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Latent Transforming Growth Factor-beta Binding Protein-1 as a Diagnostic Biomarker for the Detection of Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the most common liver cancer and a leading cause of cancer-related death worldwide. The current study aims to evaluate the diagnostic role of latent transforming growth factor-beta binding protein-1 (LTBP-1) as a biomarker to distinguish hepatocellular carcinoma (HCC) from patients with liver cirrhosis.

Methods: The study carried out on 90 individuals classified to healthy individuals (*n*=20), liver cirrhosis *(n=30)*, and HCC (*n*=40). The serum level of LTBP-1 was measured by enzyme-linked immunosorbent assay (ELISA). Receiver operating characteristics (ROC) curves and area under the curve (AUC) were calculated.

Results: The level of LTBP-1 was significantly higher in HCC patients than healthy and patients with cirrhosis. Furthermore, there was a significant ($p<0.001$) association between the level of LTBP-1 and CLIP and BCLC in HCC patients. Moreover, LTBP-1 levels were significantly ($p=0.01$) associated to child pugh grade in patients with cirrhosis and HCC. ROC curve analyses revealed that LTBP-1 showed a better diagnostic performance (AUC=0.970, Sensitivity: 82.50%, Specificity: 96.67%, PPV: 97.06%, NPV: 80.56%) in distinguishing HCC from cirrhosis patients, compared to AFP (AUC=0.810, Sensitivity: 62.50%, Specificity: 93.33%, PPV: 92.59%, NPV: 65.12%).

Conclusion: These findings suggested that LTBP-1 may be a promising biomarker for distinguish HCC from liver cirrhosis patients.

Keywords: AFP; Biomarker; Cirrhosis; Hepatocellular carcinoma; LTBP-1

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy [1]. Globally, the burden of cancer in 2012 was 14 million cases and is expected to proliferate to 22 million over the next two decades [2]. Cirrhosis, hepatitis C virus, and ingestion of fungal carcinogens as aflatoxin B1 [3-5] were the main etiologic factors of HCC. In Egypt, liver cancer forms nearly 12% of digestive organs malignancies and 2% of total malignancies. The HCC organizes approximately 70% of liver tumors among Egyptians [6].

Alpha-fetoprotein (AFP) is a glycoprotein produced by the fetal liver. Its levels markedly declined at birth, but are raised in patients with HCC, cirrhosis, chronic hepatitis, liver necrosis, or gonadal tumors [7]. AFP is the most widely used tumor marker for the detection of HCC [5,8], however, there is a limitation in the usage of AFP due to its poor diagnostic performance [9]. Therefore, new and more specific markers are necessary required for early detection of HCC [10,11].

The latent transforming growth factor-beta binding protein 1 (LTBP-1) is a secreted protein and considers as a part of the extracellular matrix (ECM). LTBP-1 targets transforming growth factor-beta 1 (TGF-β1) and localizes it to ECM by interacting with integrin and fibronectin [12-14]. It has been reported that in human malignant gliomas, the expression of LTBP-1 was gradually increased [15]. Also, previous studies showed that the immunohistochemistry staining of LTBP-1 was extremely strong in the tumor stroma of malignant mesothelioma [16], pancreatic ductal adenocarcinoma [17] and ovarian carcinoma [18]. To date, only one publication is known about the serum level of LTBP-1 in cancer patients. Therefore, the current study aimed to measure the serum levels of LTBP-1 and AFP to investigate whether LTBP-1 could improve the diagnostic performance for HCC, along with AFP in Egyptian patients.

Materials and Methods

Ethics statement and patient's groups

The current study was conducted as a cross-sectional and casecontrol based study in National Liver Institute, Menoufia University, Egypt. It was approved by the Institutional Review Board National Liver Institute (IRB number 00003413). The participants provided written informed consent to participate in this study. The IRB approved this consent procedure. The current study included 90 individuals; 40 HCC patients (11 female and 29 males with the mean age of 53 years), 30 patients with cirrhosis (7 female and 23 males with the mean age of 54 years), and 20 healthy volunteers as a control group (1 female and 19 males with the mean age of 49 years).

