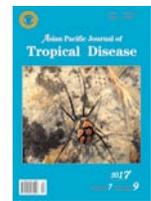


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## Evaluation of serum protein markers in diagnosis of hepatocellular carcinoma and carcinogenesis risk assessment in chronic liver disease patients

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## ABSTRACT

**Objective:** To assess the diagnostic value of the protein markers in both cirrhotic patients on top of hepatitis C virus (HCV) and in hepatocellular carcinoma (HCC) patients on top of HCV in comparison to normal controls.

**Methods:** A total number of 100 subjects including HCC, cirrhotic patients on top of HCV and normal controls were subjected to serum protein markers analysis for alpha-fetoprotein, apolipoprotein A1, apolipoprotein A2, insulin like growth factor 1 and insulin like growth factor 1 receptor by western blotting technique.

**Results:** It was found that alpha-fetoprotein alone could not be used as a screening test while apolipoprotein A2 as a serum marker could be used as a non invasive screening test to differentiate a case of HCC from cirrhotic HCV patient. The all four markers were able to discriminate normal persons from HCC and cirrhotic HCV patients effectively.

**Conclusions:** We concluded that proteomics analysis being non invasive, rapid and sensitive is a novel gate that can serve in early diagnosis and screening of HCC and cirrhotic HCV patients.

## 1. Introduction

Hepatitis C virus (HCV) is one of the major health problem, which leads to chronic infection of 350 million individuals in the world[1]. Hepatitis C is the most public health stress in Egypt where HCV prevalence is the highest in the world. Egypt Health Issues Survey (2015) showed that between 15 and 59 years of age, about 10% of Egyptians had been infected with HCV infection, while 7% of them have chronic active hepatitis C[2].

About 40%–60% of HCV infections result in chronic liver disease which could be complicated by hepatocellular carcinoma (HCC) via oxidative stress, insulin resistance, fibrosis, liver cirrhosis and HCV induced steatosis[3].

Unfortunately, HCC diagnosis usually is made when patients become symptomatic with liver function affection. At this late time, there is no beneficial treatment that would improve survival[3].

Liver function tests including alpha-fetoprotein are of little value in screening for liver disease as many serious liver diseases may be associated with normal levels and abnormal levels might be found in asymptomatic healthy individuals[4].

Also the use of liver biopsy as a screening method carries many risks for a hepatic patient. Studies found a great benefit for early screening of patients for HCC that gave them the option for receiving multiple treatment choices with improved clinical outcomes[5]. This leads to the idea of using non invasive

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The study protocol was performed according to the Helsinki declaration and approved by Cairo University Ethical committee and that informed written consent was obtained from all patients and controls.

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biomarkers with minimal risk and complications as proteomes analysis as a screening method for early detection of cases of HCC. With proper screening, we could save many patients and help them to preserve proper functioning liver.

## 2. Materials and methods

### 2.1. Study area

This study was conducted on a total number of 100 patients. They were divided into 3 well matched age and sex groups. Group 1 included 50 HCC patients on top of HCV. Group 2 included 30 cirrhotic patients on top of HCV. Group 3 included 20 healthy control subjects with no liver disease or a history of hepatitis virus infection. All patients were from Gastroenterology and Hepatology Department of Kasralainy Hospital and outpatient clinic.

Written informed consents were obtained from all patients before enrollment into the study. All patients and controls were subjected to the following: detailed history taking including demographic data and medical history; complete clinical and radiological examination with special emphasis on abdominal examination; laboratory investigation including serum alpha-fetoprotein, apolipoprotein A1 (Apo A1), apolipoprotein A2 (Apo A2), insulin like growth factor 1 (IGF1), insulin like growth factor 1 receptor (IGF1R) and serologic tests for hepatitis B virus, HCV, and HIV.

