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Diagnostic significance of Serum Clustrin and Heat Shock Protein 70 in Hepatocellular Carcinoma Egyptian Patients

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Abstract: Background: The most popular primary liver cancer and the second common reason of cancer-related mortality globally is hepatocellular carcinoma (HCC). In Egypt it is considered the second cause of cancer related mortality among men. Early detection of HCC increases the success of curative treatments. Both US and AFP combined usage improves detection rates, yet expenses and false-positive rates also increase. Increased secreted Clustrin (CLU) expression is correlated with radioresistance, chemoresistance and resistance to hormones, rendering CLU a promising antitumor therapeutic goal. A highly conserved stress response protein is heat shock protein 70 (HSP70). It protects the cell and promotes a range of stimuli to induce repair.

Aim: The objective of this research was to investigate the diagnostic value of CLU and HSP70 in HCC patients in order to improve the outcome of HCC patients through early diagnosis.

Methods: A total of 120 individuals have been enrolled in this research. They were classified into 3 groups: Group 1 involved 10 healthy individuals who served as a control group. Group 2 included 20 patients diagnosed with liver cirrhosis. Group 3 HCC group was divided into two subgroups: Group (3 A): 60 patients with proved HCC before treatment. Group (3 B): 30 patients with HCC were treated using interventional radiology and followed up for 3 months post treatment. Serum levels of AFP, Clustrin and Heat shock protein 70 were assayed using ELISA technique.

Results: The comparison between healthy control group and HCC patients without any intervention revealed that serum CLU cutoff was 132.2 ng/ml with sensitivity of 96%, specificity of 97 % and accuracy of 95 %, furthermore, serum HSP 70 cut off was 38.0 ng/ml with sensitivity of 94%, specificity of 95 % and accuracy of 98 %. While AFP cut off became 114.4 ng/ml with sensitivity of 90 %, specificity of 92 % and accuracy of 91 %. These results indicate that serum CLU level and serum HSP 70 level are better diagnostic markers for HCC detection than serum AFP level.

Conclusion: CLU and HSP70 are promising and potentially complementary candidate biomarkers for effective detection of early stageHCC.

Key words: Clustrin, Heat shock protein 70 and HCC.

INTRODUCTION

The most popular primary liver cancer and the second common reason of cancer-related mortality globally is hepatocellular carcinoma (HCC).[1] The high incidence of HCV infection, accompanied by increasing rates of obesity, alcohol abuse and uncontrolled type II diabetes, is the risk factor that mainly leads to the increase in HCC.[2]Egypt has one of the largest burdens of

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worldwide hepatitis C virus (HCV) infection; it is predicted that HCV incidence is about 4.5% to 6.7%.[3]

Radiological and/or laboratory techniques are used to diagnose HCC. Ultrasonography (US), triphasic computerized tomography (triphasic CT-scan) and dynamic magnetic resonance imaging (dynamic MRI) are primarily relays of radiological diagnosis. The US sensitivity of HCC detection is directly correlated to the size of the tumor. Another main downside of the US is that it is very reliant on operators.[4]Laboratory diagnosis of HCC, on the other hand, is developed either by measuring circulating biomarkers or by fine-needle cytology that is invasive with intra-or inter-observer variance.[5]

Surveillance is the only practical approach to improve the management of HCC. Early detection of HCC increases the success of curative treatments. [6] Serological tests include AFP with a cut off value 200 ng/ml, is suggested to provide the optimal balance between sensitivity and specificity. However, its estimated sensitivity is only 60%. Therefore, AFP is considered an inadequate screening test. [7] US is widely used for surveillance. Its specificity is greater than 90%. Both US and AFP combined usage improves detection rates, yet expenses and false-positive rates also increase.

The European Association for the Study of Liver Disease (EASL) and the American Association for the Study of Liver Disease (AASLD) have recommended guidelines for practice requiring further analysis of nodules found with dynamic imaging technology during U.S. surveillance, including contrast-enhanced U.S. or dynamic MRI, to demonstrate the various vascular supplies of HCC versus non-malignant entities.[6] In addition to biannual follow up to determine tumor doubling time.[8]

The early diagnosis of HCC is needed as only 44% of the patients are diagnosed at a localized stage of the disease, leading to limited treatment choices and poor prognosis. The identification of diagnostic and prognostic biomarkers is a significant problem, as these indicators can make it easier to diagnose HCC. In addition, these biomarkers can provide possible therapeutic targets for HCC.[9&6]

