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Estimation of Clusterin Role as a Biomarker for Virus C-related Hepatocellular Carcinoma

Mohy-Eldin A Abdel-Atti[1], Dahlia I. Badran[2*], Ohoud M. Marie[3]

Departments of [1]Organic chemistry, Faculty of science, [2]Medical biochemistry, Faculty of Medicine, [3]Biochemistry, Faculty of Science, Suez Canal university, Ismailia, Egypt

Abstract

Background: **More than 500,000 new patients are diagnosed every year with hepatocellular carcinoma (HCC) worldwide. Clusterin (apolipoprotein J) is a multifunctional glycoprotein that exists in almost all mammalian tissue and most human body fluids. Recently, few studies have been done to evaluate serum clusterin role as a biomarker for diagnosis viral related hepatocellular carcinoma.** ***Aim:*** **The aim of this study is to evaluate the potential usefulness of clusterin as a new biomarker for diagnosing virus C-related hepatocellular carcinoma.** ***Subjects and Methods:*** **Twenty-nine patients with HCC, twenty-nine patients with liver cirrhosis and thirty healthy controls were enrolled in the study. Estimation of serum clusterin was done by enzyme linked immunosorbent assay (ELISA). Alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, albumin level, bilirubin level as well as creatinine level were measured in serum by routine enzymatic method. Sera were stored at -20˚C to measure alpha fetoprotein (AFP) concentration and clusterin concentration.** ***Results:*** **Serum clusterin level was not significantly elevated in patients with HCC ($p>0.05$). Receiver operator curve showed that AFP had a greater area under curve value (0.92) than that of clusterin(0.58)** ***Conclusion:*** **our study concluded that AFP is still the gold standard tumor biomarker for HCV related HCC, and that it can be used in conjunction with ultrasonography in the early detection of HCC instead of serum clusterin.**

Keywords: alpha fetoprotein, Hepatitis C virus related liver cirrhosis, liver cancer

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer over the world and the third most common cause of cancer mortality[1,2]. In Egypt, hepatocellular carcinoma (HCC) is the second most common cancer between men and the 6th most common cancers between women[3]. This elevated incidence may be due to high prevalence of hepatitis C virus (HCV) besides its complications and the fact that people born 20 years ago or before in Egypt has not been vaccinated against hepatitis B virus (HBV)[3]. Egypt has possibly the greatest HCV prevalence over the world[4], estimated among Egyptians to be around 14%[5]. Hepatocellular carcinoma is often diagnosed at end stage where effective therapies are absent. So the surveillance of patients at risk is very important[6]. Clusterin has been known almost two decades ago[7] as a protein leading to cluster of red blood cells[8]. Clusterin, also known as apolipoprotein J, is a heterodimeric glycoprotein with a molecular weight of about 70 to 80 kDa. It is encoded by a single gene. It has multiple isoforms, which

Corresponding Author: dahliabadran@hotmail.com

are referred to nuclear or secreted, relying on their location. Secreted clusterin is the most prevalent isoform. Clusterin is present mainly in many mammalian tissues[9,10]. It has been involved in many biological processes, including apoptosis regulation, reduction of complement activation, response to damage and stress, autoimmune damage, removal of toxic substrates, and interaction with lipids[11], and is a main protein in physiological fluids including plasma, milk, urine, cerebrospinal fluid and semen. Through a given tissue, clusterin may be expressed mostly in certain cell types-for example, in epithelial cells at tissue, fluid interfaces or in specific sub- types of neurons[8]. A lot of reports suggested changed expression of clusterin whether up regulated or down regulated has a major role in tumorgenesis[12]. This study aimed to evaluate the potential usefulness of clusterin as a novel biomarker for diagnosis virus C-related hepatocellular carcinoma.

Subjects and Methods

This study was done in the chemistry department, faculty of science, Suez Canal University and the internal medicine department of Suez Canal University hospital, Ismailia, Egypt. Eighty-eight adults were enrolled in this study. An informed consent was obtained from all participants. 2) The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the ethical committee of Faculty of Science. These subjects were classified into three groups based on clinical and laboratory characteristics as the following: *Group 1:* this group included 29 diagnosed patients with virus C-related HCC (21 males and 8 females) with a mean age of 53.86 ± 6.4 years. The diagnosis of HCC was based mainly on ultrasound imaging study and Triphasic computed tomography (CT).

