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Research Article
Combination of Serum Survivin and AFP as a Potential Marker in HCC Associated with Hepatitis C Viral Infection

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Abstract

Background and Objective: Hepatocellular carcinoma (HCC) has a rising prevalence rate worldwide. This study aims to appraise the serum survivin levels in malignant and nonmalignant chronic liver diseases (CLDs) patients and to elucidate a possible correlation between survivin and the clinico-pathological characteristics of the disease. **Materials and Methods:** During the period between May, 2016 and April, 2017, 96 chronic HCV patients with or without cirrhosis were included in this study. Persons with focal lesions in Ultrasound (US) and/or serum α-fetoprotein (AFP) level ≥200 ng mL^{-1} were investigated via triphasic computed tomography scanning (CT) and/or MRI. The levels of both survivin and AFP proteins in the serum were detected using ELISA. **Results:** Survivin was undetectable in 111 out of 120 serum samples (92. 5%). The survivin-positive samples (n = 9, median level, 81.46 pg mL^{-1}, range, 9.38-223.81 pg mL^{-1}) corresponded to 9 HCC patients who have multiple focal lesions. The sensitivity and specificity of survivin for selective detection of the HCC patients over the non-malignant CLD patients (HCV group and LC group) were 85.7 and 72%, respectively, at a cut-off value -26.196 pg mL^{-1}. Also, using a combination of both markers improves the sensitivity, specificity, PPV, NPV to be 85.7, 95, 85.7 and 95% respectively. **Conclusion:** The previous results suggested that serum survivin could be a potential marker for detecting HCC development. Also, the combination between survivin and AFP is effective for increasing specificity for HCC diagnosis.

Key words: Hepatocellular carcinoma, anti-apoptosis, survivin, ELISA, AFP

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hepatocellular carcinoma (HCC) has a high incidence worldwide. It is the second leading cause of cancer-related death around the world[1].

In Egypt, liver cancer accounts for 11.75% of the malignancies that occur in the digestive organs and 1.68% of the total malignancies. The HCC represents 70.48% of all liver tumors among Egyptians[2]. The HCC occurs secondary to infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis and environmental toxins including a atoxin[1,3-4].

Treatment of HCC is effective in the early stages of the disease. Unfortunately, HCC is detected usually after a period of the tumor onset at a time when curative surgical resection or organ transplantation cannot be performed owing to advanced disease or extensive impairment of liver function[5].

In the absence of pathological confirmation, alpha-fetoprotein (AFP), besides the radiological findings, is an important tool for diagnosing HCC. However, AFP has a lower sensitivity as there are other diseases rather than HCC including liver cirrhosis, some benign hepatic focal lesions in addition to non-hepatic malignancies can cause increase in the AFP levels. Also, some HCC patients show negative results of AFP which makes it insufficient in HCC diagnosis[6] . This complication emphasizes the need to identify sensitive diagnostic and prognostic biomarkers, especially for early-stage tumors.

Survivin is a 16.5 kDa anti-apoptotic protein that suppresses the activities of caspases and regulates mitosis. Apoptosis is common in liver injury and may lead to inflammation, fibrogenesis and progression of cirrhosis[7]. Survivin promotes cell proliferation by reducing nuclear accumulation of p21, an inhibitor of cell cycle progression[8]. Survivin is normally expressed in embryonic and fetal tissues where apoptosis plays an essential role in the development of the fetus. Early studies revealed that survivin is either undetectable or expressed at a very low level in adult tissues[9], although recent evidence illustrates that survivin may have a role in normal cell cycling as it is expressed at the onset of DNA synthesis and through the G2 and M phases of cell replication in the non-neoplastic liver[10]. The role of survivin in HCC is still inadequately defined, but elevated mRNA expression has been detected in 31-88% of Japanese HCC patients[8,11] and has been accompanied by shorter survival[12]. Therefore, this study aims to assess the serum level of survivin in Egyptian patients with HCC accompanied by chronic HCV infection and to compare it with its level in non-malignant CLDs patients. Also, the present study aims to validate the diagnostic performance of survivin, as a tumor biomarker, for HCC detection in comparison with AFP.

MATERIALS AND METHODS

The current study was carried out on 120 subjects, 96 chronic liver disease patients and 24 healthy controls. Patients were recruited from the Hepatology Department of National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt from May, 2016 and April, 2017. All patients and controls were aware of the study and all patients provided a written informed consent for contributing in this study.