Heat shock protein 70 (HSP70) is a highly conserved stress response protein. It protects the cell and promotes a range of stimuli to induce repair. HSP70 is expressed under physiological and stress conditions, including carcinogenesis.[10] Hsp70 is an effective inhibitor of apoptosis and is often constitutively over expressed in tumors.[11]In cancer patients, serum HSP70 levels have been shown to be substantially higher relative to healthy people, sufferers without liver diseases and hepatitis.[12]

Clusterin (CLU) is a 449-amino acid, heterodimeric glycoprotein which is expressed and found in the majority of body fluids. Radioresistance, chemoresistance and hormonal resistance are correlated with increased expression of the secreted CLU, rendering CLU a promising goal for antitumor therapies.[13]Two proteins encoded by this gene are present in humans: secretory CLU protein (sCLU) (75-80 kDa) and nuclear CLU protein (nCLU) (55 kDa).[14&15]

PATIENTS AND METHODS

Study population and demographic information

This study was conducted at Hepato-Gasteroenterology department, Theodor Bilharz Research Institute and written informed consent was given by all participants. Within the ethical guidelines of the Helsinki Declaration of 1975.A total of 120 individuals were enrolled in this research. They have been categorized into 3 groups:

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Group I involved 10 healthy individuals who served as a control group (6 males/ 4 females) and whose age ranged from 32 to 70 years (mean ± SD =53.2±16.7).
Group II included 20 patients diagnosed with liver cirrhosis (12 males/ 9 females), and their age ranged from 43 to 58 years (mean± SD=50.5±7.5). **Group III** HCC group, which divided into two subgroups:
- **Subgroup (3A):** 60 patients with proved HCC with no history of any radiological intervention or any intervention for HCC (Before treatment), (50 males/ 10 females), and their age ranged from 46 to 66.5 years (mean± SD= 56.7±9.8).
- **Subgroup (3B):** 30 patients with HCC in group 3A were continue follow up after 3 months of treatment (interventional radiology) (25 males/ 5 females) whose age ranged from 53 to 62 years (mean ± SD =57.7±4.6).
Exclusion criteria for study and for interventional radiology included malignancy other than HCC, hepatic metastatic lesion , Patients receiving antitumor treatment, Patients with history of ischemic cardiac disease in the preceding six months, Uncontrolled hypertension, unstable angina, inadequate kidney functions (S.creatinine >1.5 mg/dl), Obesity and overweight.
In complicated cases, all patients included in this research underwent full medical history and complete clinical assessment with special liver and splenic stress (hepatosplenomegly), presence of ascites, symptoms of failure of liver cells such as jaundice, palmer erythema, lower limb edema or encephalopathy.

Laboratory investigations including:

- Complete blood picture was performed using automated cell counter, Medonic, Boule Diagnostics, Sweden. Prothrombin time (PT), Prothrombin concentration (PC) and international normalized ratio (INR) was performed using fully automated haemostasis analyser STA *Compact*®, Diagnostica Stago, Spain. Blood Chemistry including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total/ direct serum bilirubin, total protein, serum albumin and alk.phosphatase (ALP), serum creatinine and serum urea were performed on Olympus AU480 Chemistry Analyzer, Beckman Coulter, USA. Viral markers including HCV immunoglobulin G (HCV IgG) and hepatitis B surface antigen (HBsAg) were performed on ADVIA Centaur CP Immunoassay System, Siemens, Germany.
    - Serum AFP concentrations were determined using Human Alfa–feto protein (α-FP) ELISA kit, Cat. No. ELH-AFP, RayBio® Inc., Georgia, USA. Serum Clustrin and HSP70 concentrations were assayed using Human Clusterin (CLU), ELISA Kit, Cat. No. In-Hu3203 INOVA® andHuman Heat Shock Protein 70 (HSP70) ELISA Kit, Cat. No. In-Hu3299 INOVA®, both from Innova Biotech Co Limited, Beijing, China. The optical densities of standards and samples were determined using microplate reader (Tecan, Sunrise™Japan). The standard curve was created by decreasing the data using Magellan™ softwarethat can produce a log/log curve-fit.

Imaging studies:
Abdominal ultra-sonography (U/S) and Tri-phasic CT (Triphasic abdominal CT scan was performed on HCC group only. Evaluation of the severity of liver cirrhosis was obtained for all cirrhotic patients with Child Pugh Score.

