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Original article

Transthyretin as a Novel Biomarker for Diagnosis of Hepatocellular Carcinoma in Cirrhotic patients

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ABSTRACT

Background: Hepatocellular carcinoma [HCC] is one of the leading causes of cancer-related deaths worldwide. A major problem with HCC surveillance is the lack of reliable biomarkers. Serum transthyretin [TTR] may be a sensitive marker for the diagnosis of patients with liver cell damage, liver cirrhosis or hepatocellular carcinoma.

Aim of the work: This study aimed to evaluate the potentiality of serum transthyretin [TTR] as a novel biomarker for detection of HCC in cirrhotic patients.

Patients and Methods: This Current study was conducted on 70 patients with chronic liver disease. Also, 20 healthy person matched for age and sex were included as a control group. Patients were classified into 2 groups [30 cirrhotic patients with newly diagnosed HCC & 40 cirrhotic patients without HCC]. Serum TTR levels were measured using enzyme linked immunosorbent assay technique.

Results: Serum levels of TTR were significantly much lower in HCC patients when compared to cirrhotic patients without HCC or control group [p<0.0001]. Significant decrease of serum TTR in HCC patients, with portal vein invasion or nodal invasion than in HCC without vascular or nodal invasion. The diagnostic accuracy of TTR was higher than that of AFP regarding sensitivity [83.3%] and negative predictive value [81.4%] in diagnosis of HCC.

Conclusion: detection of lower level of TTR alone or in combination with other validated markers may be potentially informative biomarker for detection of HCC among cirrhotic patients at early noninvasive stage where curative treatment can be applied.

Keywords: Transthyretin; Cirrhosis; Hepatocellular Carcinoma; Alpha-fetoprotein; Noninvasive.

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* Main subject and any subcategories have been classified according to researchers' main field of study.

INTRODUCTION

HCC is a public health problem. It is the second most common cause of cancer-related mortality**.** Liver cirrhosis is the risk factor in the development of HCC. It has been estimated that HCC incidence rate to be 2%-4% per year[1-3]. Early detection of HCC by using surveillance tools have increased quality of life & patient survival by providing effective treatments[4]**.** AFP is the most widely used common biomarker for the diagnosis and screening for HCC but it has a suboptimal performance as a serological test for surveillance **[5]**. It was demonstrated that serum AFP had a low sensitivity of 41–65% and specificity of 80–94% [6]

Serum TTR [prealbumin] is a small globular non-glycosylated tryptophan-rich protein of a homo-tetrameric structure, composed of four identical subunits with two thyroxine-binding sites per tetramer**[7]**. TTR is a serum protein with multiple functional properties responsible for the transport of thyroxin and retinol binding protein complex to the various parts of the body, TTR has been associated with many other biological functions that are directly or indirectly associated with the oxidative stress and many human diseases **[8].** TTR was originally named pre-albumin. TTR is synthesized mainly in the liver and less likely caused by hepatic disease compared to other serum proteins**.** TTR had a half-life of 2 - 3 days that can reflect liver functional reserve. Liver damage and inflammatory stimulation alter pre-albumin levels **[9-11].** TTR is considered as a sensitive marker for the diagnosis of patients with liver cell damage, liver cirrhosis or hepatocellular carcinoma, reflecting the impaired liver synthetic function **[12].** **Katare et al.**[13] was the first to report that development of HCC is associated with increased level of serum TTR, where serum TTR level showed 4 times increase in HCC patients than in control. Therefore, detection of increased level of TTR alone or in combination with other validated markers may be potentially informative biomarker for detection of HCC at early noninvasive stage.

AIM OF THE STUDY

In this present study we aimed to evaluate the role of serum TTR as a novel biomarker for detection of HCC among cirrhotic patients as they are the highest risk group for HCC development.

