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RESEARCH ARTICLE

Evaluation of Serum Clusterin as a Promising Marker for Diagnosis and Staging of Hepatocellular Carcinoma

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Abstract

The prevalence of Hepatocellular carcinoma (HCC) in Egypt is extensively increasing. Clusterin has been reported to play a significant role in cancer development and progression through promoting cell survival. We aimed to evaluate CLU as a recent marker for HCC and its correlation with HCC stage in patients with chronic hepatitis C infection (HCV). The study included 15 patients with uncomplicated chronic HCV infection, 15 patients with decompensated liver cirrhosis and 40 patients with HCC on top of liver cirrhosis (furtherly subdivided according to TNM classification). Serum alpha fetoprotein and serum clustrin level were measured for all patients. Our results showed that CLU significantly decrease with decompensated liver cirrhosis compared to uncomplicated chronic hepatitis. CLU rise significantly in patients with HCC. CLU level of 182 µg/ml is a discriminating level between HCC TNM stage I (non metastatic) and TNM stage II and III (metastatic). At this level the diagnostic sensitivity is 70%, specificity 80%. The accuracy can be improved when linked to AFP at cut off value 273 ng/ml. Conclusion: CLU may be a useful marker for follow up of patients with chronic hepatitis C for diagnosis of hepatic decompensation and HCC. CLU level may be used in staging of HCC and can be a target of new therapy to prevent tumor progression.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and aggressive tumors**[1].** It is the third most common cause of cancer mortality**[2].**The prevalence of HCC in Egypt is extensively increasing. In 2001, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients **[3].**Despite recent advances in the diagnosis and treatment of HCC, the mortality rate of HCC remains high **[4, 5].**

At present, HCC surveillance with tumor markers and imaging studies such as ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI) have been recommended for patients with cirrhosis **[6, 7].**These imaging studies are expensive and the ultrasound is highly dependent on the ability of the operator.

The representative tumor markers for HCC; AFP, and PIVKA-II are not satisfactory in terms of sensitivity and specificity in early diagnosis of HCC**[1].**Therefore, more sensitive and specific serum biomarkers for early detection of HCC are desirable.

Clusterin (CLU) is a secretory heterodimeric glycoprotein of approximately 80 kDa. It is expressed in several tissues and exists extensively in human body fluids**[8].**CLU exerts an anti-apoptotic function and its expression is strongly stimulated by various stressors. It protects cells from stressors that may induce apoptosis; including cytotoxic chemotherapy and androgen or estrogen deprivation**[9].**

Clusterin has been reported to play a significant role in cancer development and progression through promoting cell survival**[10].** Some reports documented the upregulation and over expression of CLU in the majority of human cancers, such as prostate, breast, lung, bladder and colon cancers **[11-13].**

In hepatocellular carcinoma, it has been shown that clusterin is overexpressed in metastatic human HCC tissue compared to nonmetastatic HCC tissue **[12, 13, 14]**implicating a role for clusterin in HCC progression. Moreover, Chen et al. **[12]**provided evidence that CLU plays an important role in HCC invasiveness by increasing MMP-2 expression and decreasing E-cadherin expression.

A recent study showed that the down-regulation of clusterin sensitizes cells to chemotherapy and radiotherapy and decreases their metastatic potential **[15, 16].**

This study is done to evaluate CLU as a marker for HCC and its correlation with HCC stage.

SUBJECTS AND METHODS:

I.SUBJECTS

This study was performed on 70 patients with chronic HCV infection. They were recruited from the Hepatology Department of Ain Shams University Hospital. Patients were divided into 3 groups; first group had uncomplicated chronic HCV infection (15 patients). Second group had decompensated liver cirrhosis (15 patients) and third group had HCC on top of liver cirrhosis (40 patients).

Diagnosis of chronic HCV was based on the presence of HCV RNA in serum. Liver cirrhosis was diagnosed based on clinical, laboratory and ultrasonographic criteria. HCC was diagnosed by presence of hepatic focal lesions in triphasic spiral abdominal computerized tomography which show enhancement in the arterial phase and rapid wash out in the venous phase. Patients with HCC had chest CT and bone scan for further staging. They were classified into 3 subgroups according to the Tumor-Lymph Node-Metastasis (TNM) clinical staging system **[17]; Subgroup I** (n=10): This group included patients with TNM stage I. **Subgroup II (n=16):** included 16 patients with TNM stage II. **Subgroup III (n=14):** included patients with TNM stage III.

