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The Expression Level of Long Non-Coding RNA PVT1 as a Diagnostic Marker for Advanced Stages in Patients with Nonalcoholic Fatty Liver Disease

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) incorporates a wide spectrum of stages ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC). The connection of Long noncoding RNAs with NAFLD, as well as other metabolic syndromes remains relatively unexplored.

Objective: We aimed to investigate the circulatory level of lncRNA-PVT1 in patients with Non-alcoholic fatty liver disease complicated with steatohepatitis, cirrhosis, and HCC. Also, to explore their association with different clinical and laboratory variables.

Patients and methods: A case-control study enrolled 100 patients with NAFLD and 100 healthy volunteers. NAFLD patients included 53 patients with simple steatosis, 22 steatohepatitis patients, 17 cirrhotic patients, and 8 HCC patients confirmed with histopathological examination. The expression of lncRNA PVT1 was evaluated by RT PCR.

Results: The relative expression levels of lncRNA-PVT1 were significantly higher in the NAFLD group (2.29±0.37) compared to control (0.84±0.52), P<0.001 with a significant difference between the subgroups; (4.2 ± 0.1, 2.9 ± 0.16, 1.93 ± 0.14, 1.25 ± 0.18 in HCC, cirrhosis, NASH, and in simple steatosis respectively; P<0.001). There were significant direct correlations with liver function tests, lipid profile, and alpha-fetoprotein in the HCC subgroup. Alpha-fetoprotein and AST were the main predictors of lncRNA-PVT1. Cut-off values 0.94, 1.2, 1.8, 2.98 were able to discriminate simple steatosis, steatohepatitis, cirrhosis, and HCC with AUC 0.78, 0.64, 0.77, 0.81 respectively.

Conclusions: Circulating lncRNA-PVT1 could be a useful diagnostic biomarker for discriminating patients with advanced NAFLD stages.

Keywords: Carcinoma, Liver Cirrhosis, NASH, NAFLD, PVT1.

INTRODUCTION

NAFLD incorporates various stages ranging from simple steatosis to NASH, cirrhosis, and HCC [(1)]. The clinical history of NAFLD is usually benign and nonprogressive. On the other hand, NASH is a potentially fatal illness; up to 25% of patients may develop cirrhosis and develop consequences such as portal hypertension, liver failure, and hepatocellular carcinoma (HCC) [(2)]. LncRNAs are non-coding ribonucleic acids (RNAs) with a length of more than 200 base pairs. Previously, lncRNAs were thought to be worthless sections of the genome [(3)].

The significance of abnormal lncRNA expression levels in many disorders has steadily caught people's attention. While the significance of lncRNAs in cancer has been studied extensively in recent years, their relevance in the development of obesity, type 2 diabetes (T2D), and its comorbidities, such as nonalcoholic fatty liver disease (NAFLD), has only just been discovered [(4)].

lncRNA has been linked to elevated levels of steatosis, oxidative stress, inflammation, insulin resistance, and other pathogenic processes. For instance, 381 lncRNAs have been verified to be highly elevated in NAFLD[(5)], while lncRNA MEG3 has been identified as being overexpressed and involved in insulin resistance in NAFLD and hepatocyte endothelial cell aging [(6)].

The development of fibrosis in NASH patients is linked to an increased risk of liver-related morbidity and death; however, the molecular pathways driving fibrosis and cirrhosis in NAFLD patients are unknown. Long non-coding RNAs (lncRNAs) are emerging as important contributors to biological processes that drive NAFLD fibrosis onset and progression [(7)].

While many studies have been done on the pathophysiology of HCC development from hepatic fibrosis, their regulating molecular mechanisms are only poorly known. The underlying pathogenic processes involving lncRNAs that lead to HCC from chronic liver disorders and cirrhosis are yet unknown [(8)].

PVT1 is a LncRNA located in the 8q24.21 region of the human chromosome. Recently, PVT1 was found to be upregulated in diabetic cataracts and inhibits podocyte injury and apoptosis in diabetic nephropathy through FOXA1 [(9)]. In addition, PVT1 promotes the synthesis and inhibits the oxidation of fatty acids in obesity [(10)].

PVT1's possible role in cancer development has been widely investigated and reported in endometrial cancer [(11)], bladder cancer [(12)], and ovarian cancer [(13)]. Like other cancers, HCC is distinguished by genetic and epigenetic dysregulations. Among these alterations, lncRNAs may play crucial roles in the initiation and progression of HCC

Therefore, we aimed to investigate the circulatory level of lncRNAs PVT1 in patients with NAFLD-related complications including patients with steatohepatitis, cirrhosis, and HCC, and to explore their

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association with different clinical, laboratory variables, and clinicopathological features of HCC patients.

PATIENTS AND METHODS

This case-control study included a total of 100 patients with biopsy-proven NAFLD as the cause of their liver disease and 100 matched healthy subjects served as the control group. Patients were recruited from Outpatient Clinics, Internal Medicine, Tropical Medicine, and Surgery Departments, Zagazig University Hospitals.