PATIENTS AND METHODS

This study was conducted on 70 Egyptian patients diagnosed as having chronic liver disease. They were 47 males and 23 females; their age ranged from 42 to 75 years. Patients were selected from those attending Hepatogastroenterology and Infectious Disease Department, Al-Azhar University Hospital, Damietta, from August 2018 to March 2019. Also, 20 healthy normal volunteers matched for age and sex, were included as normal control group. They were 9 males and 11 females; their age ranged from 20 to 55 years. Patients with other malignances, amyloidosis, neurological disorders [e.g. schizophrenia], malnutrition, or co-morbidities [e.g. renal – cardiac – respiratory] were excluded from the study, as these conditions are known to affect serum TTR level. Patients were classified into 2 groups: Group I [HCC group]: included 30 patients with newly diagnosed HCC according to radiological criteria of triphasic abdominal CT scan and/or diagnostic AFP level. Group II [cirrhosis group]: included 40 patients diagnosed as having liver cirrhosis without HCC according to history, clinical examination, laboratory and imaging findings. This group was subdivided into 2 subgroups; Group IIa: included 20 patients with decompensated liver cirrhosis. Group IIb: included 20 patients with compensated liver cirrhosis. All participants in the study were undergone to Full history taking and clinical examination.

Sample preparation: 6ml of peripheral blood were collected from each patient as well as healthy control. Each sample was divided into two parts, the first part was collected into dry tube to prepare serum and the second part was collected into tube contain anticoagulant for hematological study. Sera were stored at -20 until used. For each collected sample; complete blood picture, liver function tests, inflammatory markers {Erythrocyte Sedimentation Rate [ESR] and C-reactive protein [CRP]}. Total Thyroxin hormone [T4] concentrations were detected in serum using ELFA technique [Enzyme Linked Florescent Assay] by the kit VIDAS T4 which is an automated quantitative test [06762 M-en-2015/01]. Hepatitis C virus antibody [HCV-Ab] and hepatitis B surface antigen [HBs-Ag] were detected using enzyme linked immunosorbent assay [ELISA].

Serum Alpha-Fetoprotein measurement: Alpha fetoprotein [AFP] level was determined using

chemiluminescence quantitative immunoassay kits and the IMMULITE automated quantitative immuno-assay analyzer [Siemens Healthcare Diagnostic Products Ltd, Munich, Germany].

TTR levels measurement: TTR level was determined using Human Transthyretin enzyme linked immunosorbent assay [ELISA] kits, Cat No EOOOOHu, standard curve range 20-2500 µg /ml, sensitivity of 14.89 µg /ml [Bioassay Technology Laboratory, Korean Biotech CO., LTD, China]. According to the manufacturer's directions, samples were allowed to clot for 10-20 minutes at room temperature and then Centrifuge at [3000 – 4000] RPM for 20 minutes. The sera were collected and transferred into plain polypropylene tubes and stored at -20 ºC until further processing. All reagents were brought to room temperature before use. Standard were reconstituted the 120µL of the standard [2560 µg /ml] with 120 µL of standard diluent to generate a 1280 µg /ml standard stock solution. Allow the standard to sit for 15 minutes with gentle agitation prior to making dilutions. Duplicate standard Points by serially diluting the standard stock solution [1280 µg/ml] 1:2 with standard diluent to produce 640 µg/ml, 320 µg /ml ,160 µg /ml and 80 µg /ml solutions Standard diluent serves as the zero standard [0 µg/ml].

Imaging part of the work included

Abdominal US: Abdominal ultrasonography was done for all enrolled subjects using ultrasound device Aplio 500 system [Toshiba, Japan].

Triphasic abdominal CT scan: Triphasic abdominal CT scan was done only for all cases of HCC using TOSHIBA model AFTEION supper S4 [4CEO9X3741, Japan].

Statistical Methods: Data was collected and analyzed with the program of [IBM**-** SPSS version 22] **[IBM- SPSS Inc., Chicago, IL].** Description of the quantitative variables in the form of Mean and Standard deviation [Mean±SD]. Description of the qualitative variables in the form of frequency and Percentage. Student's t-test: for two independent samples is used to compare quantitative variables. Correlation Coefficient [r] test: to indicate that the extent of that two variables change with one another in a linear fashion. One Way Analysis of Variants [ANOVA] test with Post hoc Tukey test: for comparison between multiple groups with quantitative continuous variables. Chi-square test [x^2 value]: was used to compare a qualitative variable between two or more independent groups. For calculated of cut -off values, the receiver operator curve [ROC] was calculated and the best cut off was that with best sensitivity and specificity. For interpretation of results, Significance level [P] value was expressed as $P < 0.05$ = significant and $P \geq 0.05$ = non-significant.

