Corroboration of Serum Apolipoprotein J (Clusterin) as a Biomarker for Evaluating Hepatocellular Carcinoma

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Background and study aim: Hepatocellualr carcinoma (HCC) is an increasing problem in Egypt. Clusterin has been reported to play a significant role in tumorigenesis. The aim of this study is to evaluate clusterin as a marker for evaluating diagnosis and metastasis potential of HCC.

Patients and Methods: Eighty patients with HCC, 30 patients with liver cirrhosis, 30 patients with chronic hepatitis and 30 healthy controls were enrolled in study. The diagnosis of HCC patients was based on computed tomography. Estimation of serum clusterin was done by enzyme linked immunosorbent assay.

Results: Serum clusterin levels were significantly increased in patients with HCC ($P<0.001$). Serum clusterin reached the lowest significant levels in cirrhotic patients. Serum clusterin was highly increased in patients with poorly differentiated tumor and in those with capsular infiltration; also it was significantly related with portal vein invasion and lymph node infiltration. In addition, serum clusterin levels were significantly increased according to the progression of Barcelona Clinic Liver Cancer and Tumor-Nodes-Metastasis staging systems. However, these findings were not observed with alpha fetoprotein (AFP). Receiver operator characteristic curve showed that clusterin had a greater area under curve value (0.95) than that of AFP (0.85). At cutoff value 128 ug/ml, serum clusterin yielded 90% sensitivity and 87% specificity for predicting HCC. While at cutoff value 100 ng/ml, serum AFP had 75% sensitivity and 80% specificity.

Conclusion: We concluded that serum clusterin is a promising useful marker for diagnosis of HCC. Higher level of clusterin was closely related to capsular infiltration, venous invasion, lymph node metastasis and poorly differentiated tumor suggesting that clusterin might be deemed as a useful marker for predicting the progression and metastasis potential of HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide [1]. Hepatocellular carcinoma is currently the main cause of death in patients with hepatitis C virus (HCV) related cirrhosis, and the issue of HCC in Egypt is extensively increasing. In 2001, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients [2]. Over a decade, there was nearly two fold increase of the proportion of HCC among CLD in Egypt with significant decline of hepatitis B virus and slight increase of HCV as risk factor [3]. Hepatocellular carcinoma is often diagnosed at advanced stage where effective therapies are lacking, so the surveillance of patients at risk is necessary [4].

Clusterin (apolipoprotein J) is a highly conserved multifunctional glycoprotein present in almost all mammalian tissue and most human body fluid [5,6]. It has a wide degree of conservation and a wide degree of tissue distribution suggesting that it has a fundamental biological role. Its action resembles that of small heat

shock protein (sHsPs) since it binds to exposed hydrophobic regions of unfolded protein and inhibit aggregation by stabilizing them in an adenosine triphosphate independent manner [7,8]. Clusterin is implicated in various physiological processes including lipid transport, reproduction, complement regulation, tissue remodeling, senescence and cell interaction [9,10]. It has also been reported to play a significant role in stress response [11], apoptosis [12] and tumorigenesis [9]. In many diseases including human cancers, the expression status of clusterin might change at mRNA and protein levels. Some reports documented that a decrease of clusterin was observed in non-melanoma skin cancer [13], esophageal cell carcinoma [14], prostatic carcinoma [15]. However, in the majority of other human cancer such as, breast [16], lung [17], bladder [18] and colon cancers [19], upregulated expression of clusterin was detected. These reports suggested that changed expression of clusterin whether upregulated or downregulated may play an important role in tumorigenesis.

OBJECTIVE

This work aimed to investigate the role of serum clusterin as a biomarker for evaluating diagnosis and metastasis potential of HCC.

