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RESEARCH ARTICLE

Serum Level of Tumor Necrosis Factor Alpha as Marker for Hepatitis C Virus- Related Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) ranks fifth among the most prevalent cancers worldwide. In Egypt, the incidence of HCC had been doubling due to hepatitis C viral (HCV) infection. New serum tumor markers are required for diagnosis of HCC instead of alpha-fetoprotein (the most widely used marker) due to its poor diagnostic accuracy. Chronic inflammation is thought to be a cause of cancer. Tumor necrosis factor-alpha (TNF-α) is an important inducer of the inflammatory response and is regarded as tumor promoter. **Aim:** To assess the diagnostic accuracy of serum tumor necrosis factor alpha (TNF-α) level as biomarker for diagnosis of HCC. **Subjects and Methods:** 60 adult patients were selected for this study. They were categorized into: (Group II) which included 30 patients with cirrhosis with hepatitis C virus and (Group III) which included 30 patients with newly diagnosed HCC group on top cirrhosis and hepatitis C virus. 20 healthy subjects, age and sex-matched, were enrolled as controls. Routine tests for liver cirrhosis & HCC were done. Serum TNF-α level was measured using enzyme-linked immunosorbent assay. **Results:** Serum TNF-α was significantly elevated in HCC group when compared with other 2 groups. Insignificant difference between Okuda & BCLC stages as regard AFP levels but there were significant difference between Okuda & BCLC stages as regard serum TNF-α level, as the level of these marker was became higher as the tumor stage became more advanced. Significant positive correlations were found between TNF-α in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand. At cut off level ≥ 21.5 pg/ml, serum TNF-α had 86.67% sensitivity, 73.33 % specificity for diagnosis of HCC. **Conclusions:** The results of the present study clearly demonstrate that serum TNF-α had a better sensitivity and specificity than AFP in differentiating patients with HCC from those with cirrhosis. TNF-α could be used as reliable biomarker for HCC in HCV patients.

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Introduction:

Hepatocellular carcinoma (HCC) ranks fifth among the most prevalent cancers worldwide, and considered the third most common cause of cancer-related death. HCC is frequently the long-term sequel of chronic hepatitis B (HBV) and hepatitis C (HCV) infection [(1)]. In Egypt, the incidence of HCC had been increasing with a doubling in the incidence rate in the past 10 years, 90% of HCC cases were attributed to (HCV) infection as Egypt has the highest prevalence rate of HCV worldwide [(2,3)].

HCC diagnosis can be achieved by measuring the serum alpha-fetoprotein (AFP) level combined with imaging techniques [(4,5)]. AFP is not yet recommended for HCC surveillance by the American Association for the Study of Liver Diseases as its sensitivity and specificity cannot be satisfactory in HCC detection [(6)]. Improvement in early diagnosis is still needed because only 30% of patients with HCC are candidates for potentially curative treatments [(7)]. Thus, the discovery of an effective, reliable tool for early diagnosis of HCC will play a main role in improving HCC patients' prognosis [(8)]. Biomarkers from body fluids such as serum, and plasma are suitable for early diagnosis of HCC because they are easily accessible [(9)].

Inflammation is a dynamic response, and an important part of the body's defense mechanisms, however, excessive inflammation itself may cause disease [(10)]. Chronic inflammation is thought to account for the development of approximately 20% of human cancers [(11)]. Tumor necrosis factor-alpha (TNF-α) is an important inducer of the inflammatory response and is regarded as an endogenous tumor promoter [(12)]. The study of inflammatory markers is of particular relevance in HCC as the development of this cancer is usually associated with chronic inflammation [(13)].

Our aim was to assess the diagnostic accuracy of serum tumor necrosis factor alpha (TNF-α) level as biomarker for diagnosis of HCC.