The studied patients and controls were categorized into the following groups:

Group I (GI) : Included 24 healthy individuals, 6 of them were males (25%) and 18 were females (75%) with median (inter-quartile range) age 53 (41.25-63) years

Group II (GII) : Included 33 patients suffering from chronic HCV infection as diagnosed by seropositivity for HCV antibodies, 18 of them were males (54.5%) and 15 were females (45.5%). Their median age was 57 years with IQR (52.25-59.5 years)

Group III (GIII) : Included 42 patients with liver cirrhosis, 27 of them were males (64.3%) and 15 were females (35.7%). The median (IQR) of age was 55.5 (49.25-65.75). All of them have HCV infection

Group IV (GIV) : Included 21 cirrhotic patients with HCC fulfilling HCC criteria on tri-phasic C.T. scan, all of them were males (100%). Their median age was 63 (58.5-72.5)

Exclusion criteria: A past history or evidence of other malignancies, autoimmune disorders, organ failure and other causes of cirrhosis (e.g., HBV, alcoholic and non-alcoholic fatty liver diseases).

All patients and controls were subjected to: (a) History taking, (b) Liver and other biochemical profiles including AST, ALT, serum albumin, total bilirubin and CBC. The kits were supplied by Spectrum Company, Cairo, Egypt, (c) Abdominal ultrasound (US) with special emphasis on the liver, presence or absence of ascites, focal hepatic lesions and spleen, (d) Serum Alpha-Fetoprotein assayed using an ELISA kit purchased from (IMMUNOSPEC Corporation, USA), (e) Individuals with a solid focal lesion in US examination and/or AFP serum level $\geq$200 ng mL^{-1} were scrutinized by tri-phasic CT and/or MRI to confirm or roll out the HCC diagnosis and (f) Serum survivin levels were performed for all cases.

Quantitation of serum survivin by (ELISA): Serum survivin was measured by ELISA kit manufactured by R and D catalog number DSV00, according to the manufacturer's instructions.

Serum alpha-fetoprotein assay: Serum alpha-fetoprotein was measured by human AFP ELISA kit manufactured by IMMUNOSPEC Corporation, USA.

Statistical analysis: In the current study, statistical analyses were conducted using SPSS 23.0. The significance level is 0.05. Data were expressed as median (IQR) for quantitative non-parametric measures and number (percentage) for categorized data. The significance of the difference between two groups was determined by using Mann-Whitney U test. Kruskal Wallis test is utilized for statistical comparison between more than two sets of data if one or both of them have a skewed distribution. Spearman's correlation test (was used to investigate the correlation between 2 quantitative variables. Chi-square (χ^2) test and Fisher's exact test were used to study the relationship between categorical variables. The ROC was constructed to obtain the most sensitive and specific cut-off value for serum survivin in diagnosing HCC.

RESULTS

The study comprised 96 chronic liver disease patients and 24 healthy controls. The demographic and clinical data of the studied cases were summarized in Table 1.

Survivin was undetectable in 111 out of 120 serum samples (92.5%). The survivin-positive samples (n = 9, median level, 81.46 pg mL^{-1}, range, 9.38-223.81 pg mL^{-1}) corresponded to 9 HCC patient who have multiple focal lesions from total 21 patients in this group. Statistical analysis of the survivin showed that the median serum survivin level was significantly higher in the HCC patients than those who suffer from HCV or liver cirrhosis as determined by Mann-Whitney U test (p-value = 0.007, p = 0.03, respectively) (Table 2). Serum survivin levels were significantly affected by sex (p = 0.036), spleen size (p = 0.044) and by the presence of ascites (0.05) or focal lesion (0.007) in all patient's groups; (Table 3). Also, survivin showed direct significant correlation with the AST, serum bilirubin levels. While it showed inverse significant correlation with albumin. Also, AFP showed direct significant correlation with the AST levels. It also showed inverse significant correlation with albumin, RBCs and platelets count (Table 4).