PATIENTS AND METHODS

This study was done in Tropical Medicine, General Surgery, Medical Biochemistry and Pathology Departments, Faculty of Medicine, Zagazig University. One hundred and seventy subjects were enrolled in this study. The subjects were classified into 4 groups:

Group 1: This group included 80 patients with established HCC (60 males and 20 females) with a main age of 55.5±6.4 years. The diagnosis of HCC was based mainly on typical imaging study and histopathology study (if available) according to American Association for Study of Liver Disease (AASLD) guideline [20]. Hepatocellular carcinoma tissue was diagnosed histologically by 2 expert pathologists when the surgical liver specimens were available (25 patients) in case of respectable hepatocellular carcinoma, while the remaining HCC patients were diagnosed according to imaging. Hepatocellular carcinoma tissues from 25 patients were histologically graded into one of three categories; well differentiated, moderately differentiated, or poorly differentiated according to criteria proposed by Liver Cancer Study Group of Japan [21]. The Barcelona Clinic Liver Cancer (BCLC) staging system was obtained which accounts for different factors of performance status, tumor burden, and hepatic function and categorizes patients into 5 stages which then help select the ideal candidates for the therapies currently available [22]. Tumor-Nodes-Metastasis (TNM) stage of HCC determined by The American Joint Committee on Cancer/United International Consensus Committee (AJCC/UICC) staging system for HCC [23] was obtained on the basis of imaging studies.

Group 2 : This group included 30 patients with viral related liver cirrhosis (16 males and 14 females) with mean age of 54.7±7.4 years. The diagnosis of liver cirrhosis was established on the basis of clinical, laboratory, imaging and histo-pathological examination.

Group 3: This group involved 30 patients(18 males and 12 females and their mean age of 53.9±9.6 years) with chronic viral hepatitis who were diagnosed by persistent elevation of ALT 3 times more than normal value for more than 6 months with no evidence of liver cirrhosis as confirmed by liver biopsy and histopathological examination.

Group 4: This group included 30 healthy age and sex matched controls with normal liver function (20 males and 10 females) with a mean age of 54.5±16 years.

All patients underwent complete history taking and thorough clinical examination, triphasic computed tomography (CT) and liver biopsy (when available) for histo-pathological examination.

Biochemical measurements:

Blood samples were drawn from all subjects after an overnight fast. Sera were separated immediately and stored at −20°C. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), Albumin, Alkaline phosphatase, bilirubin, prothrombin time and creatinine were measured in serum by routine enzymatic methods (spinreact). Serum Alph-fetoprotein (AFP) concentration was measured by ELISA kit (Biosource Europe S.A, Belgium). Serum HBsAg, HCV antibodies were measured by ELISA (Abbott laboratories, North Chicago,IL). Polymerase Chain Reaction (PCR) was used for detection of HCV RNA and HBV DNA.

Estimation of serum clusterin concentration in patients and controls:

Estimation of serum clusterin using Sandwich ELISA method according to manufacturer's instructions (Human clusterin ELISA, Bouendor laboratories Ltd Modrice Czech Republic) All diluted samples, quality controls were incubated in microtitration wells pre-coated with monoclonal antihuman clusterin antibody. After 10 min and washing, biotin-labeled second monocloncal antihuman clusterin antibody was added and incubated with captured clusterin for 60 min. After another washing, streptavidin-horse radish peroxidase (HRP) conjugate was added. After 30 min incubation and the last washing step, the remaining conjugated was allowed to read with substrate solution hydrogen peroxide and tetramethylbenzidine (TMB). The reaction was stopped by the dilution of acidic solution (0.2 M H_2SO_4) and absorbance of the resulting yellow product was measured spectrophotometrically at 450 nm. The absorbance was proportional to the concentration of clusterin. A standard curve was constructed by plotting absorbance value versus clusterin concentration of standards, and concentrations of unknown samples are determined using this standard curve.

Statistical analysis:

Data were analyzed with SPSS for version 15.0 (statistical package for the Social Science, Chicago, IL). Data were expressed using descriptive statistic (mean and standard deviation, and percentage and were analyzed using "*t*" and Chi-square tests. One way analysis of variance (ANOVA) test was done to compare of different parameters between more than two groups. AFP was expressed as median (range) and data analysis was done using Mann Whitney and Kruskal-Wallis tests. Pearson correlation coefficient was used to measure the association between clusterin and the other studied parameters. The receiver operator characteristic (ROC) curve with 95% confidence interval (CI) was performed to determine cutoff values for serum clusterin and AFP. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined. *P*-value was considered significant if <0.05 and highly significant if <0.001.