***Ethical Consideration*:**

This study was ethically approved by the Institutional Review Board of the Faculty of Medicine, Zagazig University. Written informed consent of all the participants was obtained. The study was conducted according to the Declaration of Helsinki.

All patients were subjected to thorough history taking and clinical assessment.

NAFLD diagnosis was performed according to Practice Guidance from the American Association for the Study of Liver Diseases (AASLD) and the American College of Gastroenterology (ACG) in 2018. An experienced sonographer performed an abdominal ultrasound for each patient. The radiologist performed a confirmatory liver biopsy to diagnose steatohepatitis for cases with unexplained persistently elevated liver enzymes or patients with unexplained cirrhosis or HCC (diagnosis was made in some patients following resection of focal lesions). In addition, a semi-quantitatively evaluation of steatosis, lobular inflammation, ballooning, and liver fibrosis was done, conceding with the NASH CRN scoring system [(14)].

HCC was diagnosed conceding to the American Association for the Study of Liver Diseases (AASLD) practice guidelines. Clinical staging of HCC was evaluated according to the Barcelona Clinic Liver Cancer staging classification [(15)] and Child-Pugh classification [(16)]. Clinicopathological features of HCC cases including tumor number, site, size, presence of metastasis, and portal vein thrombosis were picked up at the time of blood collection. Consequently, NAFLD patients included four subgroups: simple steatosis (n=53), NASH (n=22), cirrhosis (n=17), and HCC (n=8).

Exclusion criteria: included all patients with any chronic liver disease other than NAFLD such as chronic hepatitis B or hepatitis C, biliary cirrhosis, autoimmune hepatitis, metabolic liver diseases, alcohol consumption, and patients on medications that cause hepatic steatosis as amiodarone, corticosteroids, tamoxifen, methotrexate, and oral contraceptives. Pregnancy, patients with thyroid disease, diabetes, and malignancy were also excluded.

Sampling of blood:

Fasting serum samples were collected and stored at –20°C until analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin, and creatinine were estimated by routine enzymatic methods using full automated Cobas 8000 c702 (Roche diagnostic, Germany). Serum alpha-fetoprotein (AFP) and viral markers including hepatitis B surface antigen (HBsAg) and HCV antibodies were assessed by Electrochemiluminescence immunoassay on full automated Cobas 8000 e601 (Roche diagnostic, Germany). For RNA Extraction and Quantitative PCR, venous blood was collected from fasting participants. The supernatant was immediately collected and stored at −80°C until use.

RNA Extraction and Quantitative PCR (qPCR):

Total RNA was isolated from serum using Trizol Reagent (Invitrogen, CA, USA) following the manufacturer's manual. Reverse transcription was carried out using Prime Script™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer's manual. qPCR was consequently carried out using SYBR® Premix Ex Taq™ II (Takara) on the ABI Step One Plus system (Applied Biosystems, CA, USA) following the manufacturer's manual. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an endogenous control. The primers sequences were as follows: PVT1, 5′-ATAGATCCTGCCCTGTTTGC-3′ (forward), and 5′-CATTTCCTGCTGCCGTTTTC-3′ (reverse); GAPDH, 5′GGAGCGAGATCCCTCCAAAAT-3′ (forward), and 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse).

Statistical analysis

All data were statistically analyzed using the SPSS 26.0 software package. For quantitative variables, results were expressed as mean ± standard deviation for parametric data and median (interquartile range) for non-parametric data. Categorical variables were expressed as the number of patients and percentage (%). Significant differences between quantitative variables according to whether they followed a normal distribution were evaluated by independent sample t-test and independent sample non-parametric test, respectively. The chi-square test was used to compare the significance of categorical variables. The Pearson correlation coefficient tested the correlations of lncRNA-PVT1 levels with different clinical and laboratory variables. Receiver operating characteristic (ROC) analysis was performed to assess the accuracy of the relative expression levels of lncRNA-PVT1 for diagnosis of different NAFLD stages and HCC. A linear regression analysis was used to detect the main predictors of lncRNA-PVT1 levels in the HCC group. We considered P to be significant at < 0.05 with a 95% confidence interval (CI).

RESULTS

Clinical and biochemical characteristics of the studied groups:

We observed significantly more elevated TC, TG, LDL, PT, total bilirubin, direct bilirubin, hepatic steatosis index, alpha-fetoprotein, and serum creatinine in the NAFLD group compared to healthy control. In addition, AST, ALT, and GGT values were significantly higher in NAFLD compared to controls (P<0.001, Table 1). On the contrary, we detected significantly more reduced HDL, albumin, hemoglobin, and platelet levels in NAFLD patients compared to the control **(P<0.001, Table 1).**

Table (1): Clinical, anthropometric and laboratory characteristics of studied groups.