For all patients serum alpha fetoprotein and serum clustrin level was measured and compared.

The study protocol was explained to all patients and they provided an informed consent before enrolment. The study was approved by the ethical committee of Ain Shams University hospital.

II. SAMPLES:

Ten milliliters (10mL) of freshly venous blood were withdrawn from each subject under complete aseptic precautions. 1.8mL were placed into 3.2% sodium citrated tubes in ratio 9: 1(blood: citrate) for PT assay. The rest of the blood was placed in sterile vacutainers, and was left to clot for 30 minutes. Serum was then separated by centrifugation at 1000 x g for 15 minutes and divided into three aliquots; one was used for immediate assay of routine liver function tests and serum AFP, while the others were stored at minus 70°C for subsequent assay of serum clusterin and for real–time PCR for positive HCV strand detection. Frozen samples were allowed to thaw and brought to room temperature only before analysis. Hemolysed samples were discarded, repeated freezing and thawing was avoided.

III. METHODS:

A. **Analytical Methods:**

1. Routine liver function tests: AST, ALT, albumin, total bilirubin, direct bilirubin and alkaline phosphatase were measured on Synchron CX-5 Deltaautoanalyzer (Beckman Instruments Inc.; Scientific Insturments Division, Fullerton, CA 92634, 3100, USA).

2. Real time PCR procedure:

Extraction was first done using RNA isolation kit QIAamp viral RNA isolation kit. The real time PCR test was done, by Stratagene Mx3000P instrument, using Artus HCV RG RT_PCR kit that constitutes a ready-to-use system for the detection of HCV RNA using polymerase chain reaction (PCR). This kit (master A and B)contains reagents and enzymes for the reverse transcription and specific amplification of a specific region of the HCV genome in fluorescence detector (FAM). It also contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an Internal Positive Control (IPC) in fluorescence detector (HEX). External positive controls (HCV Standards IU/ml) are supplied which allow the determination of the pathogen load using standard curve (Absolute Quantitation). Four Quantitation Standards (QS) are supplied in the kit ranging from 10^1 IU/UL to10^4 IU/UL.

The principal of TaqMan chemistry for QPCR applications includes a third oligonucleotide in the reaction known as the probe. A fluorescent dye, typically FAM, is attached to the 5′ end of the probe and a quencher. As long as the two molecules (reporter and quencher) are maintained in close proximity, the fluorescence from the reporter is quenched and no fluorescence is detected at the reporter dye's emission wavelength. The probe is designed to anneal to one strand of the target sequence just slightly downstream of one of the primers. As the polymerase extends that primer, it will encounter the 5′ end of the probe. Taq DNA polymerase has 5′–3′ nuclease activity, so when Taq DNA polymerase encounters the probe it displaces and degrades the 5′ end, releasing free reporter dye into solution. Following the separation of reporter dye and quencher, fluorescence can be detected from the reporter dye. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase have started to have an effect on the efficiency of amplification.

3. Serum AFP Assay:

Quantitative determination of AFP was performed on ELECSYS 1010 Immunoassay Autoanalyser using AFP kit supplied by Roche Diagnostics (Roche Diagnostics, Indianapolis, IN 46256, 9115 Hague Road USA). The assay is based on an electrochemiluminescence immunoassay (ECLIA) technique (Daniel et al., 2006). According to the manufacturer the analytical range is less than 10 ng/mL (ng/mL= 1.09 IU/mL).

4. Serum CLU Assay:

The assay was done using the Quantikine ELISA kit supplied by R & D Systems (USA & Canada R&D Systems, Inc. www.RnDsystems.com). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CLU has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CLU present is bound by the immobilized antibody.After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for CLU is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CLU bound in the initial step. The color development is stopped and the intensity of the color is measured at 450 nm.

B.**Statistical Methods:**

Statistical analysis was carried out on personal computer using SPSS software version16, USA. Skewed data are expressed as median and inter quartile range (IQR). Comparative statistics was done between 2 groups by Wilcoxon's rank sum and between more than 2 groups by Kruskal–Wallis test (H test). P values<0.05 were considered significant, whereas values<0.001 were considered highly significant. Receiver operating characteristic curve (ROC) analysis was applied to assess the overall diagnostic performance of each test.