Subjects and Methods

Study Population

The current prospective study enrolled 60 adult patients with HCV related chronic liver diseases (with or without HCC) who were admitted to the Internal Medicine Department, Tanta University Hospital within the period from June 2012 to Aug 2013. Twenty healthy subjects, age and sex matched as the control group (Group I) were included. The study protocol was approved by the ethical scientific committee of Tanta University. An informed medical consent was obtained from all subjects before the study. The patients were subdivided into 2 groups, (Group II) which included 30 patients with cirrhosis with hepatitis C virus and (Group III) which included 30 patients with newly diagnosed HCC group on top cirrhosis and hepatitis C virus. Patients with liver disease of other etiology other than HCV, patients with other cancers or metastatic liver cancer and patients with acute & chronic inflammation were excluded.

Study design and biochemical assays

All subjects were submitted to detailed history and clinical assessment. Liver cirrhosis was diagnosed on the basis of history, clinical examination, laboratory findings, and abdominal ultrasonography (US). Severity of liver disease was assessed by Child Pugh score [(14)]. Patient's viral infection status was determined by assaying hepatitis B surface antigen (HBsAg), hepatitis B core antibodies (anti-HBc), anti-HCV and HCV RNA by polymerase chain reaction. HCC was diagnosed by abdominal (US), abdominal triphasic CT and serum AFP. Tumor characteristics were detected including (tumor size, focal lesion number, site, portal vein invasion). Tumor staging was done using Okuda staging system [(15)], and The Barcelona Clinic Liver Cancer (BCLC) staging system [(16)].

Fasting venous blood samples (5 ml) were collected by trained laboratory technicians. A portion of blood was allowed to clot and then centrifuged at 3500g for 5 minutes to separate the serum used for assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, direct bilirubin, Albumin, AFP, and viral infection status. Serum aliquots were stored at -80 C o until assayed and thawed immediately before the measurements of TNF-α level. Another portion of blood was collected in vacutainer tubes containing citrate to separate plasma used for the assay of prothrombin time. AST, ALT, total bilirubin, direct bilirubin and albumin were assayed using Beckman CX4 chemistry analyzer (NY, USA, supplied by the Eastern Co. For Eng. & Trade-Giza, Egypt). AFP was measured using Abbott, Axyam (USA, Supplied by al kamal company Cairo, Egypt). Circulating HBs-Ag, anti-HBc and anti-HCV antibodies were tested by ELISA, using third generation kits (DiaSorin, Italy). HCV RNA by PCR assay was carried out by real time PCR using the real time PCR Step One instrument and software (Applied Biosystems).

Serum TNF-α was measured by an enzyme-linked immunosorbent assay technique, using Quantikine Human (TNF-α) Immunoassay (R&D System Inc., Minneapolis, MN, USA). This assay employs a quantitative sandwich enzyme immunoassay technique. Level of TNF-α was calculated by interpolation from a reference curve generated in the

same assay with reference standards of known concentrations. All assays were performed in duplicate according to the manufacturer's instructions.

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 17 software (SPSS Inc, Chicago, ILL Company). Categorical data were presented as number and percentages while quantitative data were expressed as mean and standard deviation. Comparison of continuous data between two groups was made by using unpaired t test for parametric data and Mann-Whitney test for nonparametric data. Comparison of continuous data between more than two groups was made by using one way ANOVA for parametric data and Kruskal-Wallis test for nonparametric data. Chi square test was used for comparison between Categorical data. Spearman test for correlations between different parameter (nonparametric) was used. ROC curve were used for estimation of sensitivity, specificity, cut off level, positive predictive value and negative predictive value. The accepted level of significance in this work was stated at 0.05 ($P < 0.05$ was considered significant).