Specimens

Peripheral venous blood samples will be collected under complete aseptic condition after overnight fasting. The blood sample of each individual was added in serum-coagulated tubes. Blood samples were centrifuged at 4°C for 10 min at 1000 g to separate the serum and blood cells. The supernatants were collected and divided. All samples were aliquoted, frozen and stored at (-80°C) till the time of examination.

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Laboratory diagnosis

The serum level of AST, ALT, Albumin, Bilirubin (direct and total), Alkaline phosphatase (ALK), Gamma glutamyl transpeptidase (GGT), Urea, and Creatinine were measured using Intrgra-400 (Roche-Germany). Fibrintimer machine (Roche - Germany) was used to detect prothrombin concentration. HBV antigen and HCV antibodies were determined by enzyme immunoassay (EIA) (COBAS-core - Germany). The serum level of AFP was measured by automated Elecyes (Roche - Germany).

Assessment of human LTBP-1 concentration by ELISA method

The concentrations of LTBP-1 in the serum samples were measured using an ELISA kit (Zhejiang Kono Biotech co., Ltd, Zhejiang, China) according to the manufacturer's instructions. Briefly, microtiter plate was firstly coated with human LTBP-1 antibody followed by serum sample (100 µl) was added to each well then, the conjugate (50 µl) was added to each well and mixed well. The plate was incubated at 37°C for 1 h, after incubation the plate was washed, and excess serum sample was removed. The absorbance of the developed color was immediately measured at 450 nm using a microplate reader. The concentrations of LTBP-1 in samples were determined using standard curve.

Statistical analysis

The statistical analysis was performed by SPSS software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD or count and percentage. Differences of continuous variables between groups (HCC versus cirrhosis, HCC versus healthy and healthy versus cirrhosis) were assessed by t-student test, analysis of variance or Mann-Whitney U test as appropriate, whereas categorical variables were compared by $\chi 2$ tests. A two-sided $P<0.05$ was considered statistically significant. The univariate analysis was performed between the HCC and non-cancer group including AFP and LTBP-1. Significant variables ($P<0.05$) from the univariate analysis were accessed into multivariate analysis by a forward logistic regression to identify independent risk factors, and independent risk factors were used to construct logistic model. The predicted probability of predicting HCC was used to construct ROC curve. The diagnostic efficacy of each panel was assessed by AUC. The optimal cut-off values for diagnosis were selected using Youden's index, which were maximal values at the sum of the sensitivity and specificity. The best panel for HCC diagnosis constructed from the training set was applied into validation set. Likewise, AUC was used to test the diagnostic efficiency.

Clinicopathological parameters of the study cohorts

The clinicopathological parameters of healthy, patients with cirrhosis and HCC groups were detected and presented in Table 1.

Levels of AFP and LTBP-1 in patients with liver cirrhosis and HCC

Figure 1 revealed that patients with liver cirrhosis had elevated levels of serum LTBP-1 and the main value was 16.4 ng/ml compared to 12.1 ng/ml in healthy control. However, patients with HCC had the maximum elevated levels of serum LTBP-1 with value 30.4 ng/ml. The level of serum LTBP-1 in patients with HCC was significantly ($P<0.001$) higher than those of healthy controls and liver cirrhosis.

Concerning AFP, patients with liver cirrhosis had elevated levels of serum AFP (15.2 ng/ml) compared to healthy control (5.6 ng/ml). Besides, patients with HCC had the maximum elevated levels of serum AFP equals 8108.2 ng/ml. The statistical difference of serum AFP between liver cirrhosis and healthy controls was ($P=0.019$), whereas the statistical difference ($P=0.029$) was observed between liver cirrhosis and HCC groups (Figure 1).

