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Study on molecular expression of long non-coding RNA Glypican3 in hepatocellular cancer patients

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the main cancers in the world with a high mortality rate. The molecular mechanisms of HCC are poorly understood. Long non-coding RNAs (lncRNAs) have a role in HCC pathogenesis. Glypican3 (GPC3) is a cell surface oncofetal proteoglycan that is expressed in HCC, and its overexpression predicts a poorer prognosis. We aimed to assess the levels of alfa fetoprotein (AFP), lncRNA AF085935 gene expression, and GPC3 protein in HCC patients.

Patients and methods: The patients were classified into three groups: HCC group, cirrhotic group, and healthy control group. For all groups, we performed clinical examinations, laboratory investigations, and imaging. The levels of AFP, GPC3 protein, and LncRNA gene expression were estimated. A statistical analysis was done.

Results: Levels of GPC3 and LncRNA gene expression were significantly higher in the HCC group versus other groups. LncRNA gene and GPC3 levels are excellent for the detection of HCC with a sensitivity of 96% and 87%, respectively. Specificity was 81% and 64%, respectively. Linear regression analysis showed that LncRNA gene expression and GPC3 protein are significant predictors for HCC ($p = 0$ and $p = 0.001$, respectively). Log rank analysis based on GPC3 and LncRNA gene expression levels in HCC patients showed that high expression of GPC3 and LncRNA is associated with shorter overall survival than those with low expressions (p value < 0.001).

Conclusion: In our study, LncRNA gene expression and GPC3 levels are good diagnostic and prognostic biomarkers for HCC patients.

Keywords: lncRNA gene, Glypican3 (GPC3), Alpha fetoprtotein (AFP), Biomarker, Prognosis, Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and is a leading cause of cancer-related deaths worldwide. Cirrhosis remains the most important risk factor for the development of HCC regardless of the etiology [1]. In Egypt, HCC constitutes a significant public health problem where it is responsible for 33.63% and 13.54% of all cancers in males and females, respectively [2]. Although the clinical diagnosis and management of early-stage HCC have improved significantly, still advanced HCC is a highly aggressive tumor with a poor response to common therapies [3]. A number of serum markers have been proposed for detecting HCC. AFP is the most frequently used. However, sensitivity ranges from 25% for tumors smaller than 3 cm to 50% for lesions larger than 3 cm in diameter [4].

Glypican3 (GPC3) is a cell surface oncofetal proteoglycan. Whereas GPC3 is abundant in fetal liver, its

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expression is hardly detectable in adult liver. Importantly, GPC3 is overexpressed in hepatocellular carcinoma (HCC), and several immunohistochemical studies reported that overexpression predicts a poorer prognosis for HCC patients [5]. Therefore, GPC3 would serve as a useful molecular marker for HCC diagnosis and also as a target for therapeutic intervention in HCC [5]. GPC3 regulates the signal pathways, including Wnt, Hedgehogs, bone morphogenetic proteins, and fibroblast growth factors. GPC3 has been shown to activate the canonical Wnt pathway in 18% of HCC and subsequent accumulation of β-catenin in the cytoplasm [6]. GPC3 also binds to insulin-like growth factor-II (IGF-2) and its receptor (IGF-1R), mediating enhancement of IGF-related signaling, cell proliferation, and prevention of apoptosis, thus supporting the HCC carcinogenesis [7].

Long non-coding RNAs (lncRNAs) are a class of newly found non-coding RNAs widely depicted in the genome [8]. Many lncRNAs have been shown to play key roles in organ development and cancer pathogenesis [9]. The aberrantly expressed lncRNAs in HCC tissues include 232 downregulated lncRNAs and 930 upregulated lncRNAs. They proved to correlate with poorer survival in HCC patients. Also, they affect the gene expressions involved in HCC proliferation, differentiation, and cell cycle, indicating an essential role of lncRNAs in hepatocarcinogenesis [10]. Additionally, alternative splicing of lncRNAs promotes HCC [11].

This work aims to assess non-invasive diagnostic and prognostic tools based on measuring the plasma levels of lncRNA *GPC3* gene expression, *GPC3* protein, and AFP in HCC and to correlate their levels with HCC progression.