Table 1: Demographic data and biochemical parameters of the patients and controls

	Variable			
Groups	Healthy controls group (n = 24)	HCV patients group (n = 33)	Liver cirrhosis patients group (n = 42)	HCC patients group (n = 21)
Age (years)	53 (41.25-63)	57 (52.25-59.5)	55.5 (49.25-65.75)	63 (58.5-72.5)
Gender male/female percentage of male	6/18 (25%)	18/15 (54.5%)	27/15 (64.3%)	21/0 (100%)[a**]
RBCs (10^6 μL^{-1})	4.85 (4.49-5.52)	4.9 (4.3-5.4)	4.5 (3.9-5.24)	3.9 (3.2-4.3)[a*,b*]
Hemoglobin (g dL^{-1})	13.7 (12.55-14.58)	14.1 (13.3-16.1)	13.6 (12.5-15.2)	11.6 (8.6-13)[a*,b**,c*]
Platelets count (10^3 μL^{-1})	273 (258-319)	165 (140-252)[a**]	80 (66-167)[a****,b**]	122 (92-195)[a***]
WBCs (10^3 μL^{-1})	5.85 (5.4-7.65)	5.5 (4.3-7.7)	5.7(3.55-7.55)	5.9 (4.1-8.4)
ALT (U L^{-1})	31 (25-36)	40 (26-83)	72 (60-77)[a***]	60 (28-75)[a*]
AST(U L^{-1})	28.5 (23.5-34.5)	40 (33-52)[a*]	92 (48-103)[a***,b*]	100 (37-117)[a**]
Total bilirubin (mg dL^{-1})	0.425 (0.325-0.537)	0.4 (0.35-0.55)	1.3 (0.55-2.4)[a*]	1.95 (0.5-9.33)[a*]
Serum albumin (g dL^{-1})	3.95 (3.8-4.18)	4.2 (3.58-4.68)	3.3 (2.65-4.25)	3 (2.07-3.2)[a**,b*]

Parameters are presented as medians (inter-quartile range) for quantitative variables and as total number (%) for categorical variables. Units are in parentheses. [a]Significant difference from healthy controls, [b]Significant difference from HCV patients, [c]Significant difference from liver cirrhosis patients, *p≤0.05, **p≤0.01, ***p≤0.001. RBCs: Red blood cells, WBCs: White blood cells, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, n: Number of subjects

Table 2: Comparison of alpha fetoprotein and survivin in the all studied groups

	Variable			
Groups	Healthy controls group (n = 24)	HCV patients group (n = 33)	Liver cirrhosis patients group (n = 42)	HCC patients group (n = 21)
AFP (ng mL^{-1})	1.55 (1.34-1.96)	6.9 (3.8-11.8)[a**]	7.1(4.4-15.48)[a***]	100(4.4-142)[a***]
Survivin (pg mL^{-1})	-19.45 (-22.6 to-19.45)	-37.91(-48.73 to -28.9)[a**]	-27.1 (-32.95 to -24.84)[a**,b*]	-9.07(-25.29 to 81.05)[b**c*]

Parameters are presented as medians (inter-quartile range) for quantitative variables and as total number (%) for categorical variables. Units are in parentheses. [a]Significant difference from healthy controls, [b]Significant difference from HCV patients, [c]Significant difference from Liver cirrhosis patients,*p≤0.05, **p≤0.01, ***p≤0.001. AFP: Alpha fetoprotein, n: Number of subjects

Table 3: Survivin levels of all the studied patients vs. various clinical parameters (n = 96)

Clinico-pathological features	Number	Survivin (Pg mL^{-1})	p-value
Sex			
Male	66	-26.19 (-32.95 to -17.18)	0.036*
Female	30	-37.91 (-48.72 to -26.19)	
Liver size			
Normal	33	-48.72 (-99.19 to -41.51)	0.084
Enlarged	57	-28.90 (-37.91 to -16.28)	
Shrunken	6	-24.39 (-25.29 to -23.49)	
Spleen size			
Normal	27	-34.30 (-45.12 to -28.9)	0.044*
Enlarged	69	-25.29 (-36.11 to -16.28)	
Ascites			
Negative	69	-32.50 (-41.51 to -25.29)	0.05
Positive	27	-25.29 (-32.5 to -3.66)	
Focal lesions			
Absent	75	-28.90 (-39.71 to -25.29)	0.007*
Present	21	-9.07 (-25.29 to 81.05)	

Data are expressed as median (IQR)

Table 4: Correlation between survivin and AFP and other parameters in all studied patients

	Concentration of survivin (pg mL^{-1})		Concentration of AFP (ng mL^{-1})	
Parameters	r-value	p-value	r-value	p-value
Survivin (pg mL^{-1})	1.000	-	0.333	0.089
AFP (ng mL^{-1})	0.333	0.089	1.000	-
Age (years)	-0.05	0.812	0.286	0.197
WBCs (10^3 µL^{-1})	0.173	0.351	-0.02	0.326
RBCs (10^6 µL^{-1})	-0.117	0.529	-0.438*	0.025
Hemoglobin (g dL^{-1})	-0.038	0.837	-0.312	0.121
platelet count (10^3 µL^{-1})	-0.166	0.372	-0.489*	0.011
total bilirubin (mg dL^{-1})	0.639*	0.014	0.384	0.217
serum albumin (g dL^{-1})	0.484*	0.026	-0.5*	0.034
AST (U L^{-1})	0.364*	0.044	0.398*	0.044
ALT (U L^{-1})	0.302	0.099	0.211	0.301