Correlation between the protein level of LTBP-1 and the studied clinicopathological parameters in patients with cirrhosis and HCC

The correlation between different studied parameters and the serum protein level of LTBP-1 in cirrhosis and HCC were illustrated in the Table 2. In HCC patients, there were positive correlations among LTBP-1 and AST (**$r=0.361$, $p=0.022$**), total bilirubin ($r=0.493$, $p=0.001$), direct bilirubin ($r=0.509$, **$p=0.001$**), urea (**$r=0.399$, $p=0.011$**), and INR ($r=0.401$, $p=0.010$). In contrast, there were negative correlations with albumin (**$r=-0.488$, $p=0.001$**), prothrombin concentration ($r=-0.447$, $p=0.004$). however, there is no significant correlation between LTBP-1 and these parameters, in patients with cirrhosis (Table 2).

ROC curve analysis for AFP and LTBP-1 in discriminating HCC from patients with liver cirrhosis

The diagnostic performance of LTBP-1 in distinguishing HCC

Variable	Groups			*P*- value
	Healthy (N=20)	Cirrhosis (N=30)	HCC (N=40)	
Sex (M/F)	19/1	23/7	29/11	-
Anti-HCV/HBsAg	0/0	30/0	40/0	-
Age (Years)	51 ± 4	54 ± 6	53 ± 5	$P_1<0.001$
				$P_2=0.03$
				$P_3=0.47$
ALT (U/L)	16.4 ± 4.5	42.2 ± 25.3	50.6 ± 28.7	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.2$
AST (U/L)	15.4 ± 3.6	76.1 ± 47.3	89.8 ± 65.2	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.31$
Albumin (g/dl)	4.4 ± 0.4	2.7 ± 0.6	2.7 ± 0.7	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.02$
GGT (U/L)	18.9 ± 5.8	67.3 ± 72.2	112.9 ± 110	$P_1=0.001$
				$P_2<0.001$
				$P_3=0.041$
ALK P (U/L)	60.2 ± 9.1	130.2 ± 71.0	194.3 ± 147.8	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.02$
Bilirubin T (mg/dl)	0.55 ± 0.17	4.1 ± 5.89	4.33 ± 5.76	$P_1=0.003$
				$P_2<0.001$
				$P_3=0.041$
Bilirubin D (mg/dl)	0.12 ± 0.04	2.74 ± 5.03	2.76 ± 4.23	$P_1=0.008$
				$P_1<0.001$
				$P_3=0.984$
PT	98.9 ± 2.4	51.3 ± 10.1	55.5 ± 14.3	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.156$
INR	1.01 ± 0.02	1.56 ± 0.26	1.53 ± 0.33	$P_1<0.001$
				$P_2<0.001$
Urea (mg/dl)	26 ± 7	75 ± 57	95 ± 65	$P_1<0.001$
				$P_2<0.001$
				$P_3=0.187$
Creatinine (mg/dl)	0.73 ± 0.13	1.15 ± 0.78	1.65 ± 1.15	$P_1=0.007$
				$P_2<0.001$
				$P_3=0.034$

Table 1: Clinicopathological parameters of the study cohorts.

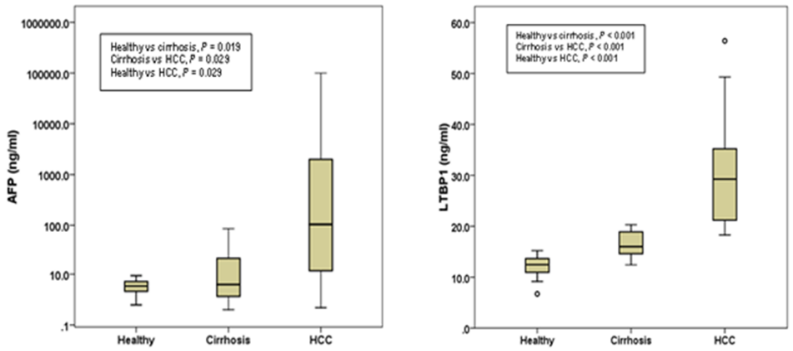

Figure 1: Box plots show the distribution of serum concentrations Levels of AFP and LTBP-1 in healthy and patients with liver cirrhosis and HCC.