Group 2: this group included 29 diagnosed patients with virus C-related liver cirrhosis (16 males and 13 females) with a mean age of 55.14 ± 7.05 years. The diagnosis of cirrhosis was based mainly on clinical examination, laboratory characteristics and ultrasound imaging study. *Group 3:* this group included 30 healthy blood donors with normal liver functions (28 males and 2 females) with a mean age 33.90 ± 4.4 years. All the studied groups underwent thorough history taking, clinical examination and serological testing for anti-HCV and hepatitis B virus surface antigen (HBsAg) by sandwich enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Rabbit Labs Co., UK). Polymerase chain reaction (PCR) was used for detection of HCV RNA and HBV DNA. Hepatitis C-related cirrhosis patients are those who had (1) positive serum anti-HCV antibodies; (2) cirrhosis compatible with HCV origin proved on ultrasound; and (3) absence of HCC defined by the absence of a focal liver mass on ultrasonography. The diagnosis of HCC was based on the criteria published by the Egyptian Society of Liver Cancer (ESLC) in 2011. These included the presence of hepatic local lesion in high risk patients (cirrhotic patients) plus either serum AFP ≥ 200 ng/ml, or a triphasic CT-scan showing typical criteria for HCC[13].

Biochemical measurements: after an overnight fast, blood samples were withdrawn from each subject. Alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, albumin level, bilirubin level as well as creatinine level were measured in serum by routine enzymatic method. Sera were stored at -20˚C to measure AFP and clusterin.

Estimation of serum clusterin: This was done using sandwich ELISA method according to manufacturer's instruction *(Human clusterin ELISA, WUHAN EIAAB science Co., Ltd).* The assay had a detection range of to

the concentration of clusterin. A standard 1.56-100ng/ml. The absorbance was proportional curve was constructed by plotting absorbance value versus clusterin concentration of standards, and concentrations of unknown samples were determined using this standard curve.

Statistical analysis

Data were analyzed with SPSS version 16.0 (statistical package for the Social Science, Chicago, IL). Descriptive measures were done for each variable in every group (table 1). One-way analysis of variance (ANOVA) test was done to compare different parameters between more than two groups (table 2) and data analysis was done using Mann-Whitney test. The receiver operator characteristic (ROC) curve (ROC) with 95% confidence interval (CI) was performed (figure 1) to determine cut off values for serum clusterin and AFP. The Youden's index [Youden index = (sensitivity + specificity) – 1] was calculated[14]. The best cut off values had the highest Youden index. 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

This study comprised 88 subjects (29 HCC patients: mean age 53.86 ± 6.4 years; 21 (72.4%) males and 8 (27.6%) females, 29 cirrhosis patients: mean age 55.14 ± 7.05 years; 16 (55.17%) males and 13 (44.83%) females and finally 30 healthy individuals: mean age 33.90 ± 4.4 years; 28 (93.3%) and 2 (6.7%) females). Table 1 shows the clinical characteristics of healthy control, patients with cirrhosis and HCC. All subjects in the control group had normal liver functions. All the measured parameters (ALT, AST, total and direct bilirubin, albumin, and creatinine) were significantly elevated in HCC patients compared to both healthy control and cirrhosis patients.

Table 1: Clinical characteristics of healthy control, patients with cirrhosis and HCC

Parameters	Healthy control (N = 30)	Cirrhosis (N = 29)	HCC (N = 29)
Age (years) ± SD	33.90 ± 4.4	55.14 ± 7.05	53.86 ± 6.4
Sex (n and %) Males Females	 28 (93.3%) 2 (6.7%)	 16 (55.17%) 13 (44.83%)	 21 (72.4%) 8 (27.6%)
ALT (U/L)	17.23 ± 7.79	40.83 ± 33.79	159.97 ± 304.53
AST (U/L)	17.87 ± 4.9	76.07 ± 123.58	281.38 ± 577.59
Total bilirubin (mg/dl)	0.29 ± 0.07	2.89 ± 2.56	7.84 ± 7.16
Direct bilirubin (mg/dl)	0.11 ± 0.05	1.93 ± 2.27	5.96 ± 6.19
Albumin (g/L)	3.47 ± 0.67	2.1 ± 0.44	2.1 ± 0.56
Creatinine (mg/Dl)	0.58 ± 0.18	1.65 ± 0.92	2.36 ± 2.39