Comparison of diagnostic efficacy of serum survivin and AFP and their combination: Using ROC curve to differentiate between HCC patients' group over the non-HCC groups (HCV group, LC group and healthy control group), survivin showed the 57.1% sensitivity, 100% specificity, 100% PPV and 91.7% NPV (Table 5, Fig. 1). When we used the curve for AFP to differentiate between HCC patients' group over the non-HCC groups, it reported that AFP to have 71.4% sensitivity, 89.3% specificity, 62.5% PPV and 92.6% NPV. When both markers used together, the sensitivity, NPV improved to be 71.4%, 93.1% (Table 5, Fig. 1).

The sensitivity and specificity of survivin for selective detection of the HCC group over the non-malignant CLD groups (HCV group and LC group) were 85.7 and 72%, respectively, at a cut-off value -26.196 pg mL^{-1}. While the sensitivity and specificity of AFP for selective detection of the HCC group over the non-malignant CLD group were 57.1 and 100%, respectively, at a cut-off value 97.915 ng mL^{-1}. Also, using a combination of both markers improves the sensitivity, specificity, PPV, NPV to be 85.7, 95, 85.7 and 95%, respectively (Table 5, Fig. 2).

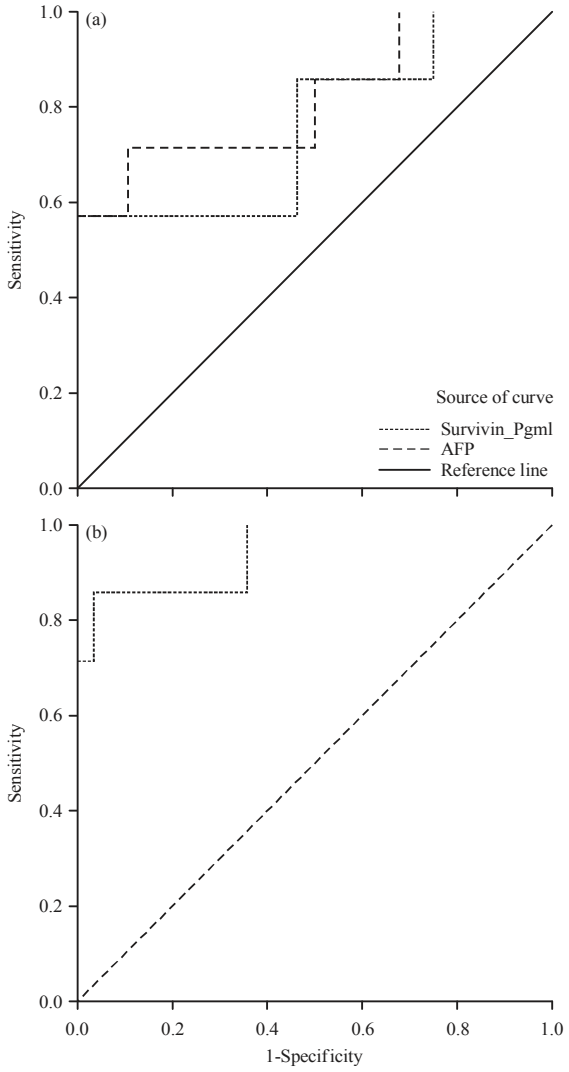

Fig. 1(a-b): Diagnostic value of survivin, AFP and their combination for detection of HCC patients. (a) ROC curves of survivin and AFP for discriminating HCC patients from the non-HCC group (HCV and LC patients and healthy control subjects). The AUC for *survivin* is 0.76 (95% CI 0.532-0.989) and 0.816 for AFP (95% CI 0.611-1) and (b) ROC curves of survivin and AFP combination (AFP+survivin) for discriminating HCC patients group from the non-HCC group (HCV and LC patients and healthy control subjects). The AUC for the combination of serum survivin and AFP in HCC diagnosis was 0.944 (95% CI 0. 845 to 1), which is higher than any one of these two factors alone

Table 5: Diagnostic sensitivity and specificity of serum survivin level of HCC patients in comparison with AFP for selective detection of HCC