Variables	Control (mean ± SD) (n=100)	NAFLD (mean ± SD) (n=100)	P
Age (years)	37.82± 7.37	38.1±5.91	0.812
Sex(male/female)	(41/59)	(45/55)	0.334
SBP (mm Hg)	122.8± 6.07	129.6± 11.2	<0.001*
DBP (mm Hg)	85.1±4.8	86.9±7.1	0.177
BMI (kg/m^2)	33.6±4.5	34.3±3.3	0.136
TC (mg/dL)	141.3± 26.34	189.6± 5.48	<0.001*
TG (mg/dL)	130.6± 12.51	143.6± 3.01	<0.001*
LDL (mg/dL)	122.9± 22.9	156.2± 4.79	<0.001*
HDL l (mg/dL)	53.1±6.67	36.9±6.775	<0.001*
FPG (mg/dL)	87.9±3.97	86.1±4.1	0.654
PT (seconds)	11.9±0.68	17.9±2.245	<0.001*
Total bilirubin (mg/dl)	0.9±0.13	1.7±0.18	<0.001*
Direct bilirubin (mg/dl)	0.25±0.07	0.91 ±0.26	<0.001*
Albumin (g/dl)	4.5±0.8	3.8±0.13	<0.001*
AST(IU/L)	33.3±3.126	93.5±20.6	<0.001*
ALT (IU/L)	18.06± 2.5	113.5±21.6	<0.001*
GGT (IU/L)	32.7±1.50	79.5±5.6	<0.001*
Serum ferritin (ng/ml)	18.02±3.06	17.02±4.06	0.902
Hepatic steatosis index	34.9±1.18	49.4±3.65	<0.001*
Alpha-fetoprotein(ng/ml)	8.19±0.9	198.1 ±6.23	<0.001*
WBC count (cell×10^3/µl	6.3±0.9	6.7±0.4	0.547
Hemoglobin (g/dl)	13.6±0.71	10.5±2.56	<0.001*
Platelet(cell×10^3/µl)	236.8±38.1	121.4±6.7	<0.001*
Creatinine (mg/dl)	0.74±0.13	1.8±0.18	<0.001*
LncRNA PVT1	0.84±0.52	2.29±0.37	<0.001*

NAFLD: non-alcoholic fatty liver disease; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; TG: triglycerides; BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; PT: prothrombin time; FPG: fasting plasma glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; WBC: white blood cell; lncRNA PVT1: long noncoding RNA plasmacytoma variant translocation1. *P < 0.05 when compared with the control group

The relative expression levels of lncRNA-PVT1 in investigated subjects.

Current results revealed statistically significant higher values of the relative expression levels of lncRNA-PVT1 in NAFLD (2.29 ± 0.37) compared to the control group (0.84 ± 0.52); **(P<0.001, table 1, figure 1a).** Furthermore, there was a statistically significant difference between the subgroups as regards the relative expression levels of lncRNA-PVT1; (values were 4.2 ± 0.1, 2.9 ± 0.16, 1.93 ± 0.14, 1.25 ± 0.18 in HCC, cirrhosis, NASH, and in simple steatosis respectively; P<0.001 **(Table 2, figure 1b).**

Clinical and biochemical characteristics of NAFLD subgroups

We noted statistically significant differences between investigated subgroups regarding levels of TC, LDL, HDL, total bilirubin, direct bilirubin, albumin, AST, ALT, GGT, alpha-fetoprotein, hemoglobin, platelets, and serum creatinine **(P <0.05, Table 2).**

Table (2): Clinical, anthropometric and laboratory characteristics of Nonalcoholic fatty liver disease subgroups.

Variables	Simple steatosis(n=53)	NASH (n=22)	Cirrhosis (n=17)	HCC (n=8)	P-value
Age (years)	36.5±7.18	37.2±4.14	37.8 ±5.26	39.1 ±6.26	0.364
SBP (mm Hg)	131.1±11.5	131.5±16.4	132.5±3.37	133.5±3.3	0.169
DBP (mm Hg)	84.1±8.31	87.6±6.17	85.1 ±5.16	85.1 ±7.7	0.484
BMI (kg/m^2)	35.5±6.33	34.06±2.98	33.6±1.71	33.6±2.2	0.542
TC (mg/dL)	184.4±6.9	150.4±22.8	209.1±16.5*	211±22.5#*	<0.001*
TG (mg/dL)	132.5±21.2	135.1±13.7	134. ±11.7	132.1 ±12.9	0.342
LDL (mg/dL)	160.4±5.8	190.9±19.9#	199.1±14.3#	201.9±14.3#	<0.001*
HDL (mg/dL)	44.4±4.29	33.6±6.178#	31.6±5.08#	31.5±5.12#	<0.01*
FPG (mg/dL)	84.5±5.6	88.1±2.6	85.1±7.7	86.1±5.7	0.642
PT (seconds)	10.92±0.3	11.9±0.25	15.86±0.23#	18.86±2.5#$	<0.001*
Total bilirubin (mg/dl)	0.9±0.22	1.1±0.2	1.5±0.1*	2.8±0.3#*$	<0.01*
Direct bilirubin (mg/dl)	0.5±0.08	0.8±0.24	0.9±0.16*	1.8±0.4#*$	<0.001*
Albumin (g/dl)	4.5±0.8	4.2±0.31	3.8±0.8#	3.3±0.73#	<0.001*
AST(IU/L)	72.6±4.13	125.2 ±4.42#	111.9±6.3#*	105.9±6.3#*	<0.001*
ALT (IU/L)	71.4±12.7	158.6±13.3#	120.3±8.2#*	100.7±6.2#*	<0.05*
GGT (IU/L)	63.1±4.62	100.3±1.76#	130.5±12.6#*	133.5±12.6#*	<0.05*
Serum ferritin (ng/ml)	19.4±2.5	23.3±2.48	21.25±4.2	22.25±2.6	0.865
Hepatic steatosis index	46.6±6.31	50.6±7.41	45.1±2.14	46.1±2.14	0.811
Alpha-fetoprotein(ng/ml)	11.19±0.9	13.1 ±6.23	22.19±0.9 #	198.1 ±6.2#*$	<0.001*
WBC count ($cell\times10^3/\mu l$)	6.9±1.9	6.7±0.4	6.3±0.9	6.1±1.4	0.765
Hemoglobin (g/dl)	10.6±1.71	10.5±2.5	9.9±1.7#	9.2±2.56#	<0.001*
Platelet($cell\times10^3/\mu l$)	199.8±8.1	188.4±6.7	101.8±8.1#	84.46.7#*$	<0.001*
Creatinine (mg/dl)	0.74±0.13	0.9±0.08	1.8±0.23#	1.9±0.28#*	<0.001*
LncRNA-PVT1	1.25±0.18	1.93±0.14#	2.9±0.16#	4.2±0.1#*$	<0.001*

