ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Serum Levels of Soluble Fas and Soluble Fas Ligand as Markers for Hepatocellular Carcinoma in Hepatitis C Virus Patients

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Manuscript Info *Abstract*

Manuscript History:

Received: 25 July 2014
Final Accepted: 27 August 2014
Published Online: September 2014

Key words:

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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. The Fas receptor/ ligand system, including soluble forms, is the most important apoptosis initiator in the liver. Dysregulation of this pathway may contribute to abnormal cell proliferation. **Aim**: To assess the diagnostic accuracy of serum soluble Fas (sFas) and soluble Fas Ligand (sFasL) levels as biomarkers for diagnosis of HCC. **SubjectsandMethods**: 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 sFas and sFasL levels were measured using enzyme-linked immunosorbent assay. **Results**: Serum sFas and sFasL levels were 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 sFas, and sFasL levels, as the levels of these markers were became higher as the tumor stage became more advanced. Significant positive correlations were found between sFas and sFasL in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand. Significant positive correlations were found between sFas, and sFasL with each other. At cut off level ≥ 1770 pg/ml, serum sFas had 76.67% sensitivity, 83.33 % specificity for diagnosis of HCC. Regarding serum sFasL level for diagnosis of HCC, it had 86.67% sensitivity, 83.33% specificity at cut off level ≥ 1310 pg/ml. **Conclusions**: The results of the present study clearly demonstrate that serum sFas and sFasL had a better sensitivity and specificity than AFP in differentiating patients with HCC from those with cirrhosis. sFas and sFasL could be used as reliable biomarkers for HCC in HCV patients.

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)].

Apoptosis is an important mechanism for controlling the balance between cell proliferation and cell death. Related proteins and their receptors on the cells, which associate with the inhibition or augmentation of cell death, regulate apoptosis. One of the best-characterized systems is the Fas-Fas ligand system [(10,11)]. Fas consists of 2 isoforms, membrane anchored (mFas) and soluble (sFas). The membrane isoform (mFas) induces apoptosis in normal or tumor cells, whereas the soluble isoform (sFas) is thought to block Fas-mediated apoptosis by binding and subsequent inactivation of FasL [(12)]. FasL is belonged to tumor necrosis factor (TNF) family. Soluble FasL (sFasL) is generated from its membrane-bound FasL by a metalloproteinase-like protease [(13)]. Circulating isoforms of FasL might prevent the recognition of tumor cells by the cytotoxic T-cells by imitating tumor cells as immune-privilege sites [(14)]. The impact of apoptosis in chronic HCV infection is not well understood, it may be harmful by triggering liver fibrosis [(15)]. The Fas receptor/ ligand system, including soluble forms, is the most important apoptosis initiator in the liver [(16)]. Dysregulation of this pathway may contribute to abnormal cell proliferation and cell death [(17)]and is regarded as one of the mechanisms that prevent the immune system to reject tumor cells [(18)].

Our aim was to assess the diagnostic accuracy of serum soluble Fas (sFas) and soluble Fas Ligand (sFasL) levels as biomarkers for diagnosis of HCC.

SubjectsandMethods

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 [(19)]. 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 to exclude other etiology other than HCV. 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 [(20)], and The Barcelona Clinic Liver Cancer (BCLC) staging system [(21)]. Five patients with BCLC staging system stage A undergone hepatic lobectomy. The liver biopsy specimens were collected intraoperative after the patients were surgically operated for hepatic lobectomy and two specimens were obtained, one from the tumor tissue and the other from the surrounding non tumor tissue. All specimens were fixed in formalin fixed paraffin embedded then sectioned and stained by Haematoxylin& Eosin for routine histopathological examination.

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 ℃ o until assayed and thawed immediately before the measurements of sFas, & sFas-L levels. 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). CirculatingHBs-Ag, anti-HBc and anti-HCV antibodies were tested by ELISA, using third generation kits (DiaSorin, Italy). Serum level of sFas-L was determined with a commercially available assay, sFas-L enzyme-linked immunosorbent assay kit (Cat. No: BE5192, Assaypro, Hamburg, Germany). Serum level of Fas Ligand was measured by an enzyme-linked immunosorbent assay technique, using Quantikine Human (sFas) Immunoassay (R&D System Inc., Minneapolis, MN, USA). This assay employs a quantitative sandwich enzyme immunoassay technique. Levels of sFas, and sFas-L were 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. Abdominal ultrasonography was also done for all patients.