ALT= alanine amino transferase; AST=aspartate amino transferase

Table 2 shows a comparison between the studied groups according to serum levels of CLU and AFP demonstrating that serum AFP (ng/ml) was statistically significantly elevated in the HCC patients compared to both the cirrhosis patients (877.7 ± 2031.7 vs. 8.41 ± 10.22), and to the control group (877.7 ± 2031.7 vs. 3.16 ± 1.97), and also elevated in the cirrhosis patients compared to the control group (8.41 ± 10.22 vs. 3.16 ±

1.97). To evaluate the diagnostic performance of serum clusterin in relation to serum AFP in diagnosing HCC, the ROC curve was used. The area under the curve of AFP (0.920, p <0.05) was bigger than that of clusterin (0.582, p >0.05). Youden's index was calculated to get the best cut off value (COV). The optimal COV for serum AFP was 12.55ng/ml (this offered a diagnostic sensitivity of 86.2% and a diagnostic specificity of 91.5%). On the other hand, the best COV of clusterin was 2.26 ng/ml and this offered a diagnostic sensitivity of 55.2% and a diagnostic specificity of 69.5%. The area under the curve of AFP (0.920, p <0.05) was bigger than that of clusterin (0.582, p>0.05).

Table 2: Comparison between studied groups according to serum levels of CLU and AFP

Variable	Healthy control (N = 30)	Cirrhosis (N = 29)	HCC (N = 29)	P (F)
CLU (ng/ml)				
Range	0.85 - 6.607	0.521 - 7.463	0.564 - 5.593	>0.05 (0.358
Mean ± SD	2.15 ± 1.26	2.25 ± 1.45	2.44 ± 1.24	
Median	1.79	1.96	2.28	
Z1	-	-0.197	-1.198	
Z2	-	--	-0.964	
AFP (ng/ml)				
Range	1.2 - 9.0	1.5 - 39.7	2.3 - 11116.0	<0.05 (5.436)
Mean ± SD	3.16 ± 1.97	8.41 ± 10.22	877.7 ± 2031.7	
Median	2.55	4	405	
Z1	-	-2.543	-5.922	
Z2	-	-	-5.117	

Alpha fetoprotein; AFP, clusterin; CLU, Z1: Z for Mann-Whitney test between control and other groups, Z2: Z for Mann-Whitney test between cirrhosis and HCC groups, Statistically significant at P < 0.05

Table 3: comparison of serum clusterin and AFP in HCC patients according to Child-Pugh classes

Child-Pugh classes	A	B	C	p
AFP (ng/ml) mean ± SD	590.9± 411.3	535.357±420.8	619.51±565.292	>0.05
CLU (ng/ml) mean ± SD	0.8214± 0.462	1.9765±.9006	2.4009±1.072	>0.05

AFP has better positive predictive value and negative predictive value than clusterin demonstrating that AFP is a better biomarker than clusterin (table 4). The receiver operator characteristic curve (ROC) curve with 95 % confidence interval (CI) was performed to determine cut off values for serum clusterin and AFP (Figure 1). Figure 2 shows serum clusterin levels in the studied groups: HCC patients, cirrhosis pa tients and healthy control. Serum clusterin level showed no significant statistical difference among different groups (p > 0.05) (figure 2). Table 3 shows a comparison of serum clusterin and AFP in HCC patients according to Child-Pugh classes demonstrating that there was no significant statistical difference in serum CLU among the different Child-Pugh classes in the HCC group.

Discussion

HCC is the fifth most common cancer worldwide and the third most common cause that leads to cancer mortality[1,2,15]. In Egypt, HCC is the second most common

cancer in men and the 6th most common cancers in women[3], representing a great burden on the community due to its increasing incidence[16]. Cirrhosis as well as HCC takes place routinely in a portion of patients who develop chronic infection with HBV or HCV. HCCs arise with rising frequency in livers that are the site of chronic hepatitis[17,18] as well as cirrhosis[19-22]. For decades, screening for AFP (AFP) is the most commonly used biochemical blood test to detect liver cancer, although has a poor diagnostic accuracy and ethnic variability[16]. However, up to date the early diagnosis of HCC represents a great challenge especially for those who have small nodular lesions with no signs or symptoms which make the disease have a poor prognosis. Thus, the search for additional markers with increasing sensitivity and specificity is mandatory. Clusterin is a 75-80kDa disulfide-linked heterodimeric protein which is closely associated with the clearance of cellular debris and apoptosis[8].