NASH: Nonalcoholic steatohepatitis; HCC: hepatocellular carcinoma; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; TG: triglycerides; BMI: body mass index; LDL: low-density lipoprotein; HDL: high-density lipoprotein; PT: prothrombin time; FPG: fasting plasma glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; WBC: white blood cell; lncRNA PVT1: long noncoding RNA plasmacytoma variant translocation1.

Significant P values (P < 0.05) when compared with Simple steatosis.* Significant P values (P < 0.05) when compared with NASH.$Significant P values (P < 0.05) when compared with Cirrhosis.

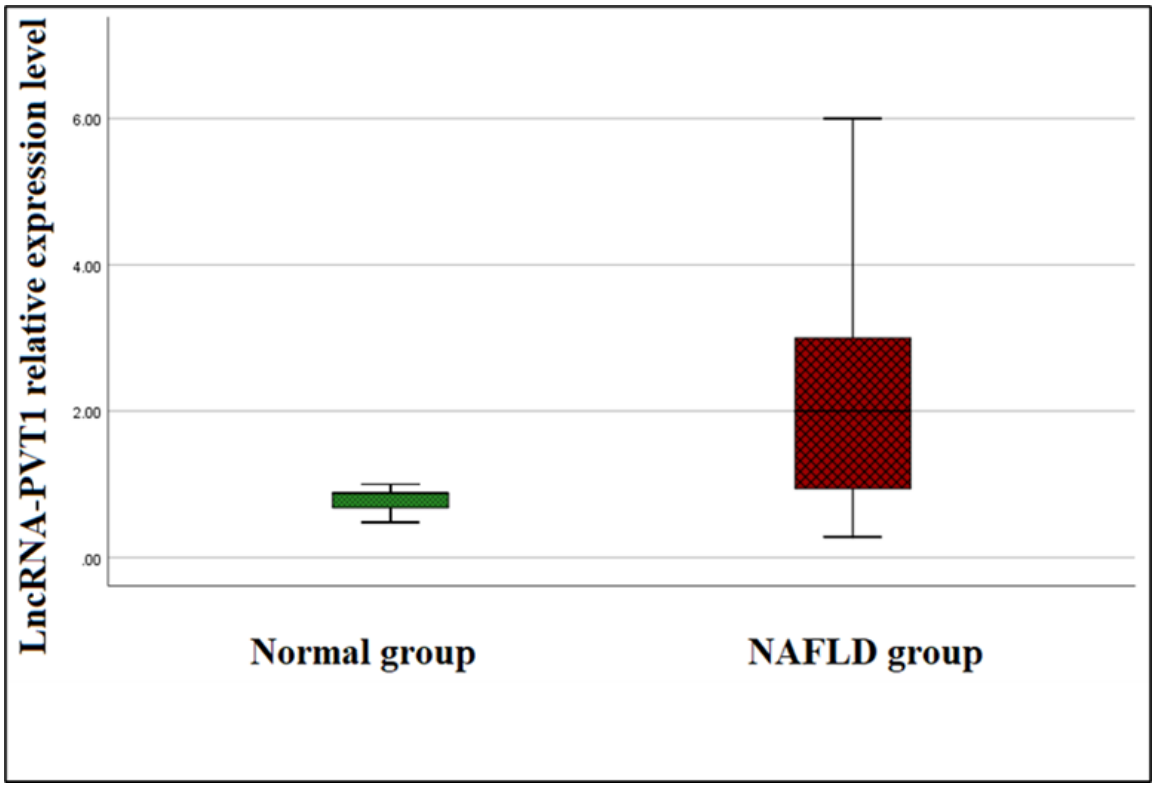

Figure (1a): Relative expression levels of lncRNA-PVT1 in NAFLD patients compared to the control group.