Results

The Demographic data of the studied groups were shown in **Table (1)**. 90% of the HCC patients were symptomatic. The performance status ranged from 0 to 3 with mean 1.3±1.368. All recruited patients were positive for HCV antibodies. Regarding Okuda staging system, 70% of HCC patients presented in stage II and 30% of HCC patients presented in stage III. Regarding BCLC staging system, 30%, 23.33%, 16.67% and 30% of HCC patients presented in stage A, B, C and D respectively. Abdominal CT showed that all HCC occurred on top of cirrhosis (100%), ascites present in 60% of the HCC patients and portal vein thrombosis (PVT) present in (40%). Regarding the focal lesion, the higher incidence of the focal lesion to be single (70%), affecting the right lobe (60%) and the size of the focal lesion ranged from 2.5 to 12.5 cm with mean 6.23±2.653. Comparison between all studied groups as regard liver functions tests were shown in **Table (2)**. The mean value of serum AFP and TNF-α was significantly elevated in HCC group when compared with the other two groups **Table (2)**. In relation to staging, there was insignificant difference between Okuda & BCLC stages as regard AFP levels but there was significant difference between Okuda & BCLC stages as regard TNF-α level, as the level of these marker was became higher as the tumor stage became more advanced, Comparison between TNF-α level in different stages of BCLC were significant except between stage B & C **Table (3)**. Significant positive correlations were found between TNF-α in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand **Table (4)**. Insignificant correlations were found between TNF-α in one hand, and age, ALT, AST, serum albumin and serum AFP on the other hand **Table (4)**. Receiving operating characteristic (ROC) analysis curves and the corresponding area under the curve were calculated for providing the accuracy of the AFP and TNF-α in diagnosis of HCC. Sensitivity (i.e., true positive rate), specificity (i.e., true negative rate), positive predictive value (PPV), negative predictive value (NPV) and cutoff values showing the best equilibrium between sensitivity and specificity were evaluated. At cut off level ≥ 105.5 ng/ml, serum AFP had 93.33% sensitivity, 46.67 % specificity, 63.64% PPV, 87.5% NPV for diagnosis of HCC. At cut off level ≥ 393.5 ng/ml, serum AFP had 33.33% sensitivity, 100 % specificity, 100% PPV, 60% NPV for diagnosis of HCC. At cut off level ≥ 21.5 pg/ml, serum TNF-α had 86.67% sensitivity, 73.33 % specificity, 76.47% PPV, 84.62% NPV for diagnosis of HCC **Table (5)Figure(1)**.

Table (1): Demographic data of the studied groups

Variable		Group I Control group (N=20)	Group II LC group (N=30)	Group III HCC group (N=30)	P
Gender	Male	15(75%)	22(73.33%)	24(80%)	0.6435
	Female	5(25%)	8(26.67%)	6(20%)	
Age (Mean±SD)		56.65±5.194	55.8 ±7.141	58.9±7.434	0.2040
Child Pugh classification	A	---------	8(26.67%)	9(30%)	0.9564
	B	---------	17(56.66%)	16(53.33%)	
	C	--------	5(16.67%)	5(16.67%)	

Table (2): Laboratory characteristics among the studied groups.

Variable	Group I Control group (N=20)	Group II LC group (N=30)	Group III HCC group (N=30)	p	P1	P2	P3
	Mean ± SD	Mean ± SD	Mean ± SD				
ALT (U/L)	29.8 ± 5.662	57.27 ± 14.934	61.17 ± 17.489	**<0.0001***	**<0.0001***	**<0.0001***	0.357
AST(U/L)	26.65 ± 5.696	50.07 ± 12.468	53.7 ± 14.399	**<0.0001***	**<0.0001***	**<0.0001***	0.3006
Total bilirubin (mg/dl)	0.77 ± 0.2003	1.39 ± 0.6873	2.72 ± 0.8185	**<0.0001***	**0.0024***	**<0.0001***	**<0.0001***
Direct bilirubin (mg/dl)	0.17 ± 0.05871	0.71 ± 0.4975	1.63 ± 0.5676	**<0.0001***	**<0.0001***	**<0.0001***	**<0.0001***
Serum Albumin (g/dl)	4.09 ± 0.4184	3.32 ± 0.48	2.79 ± 0.4513	**<0.0001***	**<0.0001***	**<0.0001***	**<0.0001***
PT (sec)	11.9 ± 0.8522	14.33 ± 2.604	16.8 ± 4.221	**<0.0001***	**<0.0001***	**<0.0001***	**0.0153***
Serum AFP (ng/ml)	5.8 ± 1.936	146.97 ± 92.919	529.7 ± 598.27	**<0.0001***	**<0.0001***	**<0.0001***	**0.0017***
TNF-α (pg/ml)	12.47 ± 7.060	18.48 ± 4.73	29.31 ± 11.226	**<0.0001***	**<0.0001***	**<0.0001***	**<0.0001***
P1 group I vs. II			**P2 group I vs. III**			**P3 group II vs. III**	