RESUTLS

Table [1] showed basic characteristics of included subjects. Males represent the higher percentage of cases in the present study. Hepatitis C virus was the underlying cause of liver cirrhosis in 88.57% [62/70] patients. While, hepatitis B virus was the underlying etiology of liver cirrhosis in 11.43% [8/70] patients only. Child A class was found in 27 patients [8 in HCC group, 3 in DLC group and 16 in CLC group], Child B in 20 patients [7 in HCC group, 9 in DLC group and 4 in CLC group], and Child C in 23 [15 in HCC group, 8 in DLC group and none in CLC group]. HCC group included 10 patients [33.3%] with BCLC stage A [early HCC], 4 patients [13.3%] with BCLC stage B [intermediate HCC], only one patient [3.33%] with BCLC stage C [advanced HCC] and 15 patients [50%] in stage D [advanced HCC]. Hematological and biochemical variables, inflammatory and tumor markers showed a significant difference among the studied patients as compared to control group, while there was non-statistically significance difference regarding thyroid hormone [T4] [P=0.054]. Serum alpha-fetoprotein had a significant much higher level in HCC patients [P< 0.001] than in cirrhotic patients or healthy controls. On the other hand, TTR showed a significant much lower level in HCC patients group [P< 0.001] when compared to cirrhotic patients or healthy controls. Also, TTR showed significant decreased level in HCC patients than in compensated cirrhotic patients [P2= 0.001]. While, there was non- statistically significant difference when compared to DLC [P1 =0.059]. In HCC patients, serum TTR level negatively correlated to WBCs counts [r= -0.368, P= 0.042], AST [r = -0.366, P = 0.043] INR [r = -0.398, P= 0.027] and ESR [r = -0.431, P=0.016], while serum TTR level positively correlated to T4 [r=0.542, P=0.002]. In decompensated cirrhotic patients, there was negative correlation between serum TTR and platelets count [r = -0.494, P = 0.031], positive

correlation between TTR and both of ALT [r=0.622, P=0.004] and AST [r=0.548, P=0.015] enzyme activity. In Compensated cirrhotic patients [group IIb], showed serum TTR was positively correlated to WBCs [r=0.452, P=0.046] and negatively correlated to ALT enzyme activity [r= -0.283, P=0.026].

Table [3]: showed significant decrease of serum TTR in HCC patients with portal vein invasion or nodal invasion than in HCC without vascular or nodal invasion.

When using the ROC curve analysis for evaluating the diagnostic performance of serum TTR in HCC, area under the curve [AUC] was 0.726. The best cut off value for serum TTR was 182 ng/ml and this offered a diagnostic sensitivity of 83.3%, a diagnostic specificity of 55%., positive predictive value of 58.1% and negative predictive value of 81.4% as showed in table [4]

When using the ROC curve for evaluating the diagnostic performance of serum AFP in diagnosing HCC, area under the curve [AUC] was 0.833 which was slightly bigger than that of TTR alone [0.726]. The best cut off value for serum AFP was 48 ng/ml and this offered a diagnostic sensitivity of 50%, a diagnostic specificity of 95%., positive predictive value of 88.2% and negative predictive value of 71.6%

When using the ROC curve for evaluating the diagnostic performance of serum combined AFP-TTR in diagnosing HCC, area under the curve [AUC] was 0.856 which is slightly bigger than that for single AFP [0.833] or single TTR [0.726]. The combined parallel approach of AFP-TTR improved the diagnostic sensitivity to 90%, specificity of 92.5%., positive predictive value of 90% and negative predictive value of 92.5% over the single use of serum TTR in HCC patients as listed in table [4].