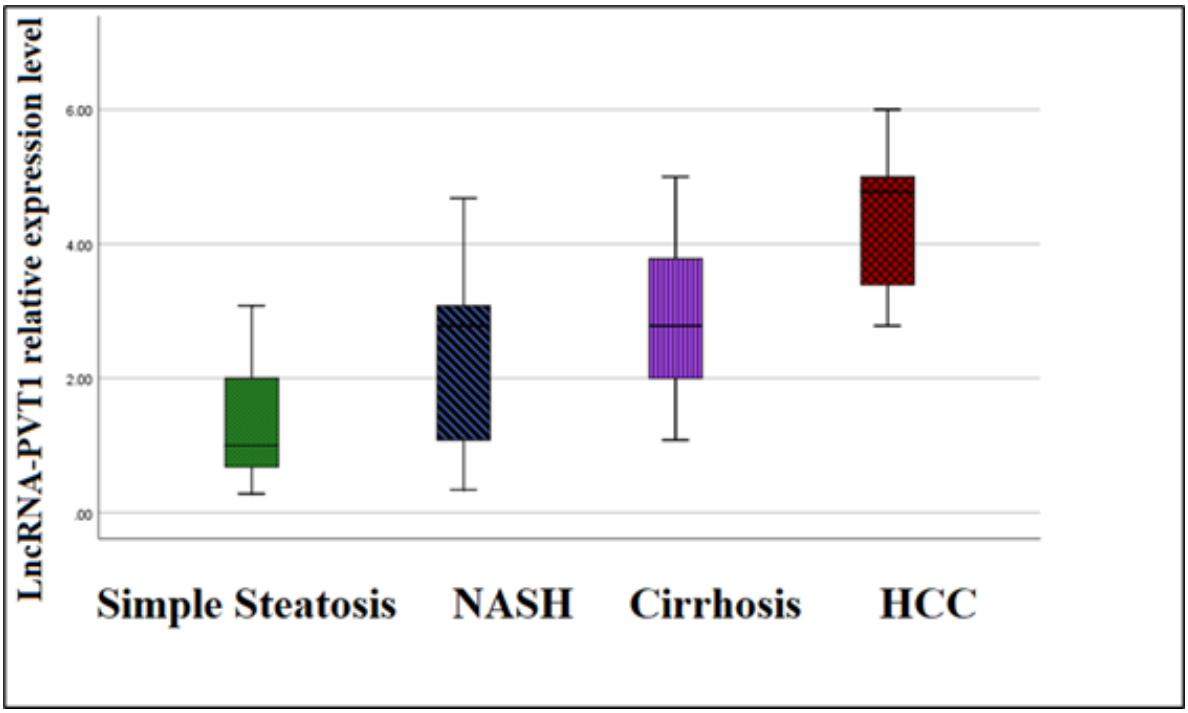

Figure (1b): Relative expression levels of lncRNA-PVT1 in NAFLD subgroups.

Association of the relative expression levels of lncRNA-PVT1 with different variables among HCC patients

The clinicopathological features of hepatocellular carcinoma patients were shown in **Table 3**. A significant relationship was found between increased expression levels of lncRNA-PVT1 and male sex & Child's class C. However, no relation was present to tumor size, the number of lesions, stage, or portal vein invasion **(as shown in table 4).**

Table (3): Clinicopathological features of hepatocellular carcinoma patients.

	HCC (n =8)- *n* (%)	*P* value
Stage		0.480
Stage I/II	3 (37.5%)	
Stage III/IV	5 (62.5%)	
Tumor size		0.157
<5 cm	6 (75%)	
>5 cm	2 (25%)	
Lymph node metastasis		<0.05*
-Absent	7 (87.5%)	
-Present	1 (12.5%)	
Distant metastasis		<0.05*
-Absent	7 (87.5%)	
-Present	1 (12.5%)	
Child-Pugh grade		0.882
-A	2 (25%)	
-B	3(37.5%)	
-C	3(37.5%)	
Portal vein thrombosis		0.157
-Negative	6 (75%)	
-Positive	2 (25%)	
Number of tumor lesions		0.889
-Single	4 (50%)	
-Multiple	4 (50%)	
Site of lesions		0.882
-Right lobe	3(37.5%)	
-Left lobe	2 (25%)	
-Both	3(37.5%)	

*Significant *P-value* (*P*<0.05)

Table (4): Relationship between the relative expression level of lncRNA-PVT1 and clinicopathological characteristics in hepatocellular carcinoma subgroup