### 2.2. Sample collection

Study was done from October 2015 until May 2016. A volume of 5 mL blood was withdrawn from each patient in clear EDTA-free tubes labeled with their name and date of collection under complete aseptic conditions to separate a volume of 3 mL serum by centrifugation. The serum was divided into six aliquots to prevent repeated freezing and thawing of the same aliquot. Only clear, non-hemolyzed samples were used. Sera were stored at  $-80^{\circ}\text{C}$  till processing.

### 2.3. Sample processing

All samples were subjected to the following: A. Protein extraction using ReadyPrep™ Protein Extraction Kit, USA and determination of protein concentration by Bradford protein assay kit, Bio Basic Inc., Canada; B. Electrophoresis and protein separation using Bio-Rad electrophoresis, USA where proteins of biological samples were separated as isolated bands; C. Western blotting using Trans-Blot, Bio-Rad, USA where transfer of protein from the gel into a polyvinylidene fluoride membrane was

followed by its blocking; D. Proteins identification using primary and secondary antibodies followed by quantification using chemoluminescence; E. Result analysis by gel imaging and gel documentation system (Gel Doc XR+, Bio-Rad, USA).

We used primary antibody that specifically binds to the protein of our research interest for the purpose of purifying, detecting and measuring followed by secondary nonspecific antibodies that are used for the indirect detection of target protein. Secondary antibody provides increased sensitivity through signal amplification, elimination of cross-reactivity in detection of primary antibodies and offers greater flexibility for labeling and detection by chemoluminescence.

We confirm that the study protocol was performed according to the Helsinki declaration and approved by Cairo University Ethical committee and that informed written consent was obtained from all patients and controls.

### 2.4. Statistical analysis

Data were coded and entered using the statistical package SPSS version 15. Data were summarized using number and percent for qualitative variables, while mean and standard deviation was used for quantitative values which are normally distributed while median and range were used for quantitative variables which are not normally distributed.

Comparisons between groups were done using *Chi*-squared test for qualitative variables. Mann-Whitney test was used for quantitative values which are not normally distributed. Correlations were done to test for linear relation between variables. Receiver operating characteristic (ROC) curve was used to test the discriminating ability of each marker to discriminate between cirrhotic HCV, HCC and controls. *P* value less than or equal to 0.05 was considered as statistically significant.

## 3. Results

No statistical significance was found due to age or sex difference. All controls serum biomarkers levels were within standard universal normal range.

On comparing level of four markers among HCC and HCV cirrhotic patients versus normal levels, we found that only Apo A2 showed higher results than normal range while the other markers showed lower level than normal as shown in Figures 1 and 2.

On measuring alpha-fetoprotein in HCC patients (level ranges between 5.1 and 412.0 ng/dL), all patients showed higher level than the normal range ( $< 5$  ng/dL).

While in cirrhotic HCV patients (level ranges between 1.5 and 164.0 ng/dL), 25% of patients showed normal level of alpha-fetoprotein (Table 1).

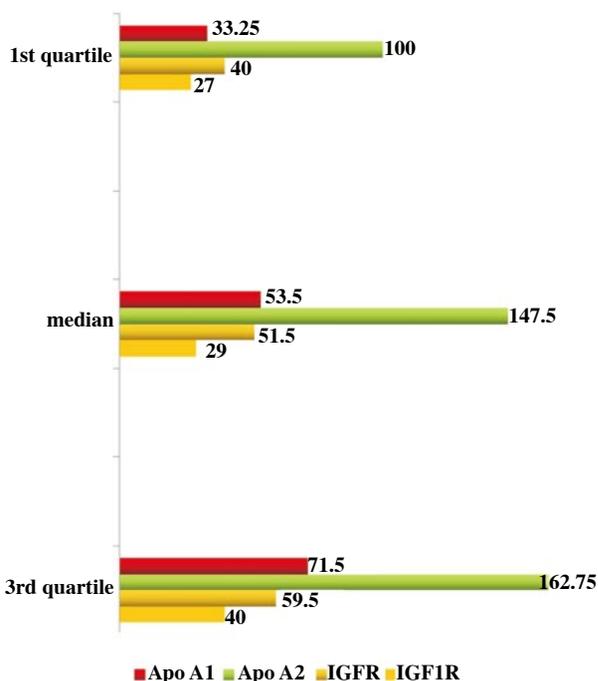


Figure 1. Four markers quantitative assay results among HCC patients.