RESULTS

Clinical characteristic of HCC patients showed that most of them were males (n = 64), their mean ages (55.5±6.4) and most of them had hepatitis C virus (80%) (Table1). Serum clusterin levels were significantly elevated in HCC patients when compared to other groups (P<0.001). Cirrhotic patients had the lowest significant level of serum clusterin when compared to other groups (Table 2 and Fig. 1).

Serum clusterin levels were investigated according to various clinico-pathological features. There were no significant differences of serum clusterin levels according to size and number of tumor nodules (P>0.05). However, serum clusterin level was significantly overexpressed in patients with capsular infiltration, portal vein invasion, lymph node infiltration and poorly differentiated tumor. On the other hand, apart from significant increased of AFP according to the progression of size and increased numbers of tumor nodules, there were no significant differences according to other clinico-pathological parameters (Table 3).

Serum clusterin levels were significantly increased according to the progression of BCLC (Table 4) and TNM (Table 5) staging systems. However, these findings were not observed with AFP.

Correlation studies between serum clusterin and other parameters showed that serum clusterin had positive correlation with AFP with absence of correlation with Child- Pugh scores (Table 6).
Clusterin had greater area under curve (AUC) = 0.95 (CI; 0.90-0.99) than that of AFP = 0.85 (CI; 0.76-0.94) (Fig. 2). At cutoff value 128 ug/ml, serum clusterin had 90% sensitivity and 87% specificity. While at cutoff value 100 ng/ml, AFP had 75% sensitivity and 80% specificity (Table 7).

Table (1): Clinical characteristic of patients with HCC, cirrhosis and chronic hepatitis.

Parameters	HCC (N = 80)	Cirrhosis (N = 30)	Ch. hepatitis (N=30)	*P*
Age (years) X±SD	55.5±6.4	54.7±7.4	53.9±9.6	0.06 (NS)
Sex (n & %):				
Males	64(80%)	20 (66.7%)	21(70%)	0.06 (NS)
Females	16(20%)	10(33.3%)	9(30%)	0.06 (NS)
Viral cause (n & %):				
HCV	64(80%)	25(83.3%)	27{90%)	0.46 (NS)
HBV	12(15%)	3(10%)	2(7%)	0.4 (NS)
Both	4(5%)	2(6.7%)	1(3%)	0.8 (NS)
Child classification (n & %):				
A	42(52.5%)	15(50%)	30(100%)	<0.001 (HS)
B	32(40%)	12(40%)	0(0%)	<0.001 (HS)
C	6(7.5%)	3(10%)	0(0%)	0.2 (NS)
Alanin aminotransferse ALT (U/L)	73.2±23.5	59.2±19.5	85.2±24.5	<0.001 (HS)
Aspartate aminotransferse AST (U/L)	71.1±21.5	60.6±17.5	79.5±22.5	<0.001 (HS)
Alkaline phosphatase (U/L)	300.3±20.3	133.2±34.2	73.1±11.3	<0.001 (HS)
INR	1.2±0.4	1.34±0.3	1.1±0.3	0.29 (NS)
Albumin (g/l)	3.5±1.1	3.4±1.3	4.1±1.4	0.02 (S)
Bilirubin (mg/dl)	2.5±0.8	1.9± 0.5	1.2±0.4	<0.001 (HS)

HS=Highly significant S=Significant NS= Non significant

Table (2): Serum clusterin and AFP levels in HCC, cirrhosis, chronic hepatitis and healthy controls.