Clinicopathological characteristics	HCC (n=8) (mean ± SD)	P-value
Sex		<0.05*
Male	4.89±1.03	
Female	4.22±1.56	
Child-Pugh grade		<0.001*
-A	1.73±1.32	
-B	2.31±1.02	
-C	3.71±1.38	
Stage		0.525
Stage I	3.11±1.64	
Stage II	4.05±1.31	
Stage III	4.21±1.21	
Stage IV	4.5±1.23	
Tumor size		0.089
<5 cm	3.75±1.71	
>5 cm	5.51±0.71	
Portal vein thrombosis		0.564
-Negative	4.14±1.52	
-Positive	4.64±1.58	
Number of tumor lesions		0.080
-Single	3.86±1.04	
-Multiple	5.26±0.98	

Pearson correlation revealed significant direct correlations between the relative expression levels of lncRNA-PVT1 and TG, LDL, PT, total bilirubin, direct bilirubin, AST, ALT, GGT, alpha-fetoprotein, and serum creatinine. On the contrary, there were significant negative correlations between the relative expression levels and HDL, albumin, hemoglobin, and platelets **(P<0.001, Table 5).**

Table (5): Pearson correlation of the relative expression level of lncRNA-PVT1 level with clinical, anthropometric, and biochemical characteristics in hepatocellular carcinoma subgroup

Variables	lncRNA-PVT1	
	R	P
Body mass index	0.089	0.377
Total cholesterol	0.020	0.844
Triglycerides	0.610	<0.001*
LDL cholesterol	0.422	<0.001*
HDL cholesterol	-0.266	0.123
Fasting plasma glucose	0.079	0.653
Prothrombin time	0.587	<0.001*
Total bilirubin	0.538	<0.001*
Direct bilirubin	0.497	<0.001*
Albumin	-0.852	<0.001*
AST	0.714	<0.001*
ALT	0.645	<0.001*
GGT	0.349	<0.001*
Hepatic steatosis index	0.079	0.653
Alpha-fetoprotein	0.588	<0.001*
WBC count	0.183	0.068
Hemoglobin	-0.840	<0.001*
Platelet	-0.363	<0.001*
Creatinine	0.283	<0.001*

LDL: low-density lipoprotein; HDL: high-density lipoprotein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; *P < 0.05

Linear regression analysis revealed that only alpha-fetoprotein and AST were the key predictors of the relative expression levels of lncRNA-PVT1 among other laboratory biomarkers in the HCC subgroup, **(P <0.001, Table 6).**

Table (6): Linear regression analyses to test the influence of the main independent variables against the relative expression level of lncRNAPVT1 level (dependent variable) in the hepatocellular carcinoma subgroup.

Model	Unstandardized Coefficients		Standardized Coefficients	T	P-value	95% C.I.	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Constant	6.885	1.546		4.452	<0.001*	3.777	9.993
Alpha-fetoprotein	-0.033	0.012	-0.131	-2.793	<0.001*	-0.057	-0.009
GGT	0.011	0.006	0.075	1.817	0.075	-0.001	0.023
AST	0.101	0.036	0.229	2.755	<0.001*	0.028	0.173
ALT	0.039	0.009	-0.034	-0.215	0.830	-0.398	0.321

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase.

The accuracy of circulating relative expression levels of lncRNA-PVT1 for diagnosis of NAFLD and NASH

The power of the relative expression levels of lncRNA-PVT1 to diagnose NAFLD among considered subjects was evaluated using ROC analysis. The cutoff value was 0.94 and the AUC was 0.789 (95% CI = 0.719–0.859), P<0.001 with sensitivity and specificity 75% and 85% **(Fig. 2a).**

LncRNA-PVT1 could discriminate NASH from simple steatosis with the cutoff value 1.2, AUC 0.649 (95% CI = 0.540–0.758), P<0.001 with sensitivity and specificity 66% and 55% **(Fig. 2b).**

The accuracy of circulating relative expression levels of lncRNA-PVT1 for diagnosis of cirrhosis and HCC among NAFLD patients

LncRNA-PVT1 levels can discriminate cirrhosis from NASH with the cutoff value 1.8, AUC 0.740 (95% CI= 0.701- 0.839), P<0.001 with sensitivity and specificity 79% and 64% **(Fig. 2c)** and also differentiated HCC from cirrhosis with the cutoff value 2.98, AUC 0.816 (95% CI= 0.733- 0.899), P<0.001 with sensitivity and specificity 91.5% and 50% **(Fig. 2d).**