Patients and methods

This is a case–control study that is performed to evaluate the molecular expression of long non-coding RNA and Glypican3 in HCC patients. This study included 144 Egyptian subjects in the age group 30–64 years. All subjects are recruited in the period from March 2018 to October 2018 from the Endemic Medicine Department, Kasr Al-Ainy Hospital, Faculty of Medicine, Cairo University. They are classified into three groups: group I included seventy patients with HCV-related HCC, group II included forty-four patients with cirrhosis, and lastly, group III included thirty healthy volunteers who served as controls. All procedures were conducted after approval of the Ethical Committee of Cairo University in accordance with the Helsinki Declaration of 1975, as revised in 2008.

All cirrhotic and HCC patients were HCV-related, and we excluded patients with concomitant hepatitis B surface antigen (HBsAg) seropositivity or autoimmune hepatitis, coinfection with the human immunodeficiency virus (HIV), and co-presence of any chronic systemic illness. Regarding all HCC patients, they were diagnosed as HCC by triphasic spiral computed tomography (CT) and/or dynamic magnetic resonance imaging (MRI) according to the international guidelines of the American Association for the Study of Liver Diseases (AASLD) for the diagnosis and management of HCC [12].

For all recruited patients, we performed laboratory investigations that included a complete liver biochemical profile, coagulation profile, and renal functions. Child–Pugh class is assessed, and we recorded the tumor characteristics (number and size of lesions, extrahepatic metastases, and vascular invasion). Serum levels of Glypican3 and AFP were estimated by enzyme-linked immunosorbent assay (ELISA) technique, and genomic analysis of long non-coding RNA expression of Glypican3 was assessed by the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) technique.

Statistical analysis of data was done by SPSS version 22. Classification of variables was expressed as frequency and percentage, and continuous variables were expressed as mean ± standard deviation. Comparisons between the groups were done using unpaired *t* test and post hoc test when comparing more than 2 groups. For comparing categorical data, the chi-square (χ^2) test was performed. Correlations between quantitative variables were done using the Pearson correlation coefficient. Survival curves were plotted by the Kaplan–Meier method, and independent prognostic factors were estimated by the Cox proportional hazards in univariate and multivariate regression models. ROC curve was constructed to detect the best cutoff value of AFP, GPC3 protein levels, and LncRNA-AF085935 gene expression for the detection of HCC. *p*-values less than 0.05 were considered as statistically significant.

Results

Our study was conducted on seventy patients with HCV-related HCC and forty-four patients with HCV-related liver cirrhosis in addition to thirty volunteered persons who served as controls. Their mean age group was high among the HCC group (57.5 years) with male predominance (85.7%). Concerning the baseline laboratory data, HCC group patients had significantly higher levels of bilirubin (1.36 ± 0.89 mg/dl), significantly lower platelet count ($132.8 \pm 63.9 \times 10^3/m^3$), and albumin level (3.3 ± 0.6 g/dl). Levels of AFP, GPC3, and lncRNA AF085935 among HCC patients were significantly higher than both the cirrhotic and control groups (109 ± 40 ng/ml, 6.3 ± 2.26 ng/ml, 1.6 ± 0.8 ng/ml, respectively). The hepatic focal lesions in HCC patients were mainly solitary (58.5%) and seated in the right lobe (78.5%). About

71.4% of cases had vascular invasion, and 64% had abdominal LN affection (Table 1).