Table 4: Predictive performance of serum AFP and clusterin as biomarkers for the detection of hepatocellular carcinoma

Statistical parameter	AFP	CLU
cut off value (ng/ml)	12.55	2.26
Sensitivity %	86.20	55.20
Specificity (%)	91.50	69.50
Positive predictive value (%)	83.29	47.08
Negative predictive value (%)	93.09	75.94
Youden's index	0.777	0.247
Area under ROC	0.92	0.582
p value	<0.05	> 0.05
95% confidence interval	0.850-0.989	0.450-0.715

In humans, clusterin is encoded by the *CLU* gene on chromosome 8 (CLU is a member of the small heat shock protein family, a molecular chaperone responsible for helping the folding of secreted proteins in ATP-independent way, and its three isoforms have been implicated differentially in pro- or anti-apoptotic processes. Through this function, CLU is involved in many diseases related to oxidative stress, as neurodegenerative diseases, aging, inflammatory diseases, and cancers[23-25]. In this study, serum CLU was measured in HCC Egyptian patients and controls (healthy controls, HCV related liver cirrhosis). Although serum clusterin level was slightly higher in the HCC group compared to the cirrhosis and the healthy control groups, such elevation showed no significant statistical difference among the three groups ($p > 0.05$). In the present study, serum AFP with a cutoff level of 12.55 ng/ml showed a higher performance than serum CLU with a cutoff level of 2.26 ng/ml in diagnostic sensitivity (86.2% vs. 55.2%), specificity (91.5% vs. 69.5%), and PPV and NPV (83.2 % vs. 47.0 % and 93.0 % vs 75.9% respectively). Similarly to our findings, Ramadan et al., concluded that serum AFP did better than serum CLU in all aspects of diagnostic performance for diagnosing HCC in a similar study done on the Egyptian population[13]. Wang et al., demonstrated in a study done on Chinese patients that there were no significant differences of serum clusterin levels between healthy subjects and HBV carriers. However, in HCC patients, the clusterin levels were significantly lower than that in healthy, HBV carriers and chronic hepatitis B patients, but statistically higher than that

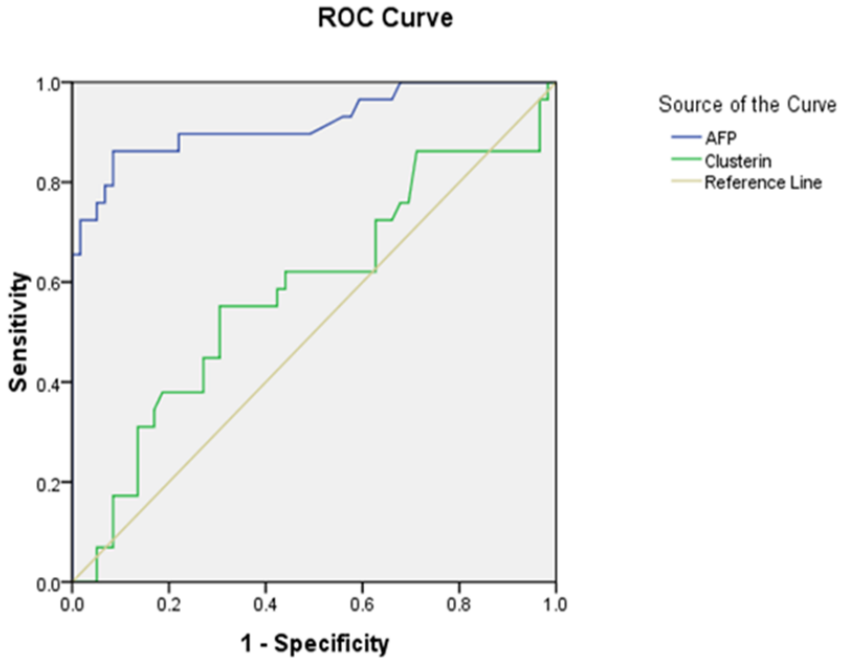

Figure 1: Receiver operating curve (ROC) comparing clusterin and AFP in diagnosing hepatocellular carcinoma. Receiver operator curve (ROC) comparing clusterin (CLU) and AFP (AFP) in patients with hepatocellular carcinoma (HCC) vs. those with and without HCC (G2 and G3)