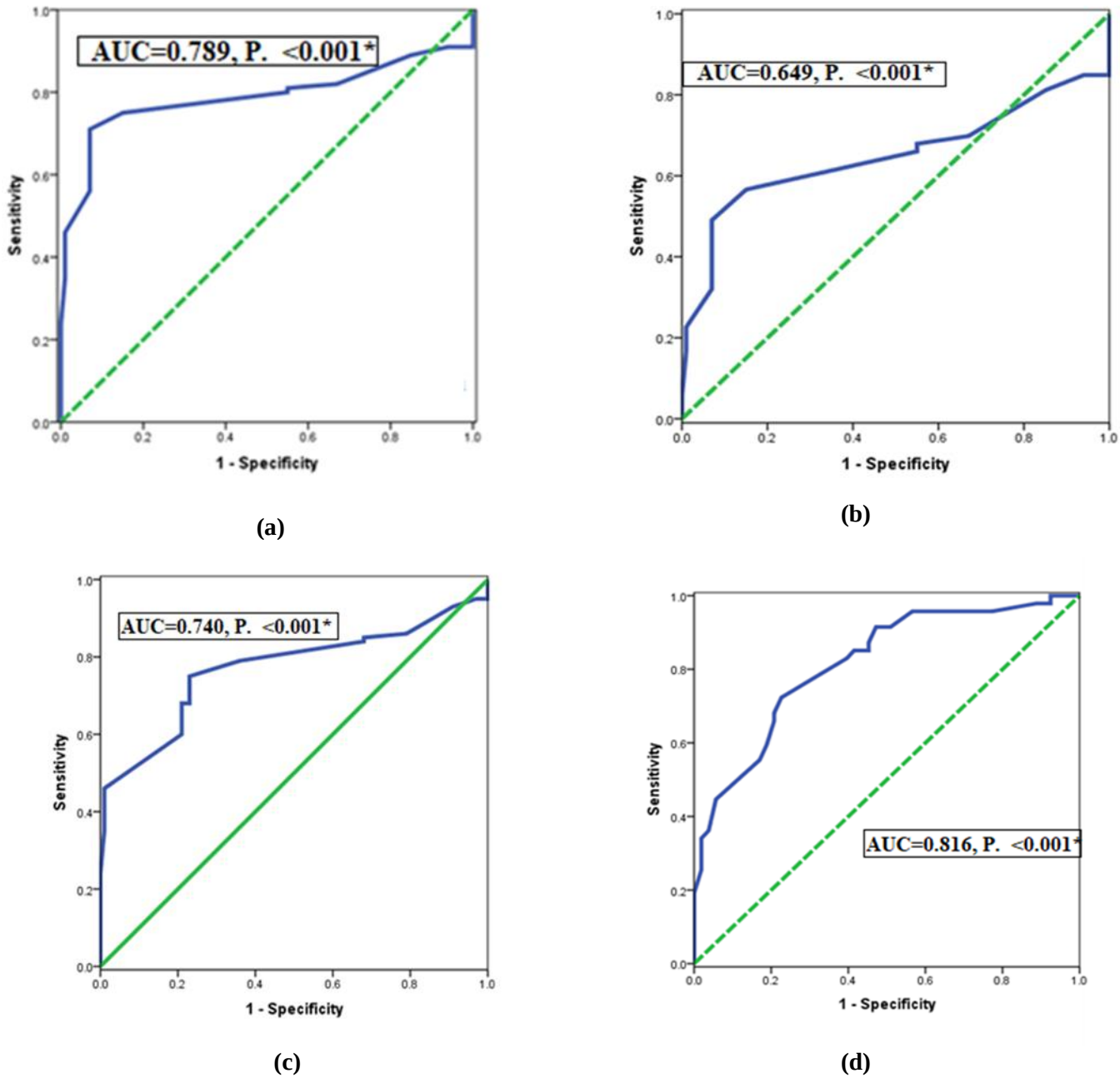

Figure (2): Receiver operating characteristic curve of the relative expression levels of lncRNA-PVT1 for differentiating different NAFLD stages: (a) for diagnosis of non-alcoholic fatty liver disease, **(b)** for differentiating steatohepatitis from simple steatosis, **(c)** for differentiating cirrhosis from steatohepatitis, **(d)** for differentiating hepatocellular carcinoma from cirrhosis

DISCUSSION

In developed countries, hepatic steatosis is the most frequent liver disease, with a frequency of 15% to 30% [17, 18]. To date, the molecular mechanisms for the pathogenesis of NAFLD and its complications are still a mystery. Research that sheds light on the functional roles of lncRNAs in developing chronic liver disease and HCC can help understand these molecular mechanisms and develop novel diagnostic biomarkers and therapeutic targets for these patients.

In the current study, we detected statistically significant higher values of the relative expression levels of lncRNA-PVT1 in NAFLD compared to control. Additionally, significantly higher values were found in the HCC group than in cirrhosis, NASH, and simple steatosis subgroups with P<0.001.

The link of long non-coding RNAs to NAFLD and other metabolic disorders (such as obesity, insulin resistance, and type 2 diabetes) remains relatively unexplored. The role of lncRNAs in NAFLD steatosis and associated fibrosis is still being studied [19].

Similar to our findings, **Zhang *et al.*** [20] analyzed the clinical diagnostic value of abnormal levels of PVT1 and miR-20a-5p and found that both can significantly differentiate healthy individuals from NFALD patients, which may be a new biomarker for the diagnosis of NAFLD. They found that PVT1 was upregulated in NAFLD patients and mice, while miR-20a-5p was

reduced. Accordingly, PVT1 and miR-20a-5p showed high clinical diagnostic values for NAFLD.

In comparison to normal controls, a study of NAFLD mice produced by high-fat diets found that 111 lncRNAs had increased expression and 180 lncRNAs had decreased expression. The discovery of these dysregulated lncRNAs in hepatic lipid regulation might imply that their expression could be used for the diagnosis of NAFLD in its early stages [21].