In studying the correlation between the studied biomarkers and the characteristics of HCC, we found that increased GPC3 levels were significantly associated with higher Child score ($p<0.001$), presence of abdominal LNs ($p<0.001$), and the multi-nodularity of focal lesions (p 0.015). Concerning lncRNA, increased expressions were significantly associated with a high Child Score ($p<0.001$) and presence of abdominal LN ($p=0.019$) and vascular invasion (p 0.003) (Table 2). When we assessed the diagnostic accuracy of the different studied parameters, the ROC curve for AFP, GPC3, and lncRNA levels in the HCC group showed that the best-chosen cutoff levels were 7.65, 4.05, and 0.93, respectively at which the sensitivity was 72%, 87%, and 96% while the specificity was 72%, 64%, and 81%, respectively. The areas under the curve (AUC) were 0.792, 0.84, and 0.813, respectively. ROC curve for AFP, GPC3, and lncRNA levels in the cirrhotic group showed lower sensitivity (60%, 62%, and 72%) and specificity (30%, 19%, and 17%) (Table 3 and Fig. 1a, b). In the linear regression

Table 1 Demographic and biochemical laboratory data among the studied groups

Variable	HCC group I, $n=70$	Cirrhosis group II, $n=44$	Control group III, $n=30$	*P1* value	*P2* value	*P3* value
Sex						
Females: *n* (%)	***10 (14.3%)***	***25 (56.8%)***	***11 (36%)***	***0.1***	***< 0.001***	***0.037***
Males: *n* (%)	60 (85.7%)[a,b]	19 (43.2%)	19 (64%)			
Age (years)	***57.5 ± 6.7***[a,b]	***42 ± 9.8***	***39.6 ± 10***	***0.5***	***< 0.001***	***< 0.001***
T-bilirubin (mg/dl)	***1.36 ± 0.89***[a,b]	***0.8 ± 0.34***	***0.83 ± 0.21***	***0.9***	***< 0.001***	***0.009***
AST (U/L)	***68.7 ± 35.5***[a]	***57.6 ± 37***[a]	***30.2 ± 6.1***	***0.008***	***0.2***	***< 0.001***
ALT (U/L)	***55 ± 29.9***[a]	***61 ± 32.7***[a]	***24.5 ± 7.4***	***< 0.001***	***0.5***	***< 0.001***
Albumin (g/dl)	**3.3 ± 0.6**[a,b]	**4.3 ± 0.76**	**4.27 ± 0.6**	***0.9***	***< 0.001***	***< 0.001***
PC (%)	**74 ± 11**[a,b]	**86 ± 10**	**87 ± 8.6**	***0.9***	***< 0.001***	***< 0.001***
Platelets x $10^3/m^3$	**132.8 ± 63.9**[a,b]	**228 ± 69**[a]	**279.6 ± 88**	***0.019***	***< 0.001***	***< 0.001***
AFP (ng/ml)	109 ± 40[a,b]	10 ± 4	5.13 ± 2.9	***0.009***	*0.001*	*0.9*
***GPC3* (ng/ml)**	6.3 ± 2.26[a,b]	3.9 ± 0.8	3.76 ± 2.06	***< 0.001***	*< 0.001*	*0.9*
lncRNA AF085935	1.6 ± 0.8[a,b]	0.95 ± 0.5	0.68 ± 0.2	***< 0.001***	< 0.001	*0.29*
Child–Pugh score of the HCC group						
A	Number of patients	%				
	25	35.7%				
B	12	17%				
C	33	47.3%				
Imaging data of the HCC group						
No. of FL		Number of patients	%			
	1	41	58.5%			
	2	12	17%			
	Multiple	17	24.5%			
Site of FL	Right lobe	55	78.5%			
	Left lobe	5	7.0%			
	Both lobes	10	15.5%			
Vascular invasion	Yes	50	71.4%			
	No	20	28.6%			
Abdominal LNs	yes	45	64.0%			
	no	25	36.0%			

Data were expressed as mean ± SD, p value < 0.05 was significant. P1 value: HCC group versus the control group; P2 value: HCC group versus the cirrhosis group; P3 value: cirrhosis group versus the control group

T-bilirubin Total bilirubin, *D-bilirubin* Direct bilirubin, *AST* Aspartate transaminase, *ALT* Alanine transaminases, *PC* Prothrombin concentration, *No FL* Number of focal lesion, *LN* Lymph node

[a] Significant difference versus control subjects

[b] Significant difference versus cirrhosis subjects

Table 2 Correlation between the studied biomarkers and the characteristics of the HCC group