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 & Pearson tests for correlations between different parameter (nonparametric & parametric respectively) were 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 values of serum AFP, sFas and sFasL were 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 were significant difference between Okuda & BCLC stages as regard sFas and sFasL levels, as the levels of these markers were became higher as the tumor stage became more advanced, Comparison between sFas and sFasL levels in different stages of BCLC were significant except between stage B & C **Table (3)**. Significant positive correlations were found between sFas, and sFasL in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand **Table(4)**. Insignificant correlations were found between sFas and sFasL in one hand, and age, ALT, AST, serum albumin and serum AFP on the other hand **Table (4)**. Significant positive correlations were found between sFas, and sFasL with each other **Table (4)**. Receiving operating characteristic (ROC) analysis curves and the corresponding area under the curve were calculated for providing the accuracy of the AFP, sFasand sFasL 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 ≥ 1770 pg/ml, serum sFas had 76.67% sensitivity, 83.33 % specificity, 82.14% PPV, 78.12% NPV for diagnosis of HCC. Regarding the diagnostic performance of serum sFasL level for diagnosis of HCC, it had 86.67% sensitivity, 83.33% specificity, 83.87% PPV, 86.21% NPV at cut off level ≥ 1310 pg/ml **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*
sFas (pg/ml)	1031 ± 43.395	1481.67 ± 251.36	2024 ± 444.4	<0.0001*	<0.0001*	<0.0001*	<0.0001*
sFasL (pg/ml)	126.1 ± 18.83	1180.33 ± 160.44	1551 ± 258.35	<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, (sFas) and (sFasL) levels in different stages of Okuda staging system and Barcelona Clinic Liver Cancer (BCLC) staging system

Variable		Serum AFP (ng/ml)		sFas (pg/ml)		sFasL (pg/ml)	
		Mean ± SD	P	Mean ± SD	P	Mean ± SD	P
Okuda staging system	Stage II	501.62±596.75	0.7173	1811.9±311.94	<0.0001*	1423±142.18	0.0004*
	Stage III	595.22±632.7		2519±281.13		1846.7±225.67	
BCLC staging system	Stage A	352.67±549.4	0.3677	1546.7±281.29	<0.0001*	1302.2±63.2	0.0001*
	Stage B	604.71±500.21		1977.1±159.66		1512.9±117.86	
	Stage C	625.4±844.93		2058±90.94		1520±116.4	
	Stage D	595.22±632.7		2518.9±281.13		1846.7±225.67	
		Comparison between sFas, and sFasL levels in different stages of BCLC were significant except between stage B&C					

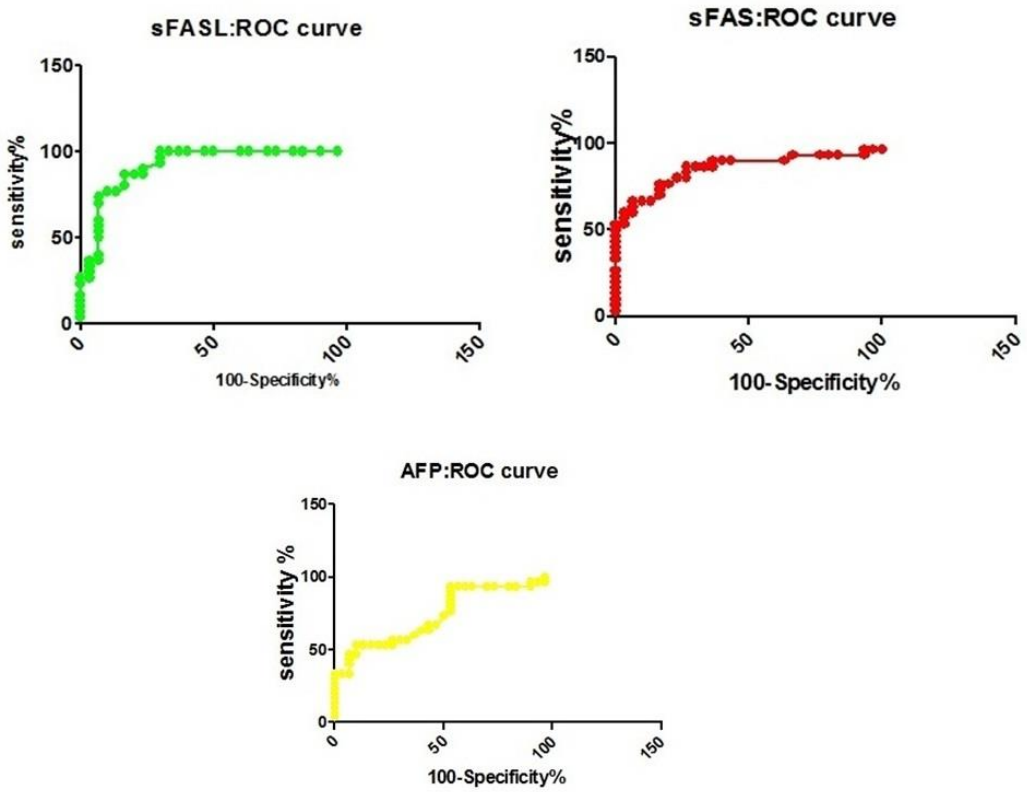
Table (4): Correlation between (sFas and sFasL) and different variables of HCC group.