Table [1]: Basic characteristics and Comparison of laboratory results among the studied groups

Variable		Group I [N=30]	Group IIa [N=20]	Group IIb [N=20]	Control [N=20]		
Age [Mean± SD]year		59.6 ± 6.0	55.6 ±7.3	54.6± 7.4	34.1±9.1		
Gender	Male	20[66.6]%	13 [65]%	14 [70]%	9 [45]%		
	Female	10 [33.4]%	7 [35]%	6 30]%	11[55]%		
Laboratory Variable	Group I [N=30]	Group IIa [N=20]	Group IIb [N=20]	Control [N=20]	Anova test	Post hoc Tukey test	
HB [g/dl]	9.6 ± 1.4	9.9 ± 1.5	10.5 ± 1.8	13.1±1.0	<0.001	P1=0.397;P2=0.046; P3= 0.026	
WBCs countx10^3/CC	7.1 ± 2.0	9.3 ± 2.8	6.2 ± 1.8	6.3 ± 1.1	0.003	P1=0.023*;P2=0.343;P3=0.002*	
Platelet count x 10^3/CC	90 ± 22.3	105 ± 25.9	108±17.3	257±47.9	<0.001	P1 =0.275; P2=0.181; P3<0.001*	
AST [IU/L]	63.5±18.2	44.8 ±11.6	38.8± 9.8	14.8 ±3.9	<0.001	P1=0.029*;P2=0.002*; P3=0.007 *	
ALT [IU/L]	44.6 ± 9.3	33.4 ±10.5	30.7 ± 7.7	13.5±3.8	<0.001	P1 =0.043*;P2=0.002*; P3<0.001*	
Total bilirubin [mg/dL]	3.4 ± 0.9	2.6 ± 0.7	0.99 ± 0.1	0.57±0.12	<0.001	P1 =0.138; P3<0.001*; P3<0.001*	
D.bilirubin [mg/dL]	1.44±0.21	0.85 ± 0.3	0.19± 0.08	0.10±0.02	<0.001	P1 =0.001*; P3<0.001*; P3<0.001*	
Albumin [g/dL]	2.6 ± 0.7	2.5 ± 0.68	3.2 ± 0.54	4.5 ± 0.34	<0.001	P1 =0.585; P2=0.045*; P3<0.001*	
INR	1.4 ± 0.37	1.38 ± 0.4	1.15 ± 0.1	1.0 ± 0.0	<0.001	P1 =0.779; P2=0.003*;P3<0.001*	
Creatinine[mg/dL]	0.95±0.29	1.1 ± 0.35	0.89± 0.27	0.59±0.13	<0.001	P1 =0.071; P2=0.498; P3=0.008*	
CPR	20.1 ± 6.5	21.6 ± 5.3	6.7 ± 1.9	2.05 ± 0.8	<0.001	P1 =0.411; P2<0.001*; P3<0.001*	
ESR	57.4±14.8	69.7 ± 15.2	37.9 ± 13	10.9±2.3	<0.001	P1 =0.062; P2<0.001*; P3<0.001*	
T[µg/dl]	8.5 ± 2.6	9.2 ± 2.2	9.6 ± 1.7	10.1 ± 1.1	0.054	P1 =0.433[P2=0.128; P3<0.001*	
AFP [IU/mL]	54.9 ± 12	28.3 ±7.1	27.5 ± 5.1	2.0 ± 0.7	P<0.001	P1 =0.001*; P2= 0.001*; P3= 0.79	
TTR [µg/ml]	141 ± 25	174 ± 14	207 ± 23	283 ± 85	P<0.001	P1 =0.059; P2=0.001*; P3=0.166	

Variables were expressed as mean±SD;
HCV-Ab: Hepatitis C Virus Antibody;
HBS-Ag: Hepatitis B Surface Antigen;HB: haemoglibin;
WBCs: white blood cells;
AST: aspartate aminotransferase; ALT: alanine aminotransferase;
INR: International Normalized Ratio; CPR: C - reactive protein; ESR:
Erythrocyte sedimentation rate, T4: Thyroxin hormone; AFP: Alpha-fetoprotien;
TTR: Transthyretin.
P1: group I versus group IIa;P2: group I versus group IIb;P3: group I versus control group;
p>0.05 is considered non-significant [NS]; p <0.05 is considered significant*.