Table (3): Comparison between AFP and (TNF-α) levels in different stages of Okuda staging system and Barcelona Clinic Liver Cancer (BCLC) staging system

Variable		Serum AFP (ng/ml)		TNF-α (pg/ml)	
		Mean ± SD	P	Mean ± SD	P
Okuda staging system	Stage II	501.62±596.75	0.7173	24.56±4.2059	**<0.0001***
	Stage III	595.22±632.7		40.4±14.661	
BCLC staging system	Stage A	352.67±549.4	0.3677	21.64±4.2536	**0.0002***
	Stage B	604.71±500.21		26.79±3.0803	
	Stage C	625.4±844.93		26.68±2.1487	
	Stage D	595.22±632.7		40.4±14.661	
	Comparison between TNF-α level in different stages of BCLC were significant except between stage B&C				

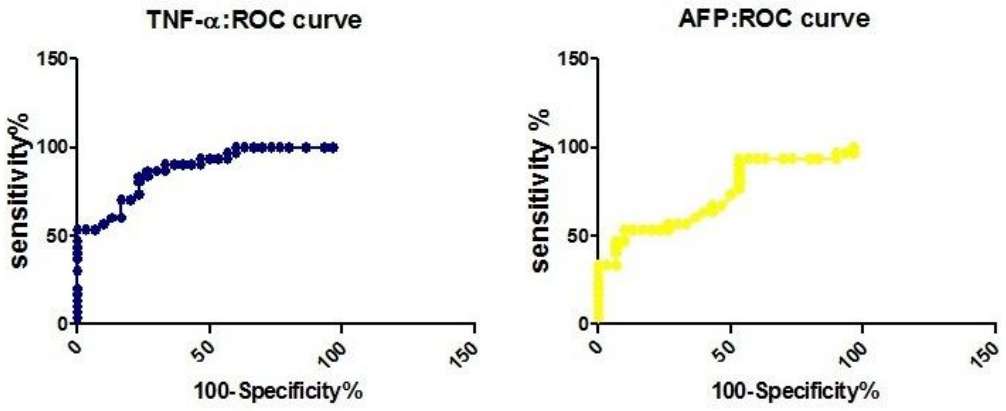
Table (4): Correlation between (TNF-α) and different variables of HCC group.

Variable	TNF-α	
	r	P
Age (years)	- 0.0296	0.8766
ALT (U/L)	- 0.0969	0.6103
AST (U/L)	0.00078	0.9967
Total bilirubin (mg/dl)	0.6922	**<0.0001***
Direct bilirubin (mg/dl)	0.6386	**0.0001***
Albumin (g/dl)	0.2685	0.1515
PT (Sec)	0.6391	**0.0001***
Tumor size (cm)	0.6824	**0.0001***
Serum AFP (ng/ml)	0.2338	0.2136

Table (5): Sensitivity, specificity, positive prediction value, negative prediction value and accuracy of serum (AFP and TNF-α) among the studied cirrhotic patients.

Variable	cutoff value	Sensitivity %	Specificity %	Positive predictive value (PPV %)	Negative predictive value (NPV %)	Area undue the curve (AUC)	95% CI
AFP	≥ 105.5 ng/ml	93.33%	46.67%	63.64%	87.5%	0.7361	0.6093-0.8630
AFP	≥ 393.5 ng/ml	33.33%	100%	100%	60%		
TNF-α	≥ 21.5 pg/ml	86.67%	73.33%	76.47%	84.62%	0.8717	0.7859-0.9575

Figure (1): ROC curve of TNF- α and AFP.



Discussion

Hepatocellular carcinoma (HCC) is the primary malignancy of hepatocyte. It is the most common primary hepatic tumor and one of the most common cancers worldwide. About 80% of people with HCC have cirrhosis. HCC is the second most frequent cause of cancer incidence and mortality among men in Egypt [(17)].