Groups	Serum clusterin level M±SD	Serum AFP level Median (range)
(G1) HCC (n = 80)	198.5±55.8	209(10-570)
(G2) Cirrhosis (n = 30)	44.4±6.9	35(4-210)
(G3) Ch. Hepatitis (n = 30)	117±18.5	10(1.7-135.1)
(G4) Healthy controls (n = 30)	113.1±18.3	2.3(1.5- 5.1)
F	122.3	106.7
P	<0.001(HS)	<0.001(HS)

AFP was expressed as median (range) and data analysis was done using Kruskal-Wallis test

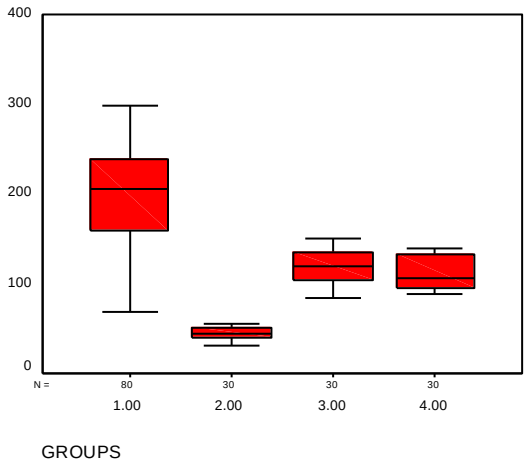

Fig. (1) : Serum clusterin in HCC, cirrhosis, chronic hepatitis and healthy controls

Table (3): Serum clusterin and AFP levels according to different clinico- pathological features.

Parameters	serum clusterin Mean ±SD	*P*	Serum AFP Median (range)	*P*
Tumor size:				
< 5 cm (n = 62)	198.9±55.9	0.73 (NS)	197(10-500)	0.005 (S)
≥ 5 cm (n = 18)	201.1±51.2		518(150-570)	
No of nodules:				
< 2 nodules (n = 66)	197.6±53.8	0.85 (NS)	200(10-518)	<0.001 (HS)
≥ 2 nodules (n = 14)	201±67.2		528(208-570)	
Infiltration of Glisson capsule:				
With capsular infiltration (n = 23)	240.3±31.5	<0.001 (HS)	200(18-570)	0.09 (NS)
Without capsular infiltration (n = 57)	180.5±54.6		238(10-538)	
Portal vein invasion:				
With portal vein invasion (n = 25)	242.3±28.4	<0.001 (HS)	286(15-570)	0.41 (NS)
Without portal vein invasion (n = 55)	177.4±53.6		208(10-538)	
Lymph node metastasis:				
With lymph node metastasis (n = 12)	260.1±58.19	<0.001 (HS)	249(20-570)	0.051 (NS)
Without lymph node metastasis (n =68)	183±51.1		207(10-528)	
Degree of differentiation (25 specimens):				
Well (n = 7)	145.5±41.5	<0.001 (HS)	169(21- 375)	0.16 (NS)
Moderate-poor (n = 18)	227.03±39.2		220 (31-538)	

NS = Non significant HS = Highly significant

AFP was expressed as median (range) and data analysis was done using Mann Whitney test

Table (4): Serum clusterin and AFP levels according to Barcelona classification.

Groups	Serum clusterin level M±SD	Serum AFP level Median (range)
Very early(n=38)	155.1±42.9	195(10-570)
Early (n=12)	207.5±19.9	323(10-570)
Intermediate (n=4)	211.5±7.5	315(130-500)
Advanced (n=16)	245.7±18.4	312.5(21-538)
Terminal (n=10)	271.8±16.6	375(19-528)
F	37.8	9.02
P	<0.001(HS)	0.06(NS)

HS= Highly significant NS= Non significant

AFP was expressed as median (range) and data analysis was done using Kruskal-Wallis test

Table (5): Serum clusterin and AFP levels according to TNM classification.

Groups	Serum clusterin level M±SD	Serum AFP level Median (range)
Stage I(n=40)	166.7±42.07	197.5(10-528)
Stage II(n=10)	171.8±56.2	208(15-570)
Stage III(n=18)	241.4±20.1	286(19-538)
Stage IV(n=12)	262.5±28.1	291.5(120-570)
F	29.13	5.2
P	<0.001(HS)	0.15(NS)

HS=Highly significant NS= Non significant

AFP was expressed as median (range) and data analysis was done using Kruskal-Wallis test

Table (6): correlation between serum clusterin and other clinical parameters.