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Statistics analysis methods:

Using an unpaired (t) test, the data is expressed as a mean ± SD or number (%) comparison among the mean values of the different parameters in the two groups. Comparison among categorical data was carried out using (Chi Square test) correlation between different variables was performed using (pearson correlation), version of the SPSS software (12) was being used in data analysis (p) value less than or equal to 0.05 was regarded to be significant and < 0.01 was regarded to be highly significant.

RESULTS

Table (1): Clinical presentations of patients of the studying groups.

	G2(N=20)	G3		P. value		
		A(N=60)	B(N=30)	A	B	C
Asymptomatic	12 (60%)	6 (10.0%)	6 (20%)	0.001**	0.09	0.001**
Rt hypochondrial pain	3 (15%)	42 (70.0%)	28 (93.3%)	0.001**	0.001**	0.001**
Anorexia & weight loss	4 (20%)	48 (80.0%)	19 (63.3%)	0.001**	0.001**	0.9
Splenomegaly	18 (90%)	24 (40.0%)	25 (83.3%)	0.09	0.09	0.9
Low grade fever	15 (75%)	18 (30.0%)	6 (20.0%)	0.001**	0.001**	0.3
L.L edema	14 (70%)	24 (40.3%)	14 (46.7%)	0.08	0.02*	0.003**
Hepatic Encephalopathy	14 (70%)	18 (30.0%)	8 (26.7%)	0.03*	0.001**	0.004**
History of Jaundice	14 (70%)	24 (40.0%)	22 (73.3%)	0.06	0.08	0.6
History of Ascites	14 (70%)	0	0	0.06	0.001**	0.001**
Radiological finding						
Ultrasound -Liver Cirrhosis	20 (100.0%)	59 (98.34%)	30 (100.0%)	0.07	0.9	0.9
Splenomegaly	18 (90%)	56 (93.34%)	29 (97.0%)	0.09	0.03*	0.1
-Ascites	14 (70%)	0	0			
-PVT	0 (0.0%)	20 (33.3%)	0 (0.0%)	0.002**	-	0.002**
-Focal lesion	0(0.0%)	60(100.0%)	-	-	-	-
CT (Focal lesion)	0(0.0%)	60(100.0%)	0%	-	-	-

All parameters are represented as frequency and percent.
P. value bearing (a) initial is significantly different between G2 and G3A groups.
P. value bearing (b) initial is significantly different between G2 and G3B groups.
P. value bearing (c) initial is significantly different between G3A and G3B groups.
*P value ≤ 0.05 significant while **P value ≤ 0.01highly significant.

Table (2): Child-Pugh classification in patients of the studied groups.

		G2 (N=20)	G3		P. value		
			A (N=60)	B (N=30)	A	B	C
Child	**A**	7 (35.0%)	12 (20.0%)	12 (40.0%)	0.001**	0.001**	0.001**
	B	9 (45.0%)	30 (50.0%)	18 (60.0%)			
	C	4 (20.0%)	18 (30.0%)	0 (0.0%)			

*P value ≤ 0.05 significant while **P value ≤ 0.01highly significant.

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Table (3): Comparison between all studied groups as regard serum clusterin (ng/ml) and heat shock protein 70 (ng/ml)

Serum clustrin assay range: 2 ng/ml – 140 ng/ml)						
G1(N=10)	**G2**(N=20)	**G3A**(N=60**)**	**G3B** (N=30	P. Value		
				A	B	c
10.9±0.5 (2 - 22)	11.2±0.6 (6 – 41)	457.2±24.0 (205 – 880)	115.5±2.9 (95 – 136)	0.01*	0.001**	0.001**
Serum HSP70 assay range: 0.8 ng/ml – 40 ng/ml						
3.5±0.3 (1 – 8.6)	6.6±2.2 (3 – 15.2)	87.8±3.7 (53 – 166)	33.8±0.5 (20 – 36.5)	0.04*	0.03*	0.001**

*P value ≤ 0.05 significant while **P value ≤ 0.01highly significant.

Regarding serum level of AFP There was highly significant increase in G3a (240.1ng/mL) compared to other groups G1 (2.1 ng/mL) *(P* value= 0.001), G2 (10 ng/mL) and G3B (80 ng/mL) (*P* value= 0.01,).
The comparison between healthy control group (G1) and cirrhotic (G2) patients revealed that CLU cutoff was 11.1 ng/ml with a sensitivity of 57%, specificity of 77 % and accuracy of 65 %. Moreover, HSP 70 cutoff was 3.5 ng/ml with sensitivity of 76%, specificity of 69 % and accuracy of 74 %, on the other hand, AFP cut off 4.4 ng/ml with sensitivity of 95 %, specificity of 92 % and accuracy of 94 %.(Fig1)