HCC is a slow progressing disease, during the initiation phase of HCC the balance between apoptosis and proliferation of hepatocytes is disrupted and favours proliferation. In response to this injury, innate immune cells migrate to the site of damage and release a plethora of proinflammatory cytokines generating an inflammatory

microenvironment, which promotes cancer progression. After chronic exposure to inflammation, hepatocytes develop mechanisms to evade apoptotic death; these results in the accumulation of damaged hepatocytes that eventually become HCC [18].

Cytokines, as the products of host response to inflammation, play an important role in the defense against viral infections. However, in HCV infection they may play a prominent role in liver damage **[19]**. Tumor necrosis factor alpha (TNF-α) is a multi-functional cytokine that regulates a variety of signaling pathways implicated in inflammation, immunity, apoptosis, cell survival, and even tumor genesis. During inflammation, TNF-α induces apoptosis repeatedly and subsequently enhances the chance of formation of anomalous cells during the process of regeneration and dysplasia **[20]**. Circulating TNF-α level increases during HCV infection **[21]**. TNF-α level correlates with the severity of hepatic inflammation, fibrosis, and tissue injury **[19]**. Persistent immune mediated hepatic injury can initiate the process of fibrosis, cirrhosis, and, eventually HCC **[22]**.

In this study, we didn't find any significant difference between HCC patients compared to either to cirrhosis patients or control patients as regards to age. In HCC patients the age ranged from (45-71) years with mean age of incidence (58.9±7.434) years old. **El Zayadi et al 2001** [23], reported that analysis of age distribution among HCC patients revealed that the most predominant age group was (40-59 years). Also, in the present study, HCC patients were more common in males than females; these results are similar to **Zakhary et al 2011**[24] who reported that males represented 70.8% of all patients in HCC group, with 83.3% of patients over 50 years.
The present study revealed that the mean values of serum AFP and TNF-α were significantly elevated in HCC group when compared with the other two groups. In relation to staging, there was insignificant difference between Okuda & BCLC stages as regard AFP levels but there were significant difference between Okuda & BCLC stages as regard TNF-α level, as the level of these marker was became higher as the tumor stage became more advanced, Comparison between TNF-α levels in different stages of BCLC were significant except between stage B & C**.** Significant positive correlations were found between TNF-α in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand . Insignificant correlations were found between TNF-α in one hand, and age, ALT, AST, serum albumin and serum AFP on the other hand.
Raghuraman et al 2005[25], found that patients infected with HCV had higher values of TNF-α as compared with healthy individuals and found that TNF-α levels had significant positive correlation with ALT level. **Aroucha et al 2013**[26], found that TNF-α higher levels significantly associated with HCC-HCV occurrence. Individuals with HCC presented higher TNF-α/IL-10 ratio than those with fibrosis. Patients with HCC were associated to higher index TNF-α/IL-10 ratio, suggesting that the unbalanced production of these cytokines may represent progression to the liver disease severity in HCV infected patients. In agreement with our study, **Shaker et al 2013**[27]**,** found that there was a higher mean level of TNF-α among cases with HCC compared to cirrhosis and control groups with highly statistically significant difference. On other hand **Shaker et al 2013**[27]**,** showed that TNF-α level showed no statically significant difference with tumor size and also showed that serum AFP had 88% sensitivity, 60 % specificity and serum TNF-α had 58% sensitivity, 64 % specificity for diagnosis of HCC.

Qiu et al 2013[28], found that TNF-α expression were negative in normal liver tissue but positive in HCC and peritumor cirrhosis tissue. Also found that there were no significant differences in the rates of positivity for TNF-α between HCC and peritumor cirrhosis tissue. The degree of differentiation of HCC was correlated with TNF-α expression. **Cheng et al 2013**[29], showed that the TNF-α 238 G/A polymorphism was significantly associated with increased risk of HCC in meta-analysis included 11 case-control studies with a total of 1,572 HCC cases and 1,875 controls.

Conclusions

The results of the present study clearly demonstrate that serum TNF-α had a better sensitivity and specificity than AFP in differentiating patients with HCC from those with cirrhosis. Serum TNF-α could be used as reliable biomarker for HCC in HCV patients.
We recommend large scale multicenter studies covering the different Egyptian population to better clarify the diagnostic performance of this new biomarker among our Egyptian patients whether alone or in combination with AFP.

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