Parameters	r	*P*
Child -Pugh score	0.27	0.08 (NS)
AFP	0.57	<0.001 (HS)
Size of tumor	0.08	0.62 (NS)
No of tumor nodules	0.22	0.16 (NS)

NS = Non significant HS = Highly significant

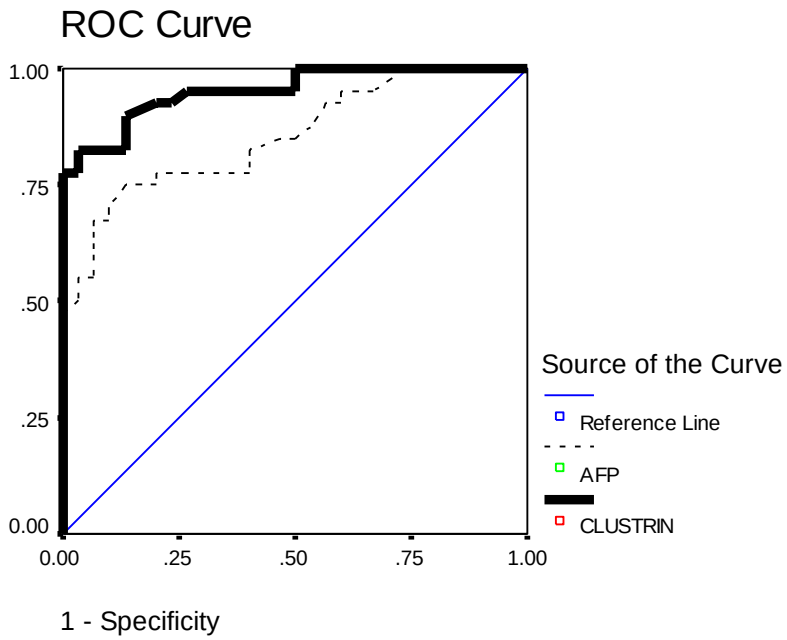

Fig. (2): ROC Curve of serum clusterin and AFP
AUC of clusterin= 0.95(CI: 0.90-0.99)
AUC of AFP= 0.85 (CI: 0.76-0.94)

Table (7): Validity of serum clusterin and AFP in the diagnosis of HCC.

	Sensitivity	Specificity	PPV	NPV
Clusterin	90%	87%	87.8	89.7
AFP	75%	80%	76.9	78.2

Clusterin cutoff value = 128 ug/ml
AFP cutoff value =100ng/ml
Cutoff values of both AFP and clusterin were determined by AUC
PPV = Positive predictive value NPV = Negative predictive value

DISCUSSION

Hepatocellular carcinoma is the sixth most common cancer and the third cause of caner related mortality in all over the world [24]. Screening of such higher risk patients with 3-6 month interval, using ultrasound (US) and AFP assay is generally recommended [25]. However, AFP has a limited role in HCC surveillance as it may increase in serum of some benign chronic liver disease patients or it may not increase in serum with some HCC patients [26]. However, El-Zayadi et al. [3] considered that α-fetoprotein is the most widely used tumor marker, but has poor diagnostic accuracy and ethnic variability. Although AFP improves detection of HCC, a significant number of HCC patients present without elevated AFP, and therefore additional markers are needed to increase the sensitivity and specificity. Therefore, early detection of HCC to improve its prognosis is an important issue for research.

Clusterin in human is a single-copy gene located on chromosome 8 p21-p12 that exhibition almost ubiquitous tissue expression pattern both during development and in adult [27]. Although number of reports has purported to explain clusterin functions in various cell types and tissue, including senescent and cancer cells, an understanding of clusterin function has remained elusive, especially in term of apoptosis and tumorigenesis [9].

Our result demonstrated that, clusterin levels were significantly higher in HCC patients than that in other different groups. Serum clusterin reached the lowest significant levels in cirrhotic patients without HCC. However, there was no significant difference of serum clusterin levels between healthy subjects and chronic hepatitis. Our data indicated that upregulated serum clusterin level in HCC patients might play a role in tumorigenesis and it could be used as a marker for early detection of cirrhotic liver that progressed to HCC. Significant lower level of serum clusterin in cirrhotic patients may be due to reduced liver cells mass or regenerating nodule can not be able to express clusterin like normal and malignant cells.