Variable	LTBP-1			
	Cirrhosis (N=30)		HCC (N=40)	
	r	*P* value	*r*	*P* value
Age (Year)	-0.083	0.662	-0.264	0.100
AST (U/L)	0.306	0.100	0.361*	0.022
ALT (U/L)	0.131	0.491	0.104	0.522
ALP (U/L)	0.217	0.250	0.205	0.203
GGT (U/L)	0.225	0.232	-0.031	0.850
Total Bilirubin (mg/dl)	0.098	0.605	0.493**	0.001
Direct Bilirubin (mg/dl)	0.011	0.952	0.509**	0.001
Albumin (g/dl)	-0.328	0.077	-0.488**	0.001
Urea (mg/dl)	-0.114	0.547	0.399*	0.011
Creatinine (mg/dl)	-0.078	0.684	0.208	0.198
Prothrombin Conc.	-0.127	0.505	-0.447**	0.004
INR	0.127	0.503	0.401*	0.010

Note: *r*: Pearson Correlation, *P*<0.05 is significant correlation, Positive *r* value is positive correlation (direct), Negative *r* value is negative correlation (inverse), **. Correlation is significant at the 0.01 level, *. Correlation is significant at the 0.05 level.

Table 2: Correlation between LTBP-1 and different clinopatholigical parameters in cirrhosis and HCC groups.

(*n*=40) from patients with liver cirrhosis (*n*=30) was evaluated by determination of Receiver operating characteristics (ROC) curve. As shown in Figure 2, ROC curve analysis for LTBP-1 revealed that at cut-off value 20.2 µg/ml, LTBP-1 exhibited area under curve (AUC=0.97) and the values of sensitivity, specificity, PPV, NPV, and accuracy were (82.5%, 96.67%, 97.06%, 80.56%, and 88.57%, respectively). On the contrary, AFP at cut-off value 42.8 µg/ml, the AUC was 0.81 and the values of 62.5%, 93.33%, 92.59, 65.12, and 75.71 were noticed for sensitivity, specificity, PPV, NPV, and accuracy, respectively as shown in Figure 2.

The association between AFP and LTBP-1 Levels and CLIP and BCLC scores in HCC patients

As shown in Table 3, the level of LTBP-1 in HCC patients was significantly (p<0.001) associated with CLIP score. Because, the LTBP-1 was gradually increased with CLIP score increasing, where LTBP-1 concentration in score 5 recorded 49.1 against to 19.4 in score 0. Conversely, AFP was not significantly (p=0.098) associated with CLIP score. Concerning to BCLC, Table 4 illustrated that there was a significant (p<0.001) association between the serum level of LTBP-1 and BCLC score in HCC patients. The LTBP-1 level was gradually increased with the progress in BCLC score, where, the level was 46.8 in score 4 against 26.6 in score 0. On the contrary, AFP level was not significantly (p=0.172) associated with BCLC score.

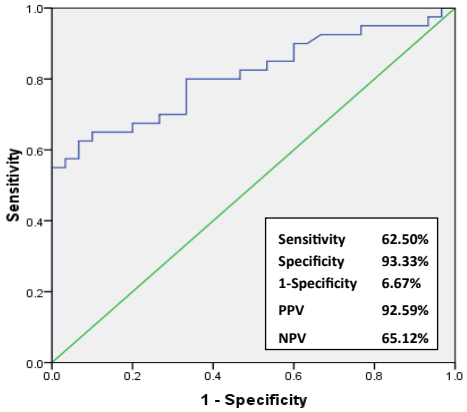

Figure 2: ROC curve analysis of AFP as a predictor of HCC between cirrhosis and HCC groups. At cut-off value 42.8 ng/ml, AFP had an area under the curve (AUC) of 0.810 and the percent of specificity and sensitivity was 93.3 and 62.5, respectively.

CLIP score	N	AFP		LTBP-1	
		Mean ± SD	*P*- value	Mean ± SD	*P*- value
0	7	117.8 ± 106.1	0.098	19.4 ± 0.7	<0.001
1	8	593.5 ± 1004.3		26.0 ± 8.3	
2	5	1337.6 ± 2325.2		26.5 ± 2.4	
3	11	15791.6 ± 32407		31.9 ± 3.6	
4	5	949.4 ± 1218.7		38.2 ± 6.8	
5	4	33403.0 ± 39891.9		49.1 ± 5.7	

Table 3: Serum levels of AFP and TBP-1 in association to CLIP score in HCC patients.