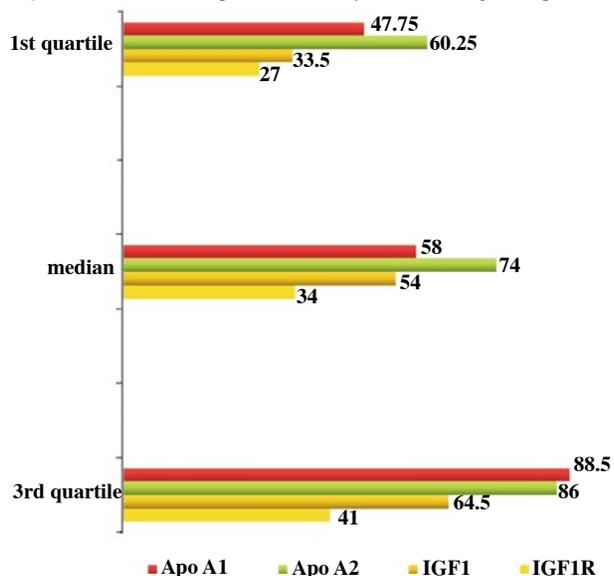


Figure 2. Four markers quantitative assay results among cirrhotic HCV patients.

Table 1

Alpha-fetoprotein quantitative assay results among HCC versus cirrhotic HCV patients.

Alpha-fetoprotein	HCC	HCV
Number	50.0	30.0
Mean	142.8	42.3
1st quartile	26.0	4.1*
Median	144.5	19.5
3rd quartile	210.0	73.5

\*: Alpha-fetoprotein showed lower level than normal. The above table showed that alpha-fetoprotein level in cases of HCC patients was higher than normal; on the other hand, in cirrhotic HCV patients it showed normal or even lower levels than normal as recorded in our cases.

3.1. ROC curve (HCC versus cirrhotic HCV)

Upon analysis of diagnostic value of all markers to differentiate

HCC from cirrhotic HCV patients we found that, concerning Apo A1, Apo A2 and alpha-fetoprotein they were able to differentiate between the two cases at cutoff levels < 54.5 mg/dL, > 127 mg/dL and > 48 ng/dL respectively with the highest positive predictive value (PPV) of Apo A2, followed by alpha-fetoprotein and the least was Apo A1 test. While IGF1 and IGF1R were not able to differentiate a case of HCC from cirrhotic HCV (Figure 3).

3.2. ROC curve (HCC versus controls)

Upon analysis of diagnostic value of all markers to differentiate HCC patients from normal person, we found that all four markers were able to differentiate between the two cases as follows.

When Apo A1, Apo A2, IGF1, and IGF1R at cutoff levels < 112.5 mg/dL, > 53 mg/dL, < 78 ng/dL and < 93.5 ng/dL respectively, the highest sensitive tests were Apo A1, IGF1 and IGF1R and the least sensitive was Apo A2 test (Figure 4).

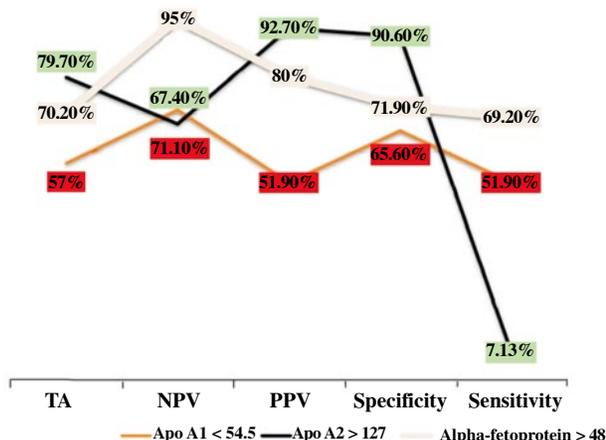


Figure 3. ROC curve (HCC versus cirrhotic HCV).