RESULTS:

The demographic data of the study population divided into 3 main groups and HCC subgroups are summarized in table (1). Male patients were more frequent than females being 80%, 73.33% and 82.5% for group of chronic hepatitis, liver cirrhosis and HCC group respectively. The mean age of patients in such groups is 51.2±7.32, 50.3±6.52 and 51.2±6.78 years, respectively.

Table (1): Demographic data of studied groups and HCC subgroups

variables		**Chronic hepatitis n= 15**	**Liver cirrhosis n= 15**	**HCC n= 40**	**TNM Stage I n= 10**	**TNM Stage II n= 16**	**TNM Stage III n= 14**
Demography	**Gender**						
	Males	12 (80%)	11 (73.33%)	33 (82.5%)	9 (90%)	13 (81.25%)	11 (78.6%)
	Females	3 (20%)	4 (26.67%)	7 (17.5%)	1 (10%)	3 (18.75%)	3 (21.4%)
	Age (yrs) mean±SD	51.2 ± 7.32	50.3 ± 6.52	51.2 ± 6.78	49.8 ± 6.57	52.5 ± 6.85	50.9 ± 6

The median AFP and CLU levels were compared between the three groups of patients (table 2, 3). The median value of AFP was highest in HCC patients; which was significantly higher than each of group of chronic hepatitis and

group of liver cirrhosis. No significant difference found in median AFP level between patients with chronic hepatitis and cirrhosis. The median level of CLU was significantly different between each of the three groups; it was highest in HCC group (189 µg/ml) and lowest in patients with liver cirrhosis (53µg/ml).

Table (2): Median AFP and CLU Levels in the Studied Groups Compared byKruskall-Wallis Test

Variable	**Chronic hepatitis n= 15**	**Liver cirrhosis n= 15**	**HCC n= 40**	**H**	**p**
AFP (ng/ml) **Median**(IQR)	82(32-90)	75(42-110)	322(135-530)	50.68	**<0.001**
CLU (µg/ml) **Median**(IQR)	90(60-164)	53(40-62)	189(110-240)	46.15	**<0.001**

p< 0.001is highly significant

Table (3): Comparison of Serum AFP and Serum Clusterin Levels in the Patients' Groups using Wilcoxon's Rank-Sum Test

Variable	**Chronic hepatitis Vs Liver cirrhosis**		**Chronic hepatitis Vs HCC group**		**Liver cirrhosis Vs HCC group**	
	Z	P	Z	P	Z	P
AFP (ng/ml)	0.48	>0.05	5.67	**<0.001**	5.66	**<0.001**
CLU (µg/ml)	3.3	**<0.001**	4.73	**<0.001**	5.67	**<0.001**

p> 0.05 is non-significant and p< 0.01is highly significant

Analysis of median AFP and CLU in HCC patients according to the TNM staging system (table 4, 5 and figure 1) show that there is a significant and progressive rise of the median of both AFP and CLU with the progression of the stage of the tumor.

Table (4): Median AFP and CLU Levels in the HCC subgroups Compared by Kruskall-Wallis Test

Parameter	**HCC (n= 40)**			**H**	**P**
	TNM Stage I n= 10	**TNM Stage II n= 16**	**TNM Stage III n= 14**		
AFP (ng/ml) **Median** (Q1-Q3)	196 (157-270)	313 (287-334)	417 (380-477)	39.6	**<0.001**
CLU (µg/ml) **Median** (Q1-Q3)	164 (120-183)	183 (172-190)	219 (198-231)	44.1	**<0.001**

p< 0.001is highly significant

Table (5): Comparison of Serum AFP and Serum Clusterin Levels in the HCC Subgroups using Wilcoxon's Rank-Sum Test