Table [2]: Correlations of serum TTR with hematological and biochemical parameters in studied patients

Variable	TTR					
	HCC		Decompensated cirrhotics		Compensated cirrhotics	
	r	P value	r	P value	r	P value
Age	-0.069	0.713	0.046	0.851	0.55	0.12
WBCs count x10^3] /cmm	-0.368	0.042	-0.035	0.885	0.452	0.046
HB [g/dl]	0.19	0.305	-0.037	0.88	-0.362	0.117
Platelets count x10^3] /cmm	-0.265	0.149	-0.494	0.031	0.288	0.218
AST [IU/L]	-0.366	0.043	0.548	0.015	-0.159	0.504
ALT [IU/L]	-0.209	0.260	0.622	0.004	-0.283	0.026
Total bilirubin [mg/dL]	-0.038	0.838	-0.159	0.515	0.07	0.77
Direct bilirubin[mg/dL]	-0.33	0.07	-0.1	0.683	0.352	0.128
Albumin [g/dL]	0.205	0.268	-0.424	0.071	-0.091	0.703
INR	-0.398	0.027	0.14	0.569	0.003	0.989
Creatinine [mg/dL]	-0.114	0.542	0.084	0.734	0.375	0.103
CPR	-0.042	0.821	0.022	0.929	-0.162	0.495
ESR	-0.431	0.016	0.101	0.681	0.018	0.94
T4 [ug/dl]	0.542	0.002	-0.114	0.642	0.141	0.552

Table [3]: Serum TTR level variations regarding CT findings, child grading and BCLC stages in HCC group

Variable		No	TTR	
			Mean [ug/mL]	P value
Hepatic focal lesion size [cm]	Small ≤ 5 cm	5	154 ± 32	0.611
	Large > 5 cm	25	141 ± 24	
Hepatic focal lesion number	One focal lesion	17	149 ± 45	0.427
	Two focal lesion	6	141±39	
	Three focal lesion	4	139 ± 31	
	Multifocal	3	134 ± 28	
Portal vein patency	Patent	15	161 ± 11	0.044
	Thrombosis	15	148 ± 17	
Abdominal lymphadenopathy	Absent	13	169 ± 11	0.005
	present	17	142 ± 23	
Spleen size	Average	9	162 ± 36	0.24
	Enlarged	21	161 ± 29	
Ascites	Absent	14	151 ± 26	0.109
	Present	16	154 ± 37	
Child-Pugh grades	A	8	169 ± 23	0.342
	B	7	161 ± 30	
	C	15	159 ± 34	
BCLC stages	A	10	126 ± 41	0.341
	B	4	122 ± 37	
	C	1	188±45	
	D	15	157±58	

Table [4]: Predictive performance of serum AFP and TTR as biomarkers for the detection of HCC

Statistical parameter	TTR	AFP	Combined AFP-TTR
AUC	0.726	0.833	0.856
Cut off value [ng/ml]	182	48	-
Sensitivity [%]	83.3	50	90
Specificity [%]	55	95	92.5
Positive predictive value [%]	58.1	88.2	90
Negative predictive value [%]	81.4	71.6	92.5

DISUCSSION

A major concern with HCC surveillance is the lack of ideal biomarkers. The ideal hepatic tumor bio-markers should have high specificity and sensitivity not to be detected in cirrhosis [14]. Number of promising biomarkers for HCC had been validated, but most of them were not applied to clinical diagnosis due to their limited practicability and high cost[15].

The normal function of TTR [prealbumin] is to transport thyroxine and the retinol binding protein [RBP]/retinol complex in the blood. Taken together, TTR might be associated with tumor micro-inflammation, resulting in a poorer prognosis [10]. Furthermore, TTR is less affected by liver disease than other serum proteins, even though it's main producer is the liver [16], however, there have been few reports concerning the significance of TTR in predicting development of HCC in cirrhotic patients.