in cirrhosis patients indicating a difference in serum clusterin between HCC patients and different control subjects[26]. We did not notice any relation between serum clusterin and the magnitude of worsening of liver functions as there was no significant statistical difference in serum CLU among the different Child-Pugh classes in the HCC patients' group which is in accordance with the results reported by others[12,13]. On the contrary, Wang et al., declared that serum clusterin might be a good predictor for early diagnosis of HCC progression from HBV related liver cirrhosis which was more sensitive and specific than serum AFP for differentiating HBV-related HCC from those with liver cirrhosis. Meanwhile, Nafee et al, reported clusterin overexpression in HCC with more sensitivity and specificity than AFP in differentiating HCC from cirrhosis as clusterin was especially related to progression and metastasis of HCC[12]. On the other hand, other studies indicated that clusterin was downregulated in the majority of cancers especially in the early stages of the tumor development[27,28]. This abnormal expression can differ even along the different stages of development of the same tumor[29] as clusterin upregulation can be observed in the late stages conferring resistance to chemotherapeutic and radio-therapeutic treatment[30]. However, Nafee et al., reported that no significant difference of serum clusterin was found regarding the tumor size and the numbers of tumor nodules[12]. So, this discrepancy of results is due to the great fluctuation of clusterin levels along the different stages of the tumor development, the different cutoff value of serum clusterin between different ethnic groups and the presence of different genetic and epigenetic factors controlling the expression clusterin gene and affecting its serum level..

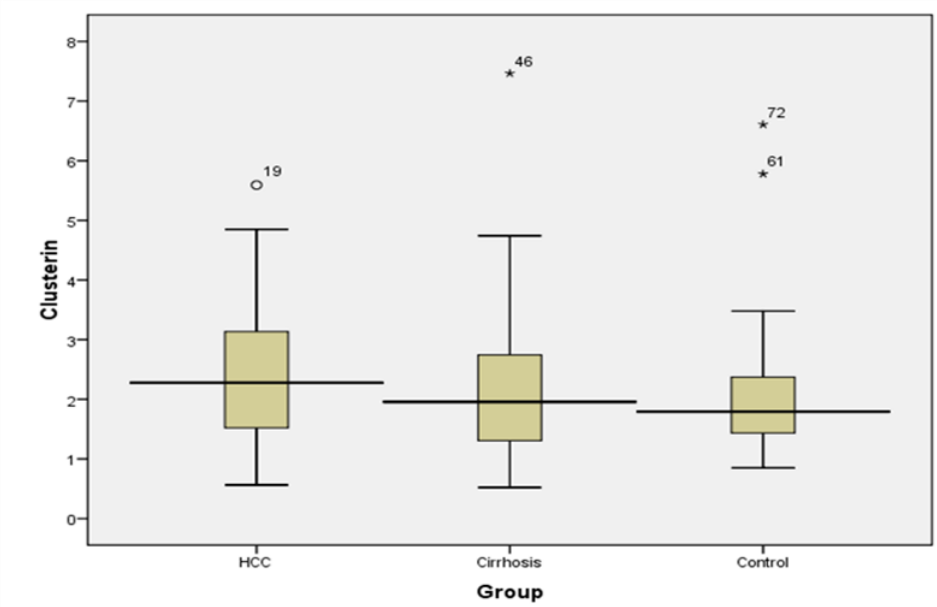

Figure 2: Boxplot of serum (CLU) in the studied groups

So more extensive studies are needed for evaluation of the different genetic and the epigenetic effects which control the expression of clusterin gene and affect its level in the blood, for better estimation of its exact role in tumorigenesis. In addition, the search for new biochemical markers useful in early screening of HCC cases and which can differentiate between liver cirrhosis and early stages of HCC is mandatory. Also, large clinical trials with continuous follow-up of serum level of clusterin is essential for the accurate estimation of clusterin role in cancers as a non-invasive, early screening test for early diagnosis of HCC. So our study concluded that AFP is still the gold standard tumor biomarker for HCV related HCC, and can be used in conjunction with ultra-sonography in the early detection of HCC cases, it as it is more sensitive (86.2%) and more specific (91.5%) than serum clusterin at cutoff value of 12.55 ng/ml, regarding the follow-up of cases, response to treatment and disease recurrence.

Conflicts of interest

The authors declare no conflict of interest.

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