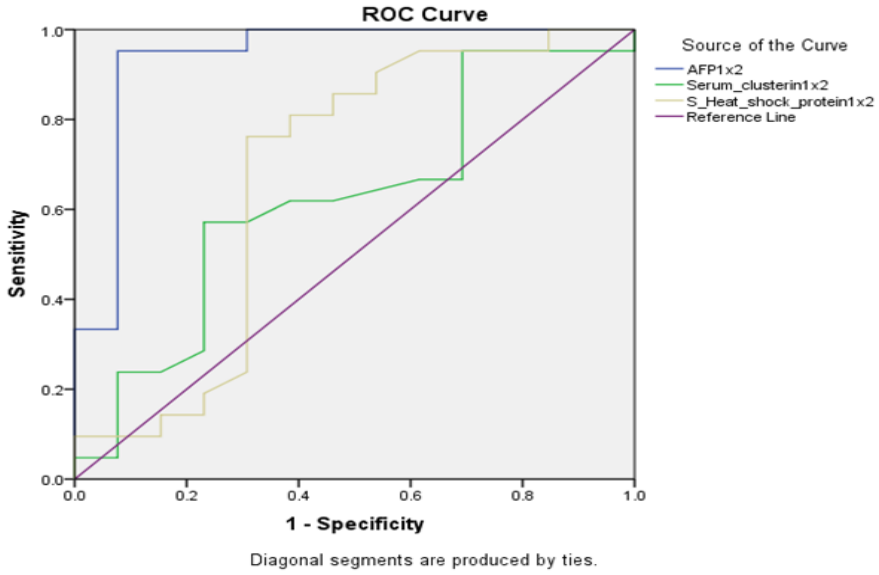

Fig. (1): Receiver operating characteristic (ROC) curve of S.CLU and S.HSP 70 as a diagnostic marker for HCC with normal or high Alpha Fetoprotein level. **(G1 X G2)**
The comparison between healthy control group (G1) and HCC patients without any intervention (G3A) revealed that serum CLU cutoff was 132.2 ng/ml with sensitivity of 96%, specificity of 97 % and accuracy of 95 %, furthermore, serum HSP 70 cut off was 38.0 ng/ml with sensitivity of 94%, specificity of 95 % and accuracy of 98 %. While AFP cut off was 114.4 ng/ml with sensitivity of 90 %, specificity of 92 % and accuracy of 91 %.(Fig 2)

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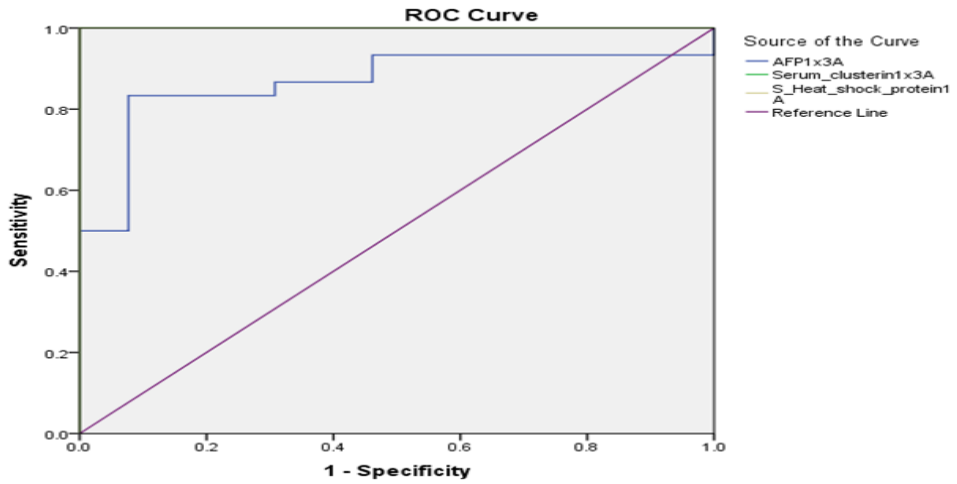

Fig. (2): Receiver operating characteristic (ROC) curve of S.CLU and S.HSP 70 as a diagnostic marker for HCC with normal or high Alpha Fetoprotein level. **(G1 X G3A)**

The above results indicate that serum CLU level and serum HSP 70 level are better diagnostic markers for HCC detection than serum AFP level.