Wang et al. [28] reported that serum clusterin levels in HCC patients were significantly lower than in those with chronic hepatitis and healthy subjects, but it was higher than in those with cirrhosis. This result disagreed to our result as serum clusterin level in our study was significantly higher in patients with HCC than in healthy subjects and chronic hepatitis patients. However, it was not surprising that serum clusterin levels were higher in HCC patients when compared to healthy subjects and chronic hepatitis patients as clusterin is normally present in all tissue and human body fluid [6] and it can also be overexpressed in human neoplasm cells including HCC. So, clusterin level could be overexpressed in HCC patients when compared to other groups. Overexpression of clusterin has also been reported in HCC in other studies [29,30]. Moreover, Kang et al. [29] studied the immunoreactive pattern of clusterin in patients with HCC and found two distinct pattern of clusterin immunoreactivity namely cytoplasmic and canalicular. They also found that cytoplasmic overexpression might be an independent predictor of poor survival, as compared with the canalicular overexpression.

Upregulated serum clusterin was also reported in other tumor like lung cancer [31], colorectal carcinoma [32], urinary bladder cancer [33], and endometrial adenocarcinoma [34]. In other type of human cancer, such breast cancer [35] and esophageal squamous carcinoma [14], down-regulated serum clusterin was frequently observed.

In this study, we investigated serum clusterin levels according to different clinico-pathological features, we found no significant difference of serum clusterin levels according to size of tumor (either more of less than 5 cm) and according to numbers of tumor nodules. This indicated that clusterin could differentiate even small HCC (≤ 5 cm) from cirrhotic liver, which is of great importance as other marker can not distinguish between early small HCC and liver cirrhosis. On the other hand, AFP was significantly increased according to the progression of size and increased numbers of tumor nodules. This indicated that small size tumor or single tumor nodule can be missed if we depend on AFP for its diagnosis as it may not rise significantly in this situation.

Serum clusterin was highly expressed in patients with capsular infiltration compared to those without, also was significantly increased in patients with portal vein invasion and lymph node infiltration. Also, we found significant higher level of serum clusterin in patients with poorly-moderately differentiated tumor than in those with well differentiated tumor. Furthermore, serum clusterin was significantly increased with the

progression of BCLC and TNM staging systems of HCC. On the other hand, these findings were not observed as regard AFP. This result indicated that clusterin overexpression could point to HCC progression and enhanced metastasis potential of HCC.

Moreover, in our study we did not observe correlation between serum clusterin and the degree of deterioration of functional liver status with advancement of Child-Pugh score which indicated that increased serum clusterin levels in HCC patients could be related to the process of carcinogenesis rather than cirrhosis or fibrosis.

Our current data also showed that the sensitivity and specificity of serum clusterin in differentiation of HCC patients from cirrhosis were 90% and 87% respectively using a cutoff value of 128 ug/ml, while at cutoff value of 100 ng/ml AFP had 75% sensitivity and 80% specificity. Analyzing of AUC showed that serum clusterin had greater AUC (0.95) than that of AFP (0.85) which suggested that serum clusterin level might be superior to AFP in diagnosis of HCC and differentiating it from cirrhosis. This result agreed with that of Wang et al. [28] who found that at cutoff value 50 ug/ml, serum clusterin had 91% sensitivity and 83% specificity. The area under the ROC curve was 0.937 for clusterin versus 0.781 for AFP.

We concluded that serum clusterin was up regulated in HCC and more sensitive and specific than AFP for differentiating HCC patients from those with cirrhosis. It was closely related to capsular infiltration, portal vein invasion, lymph node metastasis and poorly differentiated tumor suggesting that clusterin might be deemed as a useful biomarker for diagnosis and predicting the metastasis potential of HCC.

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Conflicts of interest: None.

Ethical considerations: Special consideration was given to the right to confidentiality and anonymity of all patients.The patients are free to decide whether to participate or not. Informed consent from each patient was taken**.**

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