		GPC3			lncRNA AF085935		
		Low expression ($n=34$)	High expression ($n=36$)	*p* value	Low expression ($n=34$)	High expression ($n=36$)	*p* value
Age		57 ± 7	57.8 ± 6.5	0.6	58.8 ± 7.6	56 ± 5.7	0.1
Gender	**Male**	30 (88.2%)	30 (83.3%)	0.55	29 (85.3%)	31 (86.1%)	0.9
	Female	4 (11.8%)	6 (16.7%)		5 (14.7%)	5 (13.9%)	
Child Score	**A**	25 (73.5%)	0 (0%)	< 0.001	25 (73.5%)	0 (0%)	< 0.001
	B	9 (26.5%)	3 (8%)		6 (17.6%)	3 (8.4%)	
	C	0 (0%)	33 (92%)		3 (8.9%)	33 (91.6%)	
No. of FLs	**1**	19 (55.9%)	22 (61.1)	0.015	21 (61.8%)	20 (55.6)	0.12
	2	10 (29.4%)	2 (5.6%)		8 (23.5%)	4 (11.1%)	
	multiple	5 (14.7%)	12 (33.3%)		5 (14.7%)	12 (33.3%)	
AFP	**< 20**	20 (58.8%)	15 (41.7%)	0.15	15 (44.1%)	20 (55.6%)	0.33
	> 20	14 (41.2%)	21 (58.3%)		19 (55.9%)	16 (44.4%)	
Vascular invasion	**Yes**	3 (8.8%)	5 (13.9%)	0.5	6 (17.6%)	34 (94.4%)	0.003
	No	31 (92.2%)	31 (86.1%)		28 (82.4%)	2 (5.6%)	
Abdominal LNs	**Yes**	2 (5.9%)	6 (16.7%)	< 0.001	7 (20.6%)	35 (97.2%)	0.019
	No	32(94.1%)	30(83.3%)		27(79.4%)	1(2.8%)	

The median expression level was used as the cutoff. Low expression of lncRNA and GPC3 in 70 patients was defined as a value below the 50th percentile, and high expression was defined as a value above the 50th percentile

Table 3 Diagnostic accuracy of the different studied parameters for the HCC and cirrhotic groups

Variable(s)	AUC	*p* value	95% confidence interval		Cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)
			Lower bound	Upper bound				
HCC group								
AFP	0.792	< 0.001	0.71	0.874	7.65	72	72	72
GPC3	0.84	< 0.001	0.773	0.907	4.05	87	64	75.5
lncRNA AF085935	0.813	< 0.001	0.739	0.888	0.93	96	81	88.5
Cirrhosis group								
AFP	0.3	< 0.001	0.219	0.398	4.9	60	30	45
GPC3	0.29	< 0.001	0.2	0.38	3.6	62	19	40.5
lncRNA AF085935	0.33	0.002	0.23	0.43	0.61	72	17	44.5

analysis for detecting predictor risk factors for HCC, we found that lncRNA AF085935 gene expression and GPC3 protein were significant predictor risk factors for HCC ($p < 0.001$ and p 0.001, respectively). In the cirrhotic group, we noticed that lncRNA AF085935 gene expression and GPC3 protein were non-significant predictor risk factors for cirrhosis (p 0.05 and p 0.525, respectively) (Table 4). Kaplan–Meier curve showed that patients with high expression of GPC3 and lncRNA had markedly shorter overall survival than those with low expressions ($p < 0.001$) while AFP showed no significance in overall survival (p 0.178) (Fig. 2a–c). In addition, we performed a correlation analysis between the different studied parameters in the HCC group, and there was a significant correlation between GPC3 level, lncRNA gene expression, and AFP level (Table 5).

Discussion

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [13]. A number of serum markers have been proposed for detecting HCC. AFP is the most frequently used. However, sensitivity ranges from 25% for tumors smaller than 3 cm to 50% for lesions larger than 3 cm in diameter [4]. The survival rate for HCC is very low due to the lack of reliable biomarkers, so new diagnostic measures are needed [14].