Variable	**HCC subgroup I Vs HCC subgroup II**		**HCC subgroup I Vs HCC subgroup III**		**HCC subgroup II Vs HCC subgroup III**	
	Z	P	Z	P	Z	P
AFP (ng/ml)	4	**<0.001**	4.32	**<0.001**	5.67	**<0.001**
CLU (µg/ml)	1.12	>0.05	4.67	**<0.001**	5.675.32	**<0.001**

p> 0.05 is non-

significant; p< 0.05 is significant and p< 0.01is highly significant

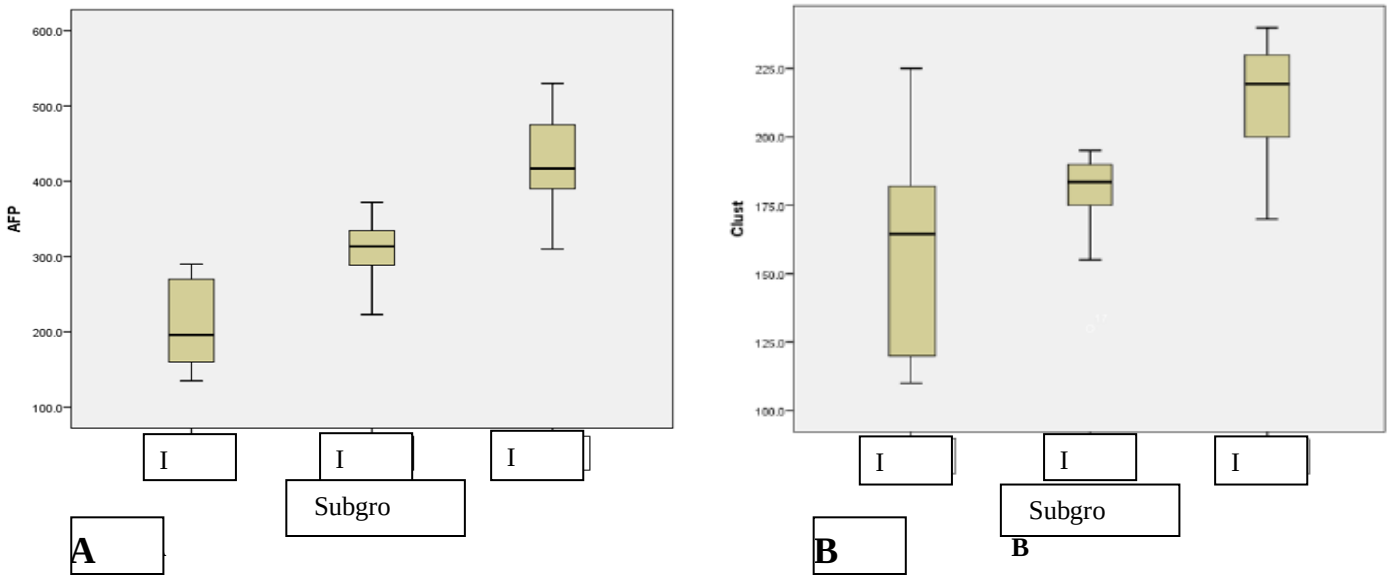
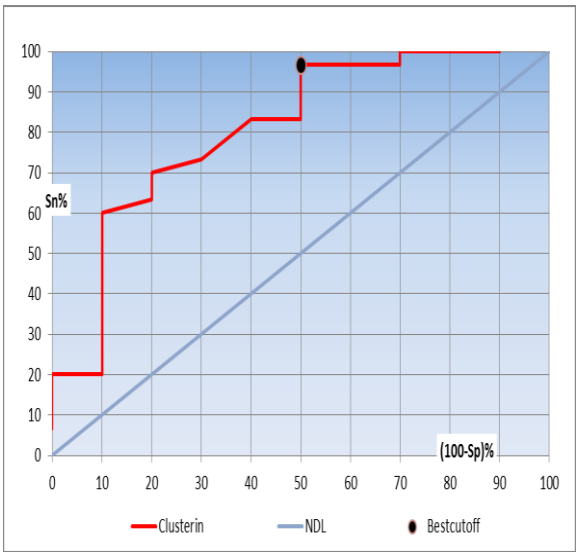
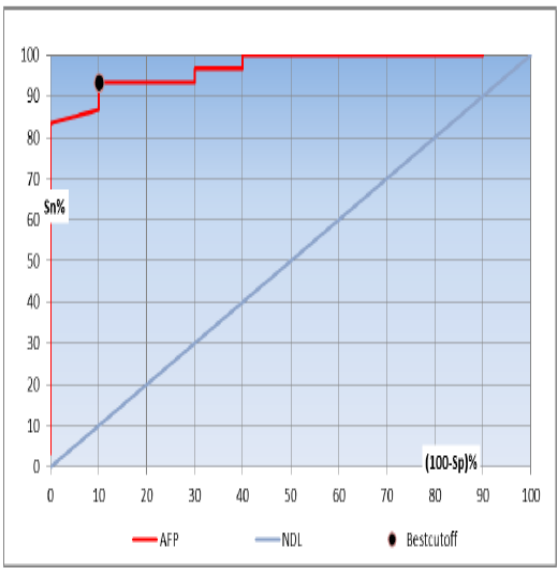