Non-alcoholic steatohepatitis (NASH) represents a progressive subtype of NAFLD and is a complex and multifactorial disease modulated by several factors, including genetic, environmental, metabolic mechanisms, and gut microbial factors. 15–25% of NASH patients develop liver-related cirrhosis and HCC [22]. In accordance with our results, many studies examined the role of other LncRNAs in NAFLD and NASH, liver-specific ablation of Blnc1 secured the Blnc1 conditional knockout mice from high fat diet-induced insulin resistance and hepatic steatosis, as well as ameliorating diet-induced NASH pathogenesis in mice. This shows that Blnc1 might be a therapeutic target for the treatment of NAFLD and NASH [23].

Another study identified 89 differentially expressed lncRNAs in a NASH minipig model[24]. MALAT1 was found to be a robust molecular driver in the pathogenesis of NASH[25]. **Atanasovska *et al.*[26]** observed that the higher expression levels of lncRNAs in NASH patients were directly correlated with NASH grade and NAFLD score.

In the current study, we found higher values of PVT1 in the cirrhosis group in comparison to NASH, and simple steatosis subgroups, previous several studies have demonstrated that lncRNAs levels were increased in conjunction with inflammation and fibrosis through many pathways, including those involved in TGF-β1 and TNF signaling, ECM deposition, and insulin resistance. Similarly, experimental studies confirmed this theory; they found the levels of lncRNAs in animal models were also significantly expressed in fibrosis-related NAFLD, including NEAT1, MALAT1, and PVT1 [27].

In fibrotic liver tissue and activated hepatic stellate cells (HSC), PVT1 expression was upregulated [28]. In addition, PVT1 can act as a ceRNA for miR-152. It competitively binds to miR-152 and inhibits PTCH1 expression through methylation, which activates the hedgehog pathway and promotes EMT and HSC activation in liver cirrhosis [29].

Many studies [29-32] demonstrated that Long noncoding RNA PVT1 was increased in HCC tissues, promoting hepatocellular carcinoma pathogenesis, progression, and inhibition of apoptotic cells. Additionally, the upregulation of PVT1 could promote HCC cell proliferation in vitro and Vivo. PVT1 gained moderate value in discriminating HCC patients from normal controls [30].

Despite the limited number of HCC cases in the current study, we attempted to pierce out the association of lncRNA-PVT1 relative expression levels with other studied variables among HCC patients. As a result, a significant relationship between increased expression levels of lncRNA-PVT1 and male sex & Child's class C was found in the HCC subgroup, however, no relation was present to tumor size, number, stage, or portal vein invasion. Conflicting results were obtained in different studies for HCC patients, in **Ding *et al.* [33],** no significant correlations were identified between PVT1 expression and clinicopathological variables such as age, gender, tumor number, tumor size, PVT, histopathological grade, or TNM stage. However, other studies [34,35] reported a positive association with male gender, race, vascular invasion, pathological grade in HCC, tumor size, Barcelona Clinic Liver Cancer (BCLC) stage, and serum bilirubin.

We detected significant direct correlations between the relative expression levels of lncRNA-PVT1 and TG, LDL, PT, total bilirubin, direct bilirubin, AST, ALT, GGT, alpha-fetoprotein, and serum creatinine. For further evaluation, we analyzed our results using linear regression analysis and we observed that alpha-fetoprotein and AST were the main predictors of lncRNA-PVT1 relative expression among other studied variables in patients with HCC. A similar observation was found in the study by **Ding *et al.* [33],** where overexpression of PVT1 was associated with a higher serum α-fetoprotein expression level (P=0.011).

Even more importantly, in this study, we attempted piercing out the cutoff value of the relative expression levels of lncRNA-PVT1 as diagnostic biomarkers of NAFLD by the ROC curve analysis, the sensitivity was 75% and the specificity was 85% for the cutoff value of 0.94. Considering NASH, the sensitivity was 66 % and the specificity was 55 % for a cutoff value of 1.2; while with a sensitivity of 79% and specificity of 64%, a cutoff value of 1.8 could differentiate cirrhosis from NASH. Interestingly, the most reliable sensitivity power was 91.5 % for a cutoff value of 2.98 in differentiating HCC from cirrhosis.

Limitation of the study:

Some limitations should be considered, including the small sample size of the study in particular the HCC subgroup of NAFLD patients as this study was conducted on the adult age group to avoid false epigenetic results which could be influenced in elderly subjects thus; further studies with larger sample size including elderly patients should be performed in the future to validate our results.

CONCLUSION

In conclusion, lncRNA-PVT1 relative expression levels are overexpressed in patients with NAFLD. Furthermore, a significantly higher level of lncRNA-PVT1 was found in progressive stages of NAFLD being more elevated in patients complicated with cirrhosis and HCC. Consequently, lncRNA-PVT1 levels could be a useful diagnostic biomarker for discriminating patients with advanced NAFLD stages. However, larger-scale

studies on patients complicated with HCC need to be further explored.

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