Based on our interest, we aimed to evaluate the levels of *GPC3* and long non-coding RNA-AF085935 gene

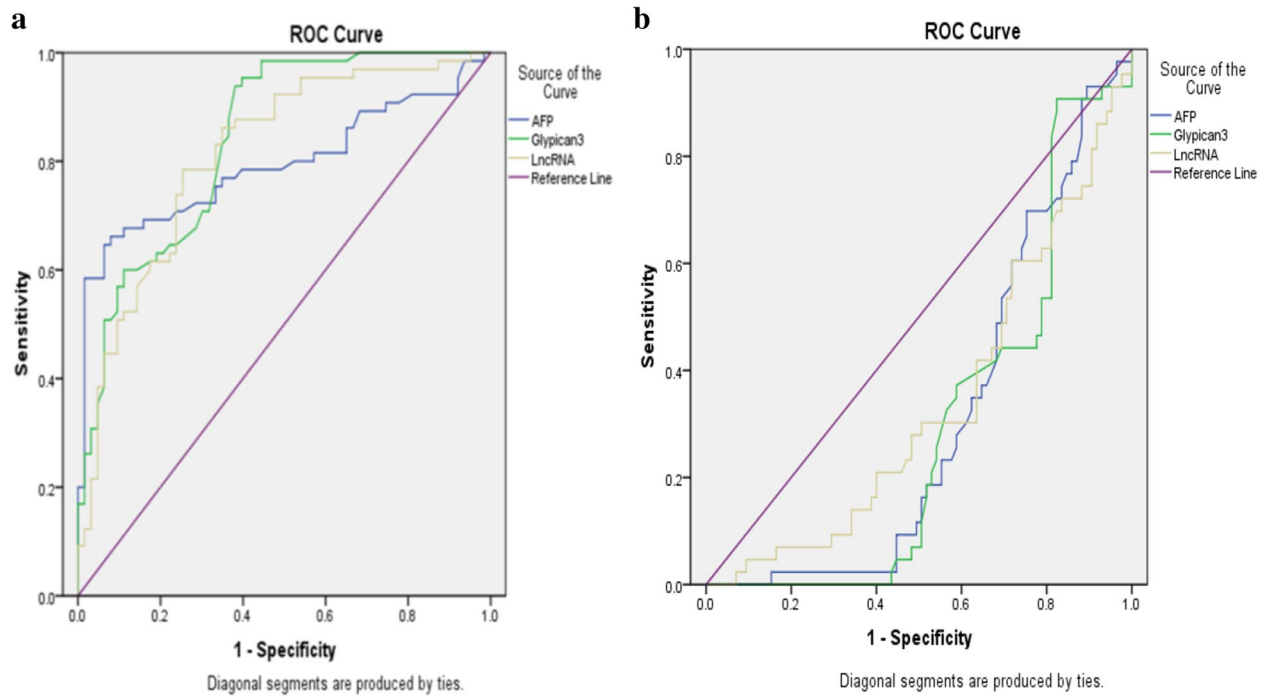

Fig. 1 ROC curve for AFP, *GPC3*, and lncRNA AF085935 gene expression in HCC 1 (**a**) and cirrhotic patients 1 (**b**)

Table 4 Linear regression model for detecting predictors risk factors for HCC and cirrhosis

	HCC group				Cirrhosis group			
	B	*p* value	95.0% confidence interval for *B*		*B*	*p* value	95.0% confidence interval for *B*	
			Lower bound	Upper bound			Lower bound	Upper bound
AFP	.001	.022	.000	.001	.004	.482	− .008	.016
GPC3	.056	.001	.024	.088	.056	.525	− .120	.233
lncRNA AF085935	.200	.000	.106	.294	.533	.05	− .034	1.031

expressions and correlate their levels with HCC progression. We found that levels of AFP and *GPC3* in HCC patients were significantly higher than those in other groups. Hippo et al. [15] and Tahon et al. [16] reported that AFP and *GPC3* levels increased significantly in HCC than in cirrhosis patients and control subjects [15, 16]. Also, Hippo et al. clearly concluded that AFP is a weak marker for HCC diagnosis and has a poor ability in differentiating HCC from non-malignant hepatopathy and that GPC3 is more sensitive than AFP in the early diagnosis of HCC [15]. In our current study, GPC3 was more accurate than AFP in the diagnosis of HCC with 87% and 72% sensitivity while specificity was 64% and 72%, respectively. This is in accordance with Wang et al. [17], Tahon et al. [16], and El-Saadany et al. [18] who similarly reported that GPC3 may be a more effective diagnostic marker for HCC than AFP [16–18]. Moreover, Wasfy and Shams-Eldeen [19] found that GPC3 had a high specificity and sensitivity in detecting HCC when compared to cirrhosis, dysplasia, and metastatic cancers [19].

