 75%, respectively. **[23]** In a another study**,** AFP/GP73 had a sensitivity of 75.8% and specificity of 79.7% with an AUROC of 0.844. vs. 0.812 for AFP with a sensitivity of 95.2% and specificity of 47.1%; in detecting early HCC. **[11]** The combined measurement of GP73 and AFP can further increase the sensitivity for the detection of HCC. [13, 24] This is not in agreement with a study which reported that a value of AFP about 12.9 ng/ml provides the optimal balance between sensitivity and specificity, and at this level the sensitivity is only 70% and the specificity is 77.5%, which is inadequate sensitivity and specificity for early diagnosis of HCC. [21,25]

In conclusion, our data revealed the possibility of using mRNA expression of GP-73 as a marker for diagnosis of HCC.

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