There was a positive association among AFP and CLU, AFP and HSP 70 (r: +0.425, +0.274 respectively) and There was a positive association among CLU and AFP, CLU and HSP 70 (r: +0.425, +0.778 respectively). Also, there was a positive correlation between HSP 70 and AFP, HSP 70 and CLU (r: +0.274, +0.778 respectively).

There was a positive association among AFP and both AST and ALT (r: +0.241, +0.258 respectively). Also, WBC count showed positive correlation with AFP (r: +0.400). In addition, there was a positive correlation between CLU and both AST (r: +0.366) and WBC count (r: +0.228). Finally, there was a positive correlation between HSP 70 and AST (r: +0.382).

DISCUSSION

HCC accounts for more than 80% of the world's primary liver cancers. HCC has a heavy disease burden and is a major cause of cancer-related mortality in several regions of the world and is reported to be the world's most common cause of cancer-related mortality.[16] For men, the risk is higher than for women, and it rises as they get older.[17]

It is believed that hepatocellular carcinoma advances through a multi-step process in which dysplastic nodules form in the cirrhotic liver, advancing with the accumulation of genetic mutations to HCC. The development of neoplasia involves many components including, insensitivity to growth-inhibitory signals, inhibition of apoptosis, unlimited replicative potential, tissue invasion through metastasis and persistent angiogenesis.[18]

HCC surveillance is one of the preventive approaches to reduce the burden of HCC via early tumor identification and effective early management. In patients with liver cirrhosis, HCC monitoring is suggested.[16]

The standard HCC monitoring test approved by the American Association for the Study of Liver Diseases is liver ultrasonography (AASLD).[16] Detection of high serum AFP levels is widely employed as an adjunct to liver ultrasonography in blood-based surveillance studies. The normal serum AFP level range is 10-20 ng/ml and the diagnostic value is commonly considered to be >400 ng/ml. Nevertheless, two-thirds of HCC sufferers with a nodule smaller than 4 cm of serum

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AFP >200 ng/ml do not have AFP in up to 20 % of HCC sufferers. However, the lack of AFP specificity and sensitivity has clarified the need for a new tumor marker to distinguish HCC from benign liver disorders. [18]

Recent studies have identified *HSP70* gene as the most abundantly up regulated gene in early HCC. Similarly, CLU has been suggested as a viable biomarker for the early detection of HCC.[19] Secretory CLU provided a potential molecular target for HCC therapy.[20]

Precision medicine with novel adjuvant therapy using *HSP70* mRNA transfected dendritic cell (DC) improved prognosis of HSP70-expressing HCC cases.[21]HCC patients with elevated CLU expression also showed poor oxaliplatin response.[22]

The aim of this research was to examine the diagnostic value of CLU and HSP70 in HCC patients in order to improve the outcome of HCC patients through early diagnosis.

The demographic data retrieved from this study revealed a significant older age difference between HCC group and other studied groups in agreement with the report from **Velazquez et al.**[23]who observed that patients over 54 years of age were four times at risk of developing HCC than younger ones. The data also revealed that 83.3 % of HCC patients are males andthis is compatible with the study conducted by El-Zayadi et al., [24] who noticed that HCC was more prevalent in males than females. The authors stated that hepato-carcinogenesis could be modulated by estrogens and androgens and attributed the higher occurrence of HCC in males due to differences in exposure to risk factors.

In the current research, there was a significant association among HCC and smoking. These results were in agreement with **Siegel et al.** [25] who recorded that 50.2% of patients with HCC had a smoking history. Heavy smokers have an increasing risk about 50% higher than nonsmokers, this may be due to presence of cytochrome P450 system which is highly inducible by smoking mainly cytochrome 1A1 that is a type of cytochrome p450 that is present in the lung and is important for metabolizing polycyclic aromatic hydrocarbons inhaled by smoking converting these procarcinogen into active carcinogen by hydroxylation reactions.[26]

Viral markers were analyzed in all subjects enrolled into the current study. The data showed that almost all HCC patients (96.7%) were HCV infected. These results are consistent with data from **Atti** [27]. While HBV is regarded worldwide as a significant risk of liver cirrhosis and HCC, over the past 2 decades, the incidence of HBV infection in Egypt has decreased, whereas HCV has risen. Egypt may have the highest prevalence of HCV globally. [28]

The Liver function tests of patients enrolled in this study showed a highly significant difference between the liver cirrhosis and HCC without radiological intervention groups regarding AST. **Wang et al.** [29] evaluated the impact of hepatitis using AST and ALT as indices for injury to hepatocytes. The significant elevation of both AST and ALT with high level of AFP may be due to their release into the blood from damaged hepatocytes, their activities have been broadly known as useful tools for detecting liver diseases like HCC. [30]Also, cases of HCC were associated with low platelet counts. This finding is in agreement with **Velazquez et al.,** [31] Furthermore; it reflects a greater hepatic dysfunction and highlights the consequences of chronic disease.