Tests	Best cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95% CI)
Healthy controls+HCV+LC patients vs. HCC patients						
Survivin	-11.776	57.1	100.0	100.0	91.7	0.76 (0.532-0.989)
AFP	17.628	71.4	89.3	62.5	92.6	0.816 (0.611-1)
Survivin+AFP	71.400	96.4	83.3	93.1	0.944 (0.845-1)	
HCV+LC patients vs. HCC patients						
Survivin	-26.196	85.7	72.0	46.2	94.7	0.836 (0.649-1)
AFP	97.915	57.1	100.0	100.0	87	0.743 (0.464-1)
Survivin+AFP	85.700	95.0	85.7	95.0	0.957(0.879-1)	

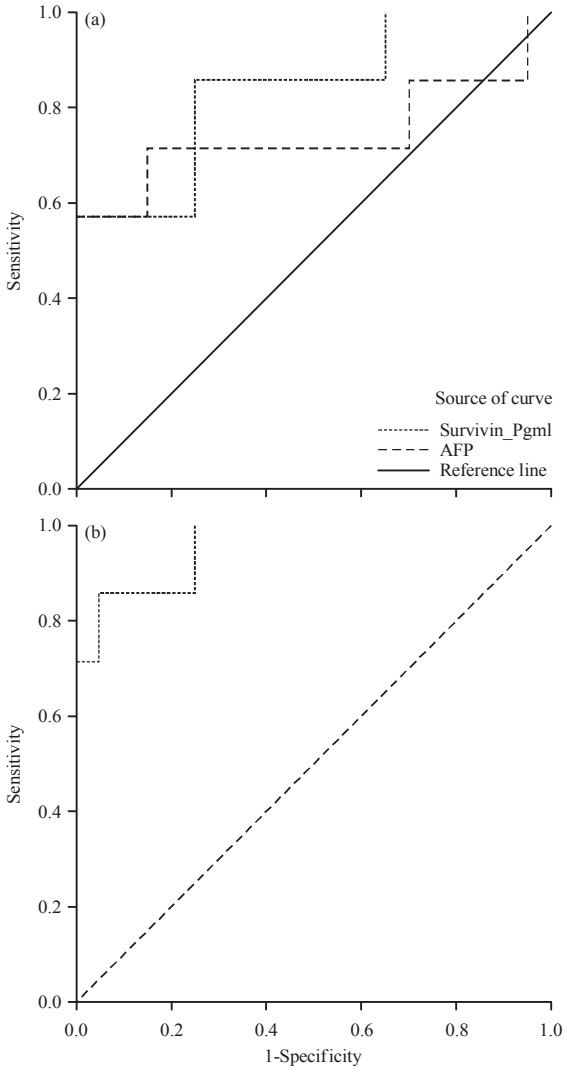

Fig. 2(a-b): Diagnostic value of survivin, AFP and their combination to differentiate between HCC patients from HCV and liver cirrhosis patients (n = 75). (a) ROC curves of survivin and AFP for discriminating HCC patient from the non-malignant CLD patients and (b) ROC curves of survivin and AFP combination (AFP+survivin) for discriminating HCC patients group from the non-malignant CLD patients

DISCUSSION

Liver cancer ranked the sixth prevalent malignant neoplasms and the third frequent cause of deaths from cancer worldwide. Also, it accounts for 7% of all deaths. Hepatocellular carcinoma (HCC) represented more than 90% of primary liver cancers and is a major global concern[13]. The HCC is strongly concomitant with hepatitis viruses B and C virus[14]. The identification and treatment of HCC before it has reached an advanced stage remains crucial to improve the prognosis of patients with this disease[15]. Thus, it is highly recommended to identify markers for screening and diagnosing HCC at an early stage. The use of AFP as a screening test for early HCC is doubtful on account of its limited sensitivity of 39-64%, specificity of 76-91% and low positive predictive value[16] of 9-32%.

Apoptosis has been well-known as a prominent mechanism in the pathogenesis of liver diseases. Apoptosis of hepatic cells occurs in acute and chronic liver diseases. Inflammation, fibrosis and regeneration of the hepatic tissue are associated with apoptotic processes. Apoptosis enables the removal of damaged cells and activates the process of liver fibrosis, which leads to the development of liver cirrhosis[17]. Survivin has a crucial role in cell division, proliferation and inhibition of apoptosis by its ability to inhibit caspases[18]. As a tumor marker, survivin has been studied in different types of cancers[19-22]. The overall survival in several tumors is significantly decreased in patients whose tumor expressing survivin in comparison with those with no expression[23].