In this study, the levels of lncRNA AF085935 in HCC patients (1.6 ± 0.8) were significantly higher than in the other groups (p value < 0.001). Additionally, lncRNA-AF085935 was more accurate than GPC3 and AFP in the diagnosis of HCC. The ROC curve for lncRNA AF085935 showed that the best-chosen cutoff level was 0.93, at which the sensitivity was 96% and the specificity was 81%. The area under the curve (AUC) was 0.813. Lu et al. [20] and Chen et al. [21] reported that their lncRNA-AF085935 expression levels were significantly higher in the HCC group compared to the other groups [20, 21]. In addition, Xiao-ting et al. [22] mentioned that lncRNA-AF085935 enhanced HCC-related cell growth and migration in vitro and in vivo via activating GPC3.

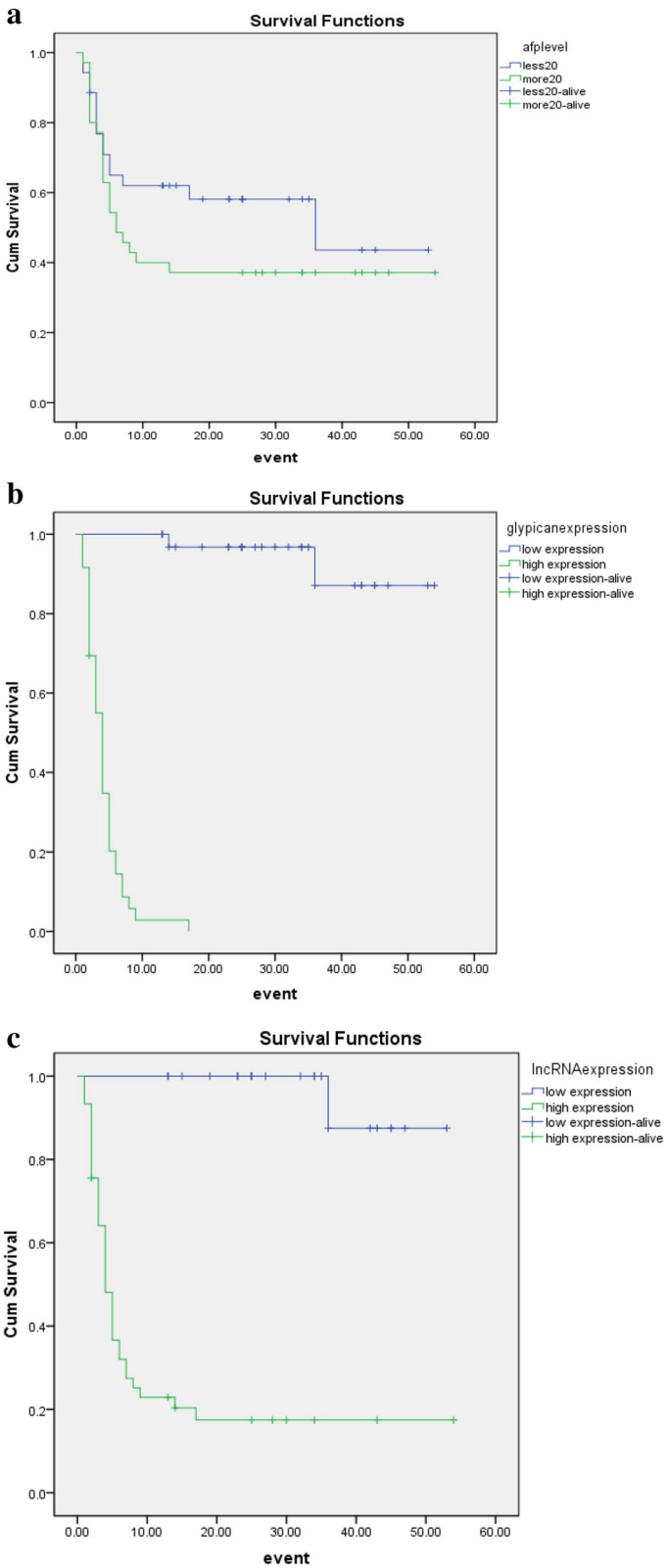

Fig. 2 **a** The survival curve corresponding to different AFP levels. **b** The survival curve corresponding to different *GPC3* gene expression. **c** The survival curve corresponding to different lncRNA AF085935 expression

Table 5 Correlation analysis between the studied parameters in the HCC group

		AFP	GPC3	LncRNA AF085935
AFP	***r***	1.000	.322**	.275*
	***p* value**		.003	.011
LncRNA AF085935	***r***	.275*	.398**	1.000
	***p* value**	.011	.000	

In agreement with Motawi et al. [23], we reported that lncRNAAF085935 expression was an independent predictor of HCC susceptibility in multivariate analysis and that lncRNA was significantly upregulated in HCC patients compared with the healthy control groups ($p < 0.001$).

In our study, linear regression analysis for risk factors predictors for HCC showed that lncRNA GPC3 gene expression and GPC3 protein are significant predictor risk factors for HCC ($p = 0$ and $p = 0.001$, respectively). Our Cox regression analyses for all variables revealed that high expression of GPC3 and lncRNA are significant prognostic factors for HCC ($p < 0.001$, HR = 124, CI 13–130 and p 0.04, HR = 1.2, CI 0.35–4, respectively). AFP level was a non-significant prognostic factor (p 0.93, HR = 0.97, CI 0.48–1.9). This is consistence with Shanshan et al. [24].

Kaplan–Meier and log rank analysis based on AFP level, GPC3, and lncRNA gene expression in HCC patients showed that patients with high expression of GPC3 and lncRNA had markedly shorter overall survival than those with low expressions ($p < 0.001$). Again, AFP level showed no significant differences in the overall survival (p 0.178). From these data, it was demonstrated that the reliability of lncRNA gene expression and GPC3 are significant prognostic factors while AFP level is a non-significant factor [25].