Figure (1): Box-Plot diagram showing: A. serum AFP levels, B. CLU levels in HCC subgroups (in ng/ml).
The diagnostic ability of both markers to differentiate early (TNM stage I) and late (TNM stage II and III) cases of HCC was studied by anothertwo ROC curves for AFP and CLU in HCC subgroups (figure 2and table 6).



Clusterin



AFP

Figure (2): ROC curve showing the diagnostic performance of CLU and AFP for discriminating HCC patients in TNM stage II and III from stage I

Table (6): Diagnostic performance of serum CLU and serum AFP in discriminating HCC TNM I from HCC TNM II & III

Variable	**Cut off**	**Sn (%)**	**SP (%)**	**P- (%)**	**P+ (%)**	**Accu (%)**
AFP (ng/ml)	273	93.3%	90%	81.8%	96.6%	92.5%
CLU (µg/ml)	182	70%	80%	47.1%	91.3%	72.5%

AFP: alpha fetoprotein, **CLU**: clusterin, **Sn**: sensitivity, **Sp**: specificity, **p-**: negative predictive value, **p+**: positive predictive value and **Accu**: accuracy

DISCUSSION:

In the course of searching for a better and more reliable diagnostic marker for HCC, previous studies reported high serum CLU values in patients with HCC suggesting that CLU might represent a useful marker for HCC and a complementary diagnostic tool **[18].**Accordingly, our study aimed to investigate the diagnostic utility of serum CLU in patients with HCC in comparison to the conventional markers, namely AFP.

Our study showed that serum CLU level drops significantly with development of liver cirrhosis compared to patients in chronic hepatitis stage. It then significantly rises with presence of HCC. The reduced liver mass in cirrhosis may explain the drop of CLU level in patients with cirrhosis **[19].** However, its significant increase with appearance of HCC can be explained by its anti-apoptotic function [9] which suggests that malignant cells when develop can over express CLU to survive and grow. This is reinforced by our findings in analysis of HCC subgroups which showed significant and progressive increase in serum CLU with advance in tumor stage.

Several reports agreed on the finding of higher CLU expression in metastatic HCC compared to non-metastatic tumors **[12-14].**CLU was also found to be involved in all aspects of tumorigenesis including tumor formation, progression, metastasis and chemo-resistance acquisition**[20].**

Although both AFP and CLU are significantly high in HCC patients, AFP is also increased in cirrhosis thus its sensitivity to accurately differentiate between liver cirrhosis and HCC is poor. In the contrary, CLU level is significantly decreased in cirrhotic patients compared to patients with chronic hepatitis. CLU level also shows significant rise with HCC development and this rise is correlated to tumor stage. Thus, the use of CLU in follow up of patients with chronic HCV and cirrhosis may be useful as follows; it can early detect development of cirrhosis due to decrease in its serum level. More important the rise in CLU level in a cirrhotic patient is an alarm of early HCC development.

Our study also showed that CLU level of 182µg/ml is a discriminating level between HCC TNM stage I (non metastatic) and TNM stage II and III (metastatic). At this level the diagnostic sensitivity is 70%, specificity 80%, positive predictive value (PPV) 91.3%, negative predictive value (NPV) 47.1% and efficiency 72.5%. The accuracy can be improved when linked to AFP at cut off value 273ng/ml.

In conclusion, CLU may be a useful marker for follow up of patients with chronic hepatitis C for diagnosis of cirrhosis and HCC. CLU level may be used in staging of HCC and can be a target of new therapy to prevent tumor progression.

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