This study is based on HCC cases that have HCV infection. The HCC developed on top of HCV-related liver cirrhosis (LC). The median age of the studied HCC cases was 63 years. This was in agreement with Baghdady *et al.*[24], who mentioned that the most frequent age of the HCC patients ranged from 42-70 years. In the present study, all the HCC studied patients were male, this was in agreement with a study conducted in Egypt about the prevalence and the epidemiological characteristics of HCC, which included 265 out of 321 male HCC patients (82.55%) and 56 out of 321 (17.45%) female patients[25] and also with Darbari *et al.*[26], who stated that HCC

occurs more frequently in men than in women. This may be due to the presence of androgen receptors in several malignancies and there is also a male predominance in risk factors. The results of this study showed that the median levels of survivin were undetectable in the majority of the patients and in all normal controls. In HCC group, the percentage of patients with a detectable serum level of survivin was 42.86% (9/21) for those who have multiple focal lesions. Moreover, there was a statistically significant difference in the serum level of survivin in HCC when compared with that in both HCV and liver cirrhosis groups. This can be explained as survivin is plentifully expressed in embryonic tissues for fetal development but its expression is undetectable in almost all terminally differentiated tissues except thymus, endometrium, testis[27]. Increased expression of Survivin inhibits cancer cell apoptosis and could promote cancer cell proliferation, resulting in rapid tumor growth[28]. Additionally, survivin may act in synergy with other factors that promote tumor progression, thereby further affecting the biological behaviors of HCC cells and promoting the malignant progression of HCC[27]. Chiu *et al.*[29] detected that the serum levels of survivin in control group were -266.44±519.86 pg mL^{-1} whereas, in HCC group the median serum level[29] was -1261.09±68.89. Matteucci *et al.*[30] displayed that serum levels of survivin was undetectable in all 62 cases of HCC serum samples that were analyzed. Also, El-Attar *et al.*[31] observed a significant increase in serum survivin level in HCV patients when compared to the control group[31].

In addition, through statistical analysis, the present study revealed that there was an association between survivin levels and sex, spleen size, the presence of ascites and focal lesions ($p<0.05$) which suggested the prognostic role of Survivin in HCC.

The expression of survivin in HCC cell lines via western blot analysis indicated that survivin was expressed by a relatively high level among different cell lines examined[32] including Hep3B, HepG2, PLC/PRF/5 and SK-Hep-1. At the tissue level, Zhu *et al.*[33] found that there was a notable increase in survivin expression between the HCC tumor tissue and liver cirrhosis tissues ($p<0.01$). Survivin protein was identified in 23 of 38 (60.5%) HCCs and 3 of 38 (7.9%) liver cirrhosis tissues. The expression of survivin in HCC was related to the metastasis of HCC ($p<0.05$) by immunohistochemistry and Western blot[33].

CONCLUSION AND RECOMMENDATION

The results of this study imply that serum survivin may be a promising tumor marker that could be added to the existing standard tests for the differentiation of late stage HCC from early stage so as to enhance the follow-up and survival rates of these patients in addition to use of curative treatments among those detected at late stage but additional and larger sample size studies are required to validate the association between high levels of survivin and HCC development. Finally, a combination assay comprising at least 2 or 3 markers in combination with imaging techniques is recommended for a more sensitive and specific diagnosis for HCC.

The small size of this study was due to the cost of kits for the biochemical investigations, we recommend to increase the number of patients in future studies.

All participants in this study are from the Egyptian population and we examined chronic liver disease induced by HCV-infection with no information if our findings would be valid for other patients with different etiologies. Thus, further investigation should be carried out in order to validate these findings.

SIGNIFICANCE STATEMENT

Hepatitis C virus is the most common cause of chronic liver disease in Egypt. The prevalence of antibodies to HCV is approximately 10 folds greater than that in the United States and Europe. This study discovered that serum survivin levels could be used as a biomarker for diagnosis and prognosis the late stage HCC and that can be beneficial for enhancement the follow-up and survival rates of these patients. Also, the present study aims to assess if the combination between survivin and AFP could improve the sensitivity and specificity of its diagnostic properties. Determination of new effective low cost and noninvasive biomarkers may be more valuable for the early diagnosis, prognosis and staging of the disease and can support clinicians in their daily routine. However, analyses tools need to be standardized and simplified in order to be useful, reliable and widely available.

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