BCLC score	N	AFP		LTBP-1	
		Mean ± SD	*P*- value	Mean ± SD	*P*- value
0	4	599.3 ± 1051	0.172	26.6 ± 11.1	<0.001
1	14	687.7 ± 1499.2		22.5 ± 4.5	
2	7	10591 ± 20256		33.1 ± 6	
3	11	21478.8 ± 37639.9		34.0 ± 6.5	
4	4	474.9 ± 909.8		46.8 ± 9	

Table 4: Serum levels of AFP and TBP-1 in relationship to BCLC score in HCC patients.

The relationship between the levels of AFP and LTBP-1 and child pugh grade in patients with cirrhosis and HCC

The relationship between serum levels of AFP and LTBP-1 and child pugh grade in patients with cirrhosis and HCC was demonstrated in Table 5. Results revealed that there was a significant (p=0.01)

Child pugh grade	N	AFP		LTBP-1	
		Mean ± SD	*P*- value	Mean ± SD	*P*- value
A	16	393.9 ± 753	0.098	18.4 ± 5.1	0.01
B	30	9810.2 ± 25830		24.6 ± 9.6	
C	24	1007.3 ± 3378.4		28.1 ± 11.9	

Table 5: Serum levels of AFP and LTBP-1 in connection to child pugh grade in patients with cirrhosis and HCC.

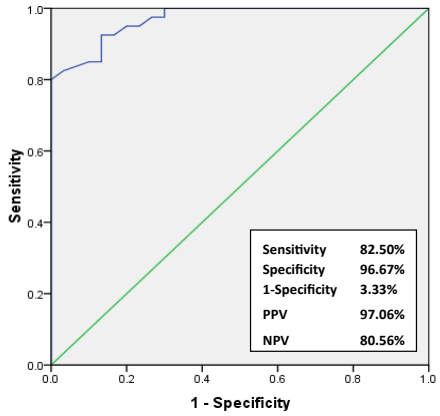

Figure 3: ROC curve analysis of LTBP-1 as a predictor of HCC between cirrhosis and HCC groups. At cut-off value 20.2 ng/ml, LTBP-1 had an area under the curve (AUC) of 0.970 and the percent of specificity and sensitivity was 96.7% and of 82.5%, respectively.

association between LTBP-1 and child pugh grade where, the maximum level (28.1) was observed in child pugh grade C compared to (18.4) in grade A. However, AFP shown not significant (p=0.098) association with child pugh grade in patients with cirrhosis and HCC.

Discussion

Transforming growth factor-beta (TGF-β1) functions as a growth inhibitor in normal cells, however, it promotes tumor progression in tumor cells by enhancing the survival, migration, and invasion [19]. LTBPs are a family of extracellular matrix (ECM). There are four isoforms of LTBPs which are LTBP1, 2, 3, and 4 [20]. The roles of LTBPs included its covalent binding with TGF-β1 which is involved in assembly, secretion, and TGF-β1 activities. Also, it promotes TGF-β1 through its binding with fibrillin microfibrils in the ECM protein to promote TGF-β1 storage [21]. Moreover, LTBPs contribute in the regulation of cell adhesion [22]. LTBP-1 is a secreted protein and may be involved in epithelial-mesenchymal cell transformation (EMT) of embryonic heart, suggesting that LTBP-1 likely contributes to malignant transformation of cells [23].

Worldwide, HCC is a dangerous problem and common spread cancer among men and women [24,25]. Eighty percent of HCC patients were HCV positive [26] and cirrhosis was the major clinical risk factor for HCC development and 90% of patients developed HCC on top of cirrhosis [27]. The high prevalence rate of HCV in Egypt encourages the authors to design the current study which aimed to investigate the role of serum LTBP-1 levels in HCC Egyptian patients to verify the possibility of using serum LTBP-1 levels as a potential biomarker in diagnosis of HCC.