Variable	sFas		sFasL	
	r	P	r	P
Age (years)	- 0.1212	0.5236	0.00581	0.9757
ALT (U/L)	- 0.1191	0.5308	0.0919	0.6291
AST (U/L)	0.05413	0.7764	0.1736	0.3589
Total bilirubin (mg/dl)	0.6357	0.0002*	0.5836	0.0007*
Direct bilirubin (mg/dl)	0.5751	0.0009*	0.4942	0.0055*
Albumin (g/dl)	0.3069	0.099	0.2202	0.2423
PT (Sec)	0.5106	0.0039*	0.3938	0.0313*
Tumor size (cm)	0.7326	<0.0001*	0.6221	0.0002*
Serum AFP (ng/ml)	0.1704	0.3680	0.2959	0.1124
sFas (pg/ml)	-------	-------	0.8547	<0.0001*
sFasL (pg/ml)	0.8547	<0.0001*	-------	-------

Table (5): Sensitivity, specificity, positive prediction value, negative prediction value and accuracy of serum (AFP, sFas and sFasL) 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%		
sFas	≥ 1770 pg/ml	76.67%	83.33%	82.14%	78.12	0.8539	0.7508-0.9570
sFasL	≥ 1310 pg/ml	86.67%	83.33%	83.87%	86.21%	0.9167	0.8446-0.9887

Figure (1): ROC curve of (sFasL, sFas 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 [(22)].

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 [(23)].

HCC shows resistance to apoptosis mediated by several death receptors. The majority of the HCC shows one or more alterations in the Fas pathway molecules, which inhibit Fas-mediated apoptosis [24]. The Fas receptor/ligand system including soluble forms is the most important apoptotic initiator in the liver [16]. Several cells in the liver had been shown to express Fas/FasL and their soluble forms sFas/sFasL which play a major role in the pathogenesis of many liver diseases [25].

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**[26], 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**[27] 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 sFas, sFasL and AFP 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 sFas and sFasL levels, as the levels of these markers were became higher as the tumor stage became more advanced, Comparison between sFas and sFasL levels in different stages of BCLC were significant except between stage B & C. Significant positive correlations were found between sFas and sFasL in one hand, and total bilirubin, direct bilirubin, prothrombin time and tumor size on the other hand . Insignificant correlations were found between sFas and sFasL in one hand, and age, ALT, AST, serum albumin and serum AFP on the other hand. Significant positive correlations were found between sFas and sFasL with each other.

Nagao et al 1999[18], **Chen et al 2001**[28] and **Peng et al 2001**[29] found that the sFas levels in HCC patients were significantly higher than those in controls. **Raghuraman et al 2005**[30], found that patients infected with HCV had higher values of sFas as compared to healthy and human immunodeficiency virus 1 infected individuals. **Hassan et al 2007**[31], showed that the mean value of serum sFas in Bilharzial fibrosis and liver cirrhosis, with and without HCC, was significantly higher than in control group. **El Bassiouny et al 2008**[32], found that sFas was significantly increased in chronic hepatitis C, liver cirrhosis, and HCC cases compared with normal controls. The increase of sFas in HCC was also significantly higher than that of chronic hepatitis C. **Zekri et al 2010**[33] found that HCC patients had also significantly higher levels of sFas when compared to controls. **Hammam et al 2012**[34], found the sFas in cirrhotic patients and HCC were significantly higher than that in normal controls and chronic hepatitis C without cirrhosis group, but there was no significant difference between cirrhotic and HCC patients.

Chen et al 2001[28], found that the serum sFas had positive correlation with the serum total bilirubin, but negative correlation with the serum albumin, prothrombin time activity and the ratio of ALT/AST. **Raghuraman et al 2005**[30], found that plasma levels of sFas in patients with chronic HCV infection showed significant positive correlation to and ALT levels.

Nagao et al 1999[18], found that immunohistochemistry revealed generation of sFas in the hepatocytes and tumor-infiltrating mononuclear cells rather than in HCC cells. Accordingly, HCC cells may eliminate Fas expression on themselves and let the hepatocytes and infiltrating mononuclear cells generate sFas to escape from the immune system.

Lapinski et al 2004[35], found that sFasL was not detected in healthy subjects, Furthermore sFasL occurred more frequently in chronic hepatitis C patients in comparison to chronic hepatitis B patients. **Nada et al 2005**[36], found that sFasL levels were higher in HCC than in chronic hepatitis or liver cirrhosis. **Hassan et al 2007**[31], found that the mean value of serum sFasL was significantly elevated in all patients with liver cirrhosis, with and without HCC, and lower, but not significantly, in patients with Bilharzial fibrosis in comparison with the control group. On the other hand **Nagao et al 1999**[18], and **Chen et al 2005**[37], found that the sFasL levels were significantly lower in patients with HCC when compared to the patients with hepatitis or liver cirrhosis.

Nakamoto et al 1999[38], found that negative association between the peripheral blood lymphocytes mortality and the serum sFasL levels. These results suggest the inhibitory effect of serum sFasL on apoptosis of PBL, which may explain the induction of immunological abnormalities with the development of HCC.

Conclusions

The results of the present study clearly demonstrate that serum sFas and sFasL had a better sensitivity and specificity than AFP in differentiating patients with HCC from those with cirrhosis. sFas and sFasL could be used as reliable biomarkers 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 biomarkers among our Egyptian patients whether alone or in combination with AFP.

Competing interests:

All The authors declare that they have no competing interests.

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