Our findings provided evidence that serum levels of lncRNAAF085935 could be useful non-invasive biomarkers for the screening of HCV-related HCC and appeared to be both reproducible and cost-effective. The molecular mechanisms by which lncRNAs function in HCC are required to be studied to explore these potential new strategies for early screening of HCC. Finally, further analysis of the roles of more differentially expressed lncRNAs in hepatocarcinogenesis is needed, and extended studies with independent larger samples are required to validate our results. Serum GPC3 level estimation can be used as a simple, rapid, non-invasive, and good diagnostic biomarker for early HCC detection as GPC3 had high sensitivity and specificity that can raise the accuracy of diagnosis.

Conclusion

We concluded that lncRNA AF085935 gene expression and *GPC3* plasma level are diagnostic and prognostic biomarkers for the detection of HCC, and also, they are strongly connected with the development and progressions of HCC.

GPC3 is a novel biomarker for the survival rate of HCC patients.

Submission declaration

This work has not been published previously and is not under consideration for publication elsewhere; its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out and, if accepted, will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright holder.

Code availability

Not applicable.

Authors' contributions

All authors have contributed significantly to finishing this work; all authors agree with the content of the manuscript. Design of the study: DS and SAEF. Acquisition of data: MMN, RmL, HIS, and HRR. Analysis of data: MMN. Interpretation of the data and drafting of the article: TME. Critical revision: AOA, MMN, and ARM. Final approval of the manuscript: all authors.

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author [Ahmed Ramadan].

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Faculty of Medicine, Cairo University Research Committee and with the 1964 Helsinki Declaration and its later amendments.
Written informed consent was obtained from the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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