This study revealed a highly significant difference in AFP levels between HCC and control group. In agreement with **Wang et al.**[29]who found increased AFP level in HCC patientscompared to control.

The current study revealed that serum CLU levels in the HCC group were higher compared to the control and liver cirrhosis groups, suggesting its function in carcinogenesis. Similarly, **Nafee et al.**[32]recorded a substantial increase in serum CLU in HCV-related HCC sufferers.

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Furthermore, **Kang et al.**[33] used tissue microarray to reveal immunohistochemical overexpression of CLU in surgically resected HCCs. Additionally, **Hsieh et al.** [34]indicated that in human hepatoma, dysregulation of the CLU gene was most probably attributed to cellular responses to external stresses or micro-environmental shifts that may have a significant impact on gene or protein expression.

The current study indicated that serum HSP 70 was higher in the HCC patients compared to both liver cirrhosis and control groups. This finding denoted the role of HSP70 in hepato-carcinogenesis. Also, **Cervello et al.** [35]and **Gehrmann et al.** [12]noticed that serum HSP70 levels in HCC patients were substantially higher relative to healthy people and hepatitis patients. The authors reported that HSP70 serum may be a beneficial biomarker for the differentiation between HCC and other hepatic diseases.

Several studies have investigated the diagnostic efficiency of biomarkers under study in HCC. In the current research, the comparison between the control group (G1) versus HCC patients without any intervention (G3A) revealed that serum CLU was more sensitive, specific and accurate than AFP. This indicated that CLU was superior to AFP as a diagnostic marker in HCC patients.

Similarly, **Wang et al.**[29] demonstrated that serum CLU with a 50 ng/ml cutoff level outperformed serum AFP with a 15 ng/ml cutoff level in diagnostic sensitivity (91% versus 67%), specificity (83% versus 76%), predictive values that are positive and negative (93% versus 88% and 77% versus 47% respectively).

Additionally, the study by **Kimura et al.** [36]performed three-step proteome analyses on serum samples of HCC patients and 83 up-regulated proteins were found, of which CLU was the most substantially over-expressed.Using another group of HCC specimens, the overexpression of CLU was verified by ELISA and the authors indicated that CLU was a possible novel serum indicator for HCC. Nafee et al., 2012 also confirmed the same finding**.**[32]

On the contrary, **Ramadan et al.** [37]concluded that serum AFP with a 137 ng/ml cutoff level outperformed serum CLU with a 135 ng/ml cutoff level in diagnostic sensitivity (77.3% versus 70.5%), specificity (100% versus 90%), predictive values that are positive and negative (100% versus 86.1% and 83.3% versus 77.6%, respectively). In addition, the authors used combined parallel method to improve diagnostic sensitivity (95.5 %) and negative predictive value (95.7 %) over the single use of AFP, but this led to a decrease in specificity of 88 % and a positive predictive value of 87.5%. Therefore, combined parallel approach improved the sensitivity at the expense of the specificity.

The present study revealed a highly significant difference regarding the serum level of HSP 70 in HCC patients before treatment and HCC patients post treatment. This finding is attributed to the ability of HSP70 to directly inhibit apoptosis as it is an effective inhibitor of apoptosis which is constitutively over expressed in tumors. The anti-apoptotic potential of HSP70 should draw attention towards the attractive role it could play in targeted therapy for cancer [38&11].

A positive association among AFP and both CLU and HSP70 was found in this study. Also, there was a positive association among CLU and HSP70. Furthermore, there was a positive correlation between both CLU, HSP70 and AST. These results were in agreement with **Ramadan et al.**[37]and **Mostafa et al.** [39].

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CONCLUSION

Serum CLU and HSP70 levels in HCC patients were significantly higher than in non-HCC patients. CLU and HSP70 are promising and potentially complementary candidate biomarkers for effective detection of HCC at early stage. AFP shouldn't be used as the sole diagnostic biomarker for HCC. The combined assay of serum AFP, CLU and HSP70 would provide a sensitive, rapid and easily accessible method for improving the diagnostic performance of laboratory screening for HCC. Further studies with large sample size are required to investigate and validate CLU and HSP70 as reliable sensitive and specific serum biomarkers for early HCC detection.

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