The clinicopathological parameters of all individual involved in the study were presented in Table 1 which clarified that a significant increase in liver enzymes and bilirubin were detected in HCC group more than that in cirrhosis group compared to the control group. This result was compatible with the previously stated that liver function tests were significantly elevated in HCC patients compared to chronic liver disease [28]. Also, in the present study, there is no significant difference of these parameters between cirrhosis and HCC groups. This finding was completely in agreement with the previously reported that hepatic functions do not distinguish between HCC from cirrhosis [29]. Moreover, it was noticed that prothrombin concentration was significantly declined in cirrhosis and HCC groups compared to control group and this may be related to the less production of coagulation factors by sickly liver. Additionally, there was weak of potassium utilization in parenchymal liver disease [30].

Due to the deficiency of effective biomarkers, there is a great challenge to screen HCC in liver cirrhosis and HCC patients. Although, the elevated AFP level was a risk factor for HCC development [31,32], it was inappropriate for HCC screening because of its poor diagnostic sensitivity (39%-65%) [5]. Results in Figure 3 revealed that HCC group had the maximum elevated levels of serum AFP followed by cirrhosis and then healthy group. These findings were in agreement with the previous studies that proven the high level of AFP in cirrhosis and HCC [33,34]. Also, the concentration of LTBP-1 in patients with HCC was gradually increased from healthy individuals to cirrhosis patients and has the highest levels in HCC patients (Figure 1). This result was compatible with the previously reported study [35]. The levels of AFP had poor statistic difference between the HCC and cirrhosis group (P=0.029), and consequently might not exactly differentiate HCC from the cirrhosis patients. In contrast, the serum levels of LTBP-1 were significantly elevated in the HCC group than that in the cirrhosis group (P<0.001) (Figure 1).

Results in Table 3 presented a significant (p<0.001) association between the level of LTBP-1 in HCC patients and CLIP score, while, AFP level was not significantly (p=0.098) associated. Additionally, there was a significant (p<0.001) association between LTBP-1 level and BCLC score as the LTBP-1 level was gradually increased with the progress in BCLC score, whereas, AFP level was not significantly (p=0.172) associated (Table 4). These findings were in consistent with that mentioned LTBP-1 levels were increased along with tumor size and the diagnostic performance of LTBP-1 was better than AFP for HCC less than 2 cm [35]. Also, similar results were proven that there was a positive correlation between TGF-β1 concentration and tumor size [36].

As revealed in Table 5, the serum levels of LTBP-1 in patients with cirrhosis and HCC were associated with more advanced child pugh grade. Conversely, AFP levels shown non-significant (p=0.098) association with child pugh grade. These findings are agreed with that reported plasma level of TGF-β1 was elevated in patients with a higher Child score [37].

Concerning to ROC analysis of AFP, it had been reported that at cut-off of ≥ 20 mcg/L AFP exhibited sensitivity range of 25%-65% and specificity to be 80%-94% [38]. These results were found to be compatible with our results which proven that at cut-off value 42.8 µg/ml, AFP showed AUC of 0.81 and the recorded values of sensitivity and specificity were 62.5% and 93.33%, respectively as shown in Figure 3. Conversely, ROC curve analysis of LTBP-1 indicated that at the best cut-off value 20.2 µg/ml, AUC was 0.97 and sensitivity and specificity were found to be 82.5% and 96.67%, respectively. These results were in consistent with the previously reported that LTBP-1 showed better diagnostic result than AFP among Chinese patients with liver cirrhosis and HCC [35].

Conclusion

The serum levels of LTBP-1 exhibited gradually increased trend in healthy individuals, liver cirrhosis and HCC patients. Serum LTBP-1 might be a potential serum marker to discriminate HCC from liver cirrhosis patients due to its high sensitivity and specificity, compared to AFP. LTBP-1 was significantly associated with CLIP, BCLC, and child pugh grade. LTBP-1 might be a promising diagnostic biomarker for HCC although; we recommended that future studies on large number of patients are required to validate these results.

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