In current study, TTR exhibited a significant much lower level [P< 0.001] in HCC patients group when compared to cirrhotic patients or healthy controls. Our result was similar to a finding of Liao et al., [17] who showed that cirrhotic patients with HCC had lower pre-albumin level. While in disagreement to result of Katare et al. who observed that serum TTR level showed 4 times increase in HCC patients than in control [13].

TTR may be a sensitive marker for the diagnosis of patients with hepatocellular carcinoma[12]. In this study, serum TTR levels were significantly much lower in HCC patients than in compensated cirrhotic patients. Few studies evaluated the significance of serum TTR as a prognostic biomarker of HCC and suggested that low concentrations may be associated with poor clinical outcome and prognosis[19-21]. In our study, serum TTR level showed significant decrease in HCC patients with portal vein or lymph node invasion than in HCC without vascular or nodal invasion. Serum TTR might be a useful biomarker for predicting the existence of portal vein tumor thrombi, as reported by Qiu et al., 2008[22] in that TTR, was down-regulated in HCC and serum TTR levels were lower in the patients with portal vein tumor thrombi [PVTT] than in those without PVTT

In this study, there was significant correlation between the serum TTR levels and T4. It can be explained by the fact that TTR is primarily responsible for the transport of thyroxin to different parts of the body and brain[23]. On the other hand, serum TTR exhibited inverse correlations with WBCs counts, AST, INR and ESR. Systemic inflammation also causes changes in the white blood cell [WBC] count, including neutrophils and lymphocytes. However, the WBC level fluctuates on a daily basis. The neutrophil to lymphocyte ratio [NLR] can more accurately indicate the inflammation status of a patient. In vitro studies have suggested that a high neutrophil count suppresses the antitumor efficacy of the host immune system[24]. Recently, a high NLR has been reported as a prognostic marker in patients with hepatocellular carcinoma[25]. Prolonged PT indicates marked liver damage [26]. AST exhibited a significant association with HCC risk. In retrospective study by Chung et al. aimed to identify the risk factors for recurrence in patients undergoing curative surgical resection and found preoperative AST level over twice upper normal values [>2N] was the only variable closely associated with tumor recurrence[27]. It is now well recognized that chronic inflammation is a risk factor for most types of cancer. Thus, inflammatory biomarkers might be used to predict cancer severity and monitor the progression[28].

In pre-operative evaluation of patients for hepatic resection HCC, prealbumin was determined at a level lower than 170 mg/dL in HCC patients [29]. In present study, analysis of ROC curve to get the best cut off value for serum TTR was 182 µg/ml and this offered a diagnostic sensitivity of 83%, a diagnostic specificity of 55%, positive predictive value of 58.1% and negative predictive value of 81.4%. These results were different from that of the study by Shimura et al.[30], where the cut off value of TTR was 11.4 mg/dL and this offered a diagnostic sensitivity of 62.5%, a diagnostic specificity of 85.7%.

Also, in this study, analysis of the ROC curve for evaluating the diagnostic performance of serum TTR in HCC, area under the curve [AUC] was 0.726 which is smaller than that of AFP alone [0.833] or that of combined TTR and AFP [0.856]. Our finding was similar to Shimura et al.[30] when using the ROC curve to determine the cut-off level of serum TTR in diagnosing HCC, area under the curve [AUC] was 0.737.

It was found in the current study that, the

diagnostic accuracy of TTR was higher than that of AFP regarding sensitivity [83.3%] and negative predictive value [81.4%] in diagnosis of HCC. These results were suggested that determining lower TTR levels alone might be of potential diagnostic value in HCC detection and has strong predictive values in differentiation of HCC and non HCC patients. While the combination between TTR and AFP might be superior in the diagnosis of HCC. Therefore, detection of lower level of TTR alone or in combination with other validated markers could be a diagnostic biomarker for detection of HCC at early noninvasive stage.

Conclusion: Serum TTR could be used in clinical practice alone or in combination with other validated markers as an accepted, reliable, and inexpensive screening tool for detection of hepatocellular carcinoma among cirrhotic patients at early noninvasive stage where curative treatment can be applied.

Financial and Non-Financial Relationships and Activities of Interest

None

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