The above figure showed that alpha-fetoprotein showed the highest sensitivity while Apo A2 showed the highest specificity to discriminate cirrhotic HCV patients from HCC cases. TA: Total accuracy; NPV: Negative predictive value.

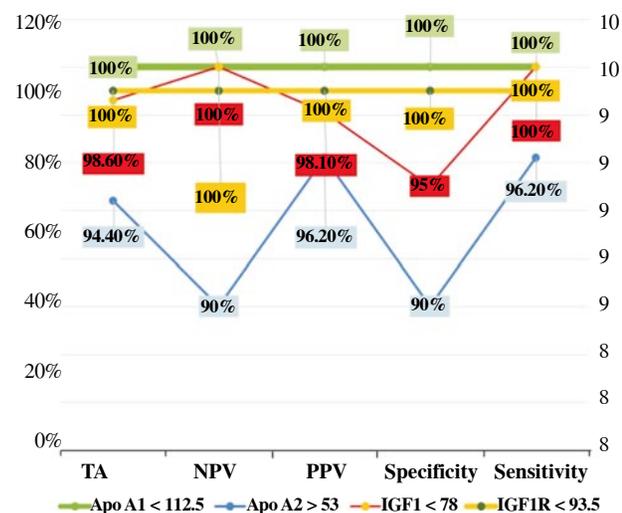


Figure 4. ROC curve (HCC versus controls).

The above figure showed that all four markers could differentiate effectively between a case of HCC and a healthy subject.

### 3.3. ROC curve (cirrhotic HCV versus controls)

Upon analysis of diagnostic value of all markers to differentiate cirrhotic HCV patients from normal person, we found that all four markers were able to differentiate between the two cases as follows. When Apo A1, Apo A2, IGF1, and IGF1R at cutoff levels < 121.5 mg/dL, > 53 mg/dL, < 71 ng/dL and < 73 ng/dL respectively, the highest sensitive tests were IGF1 and IGF1R equally followed by Apo A1 and Apo A2 tests (Figure 5).

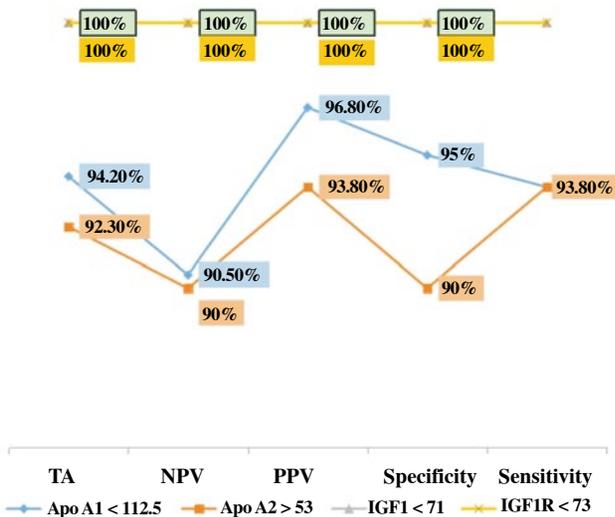


Figure 5. ROC curve (cirrhotic HCV versus controls).

The above figure showed that all four markers could differentiate effectively between a case of cirrhotic HCV and a healthy subject.

## 4. Discussion

In this study, alpha-fetoprotein measurement in HCC patients ranged between 5.1 and 412 ng/dL which was higher than the normal range (0–5 ng/dL) as stated by Nicoll *et al.*[6]. Both Lin *et al.*[7] and Ebrahim *et al.*[8] agreed with this result and recorded alpha-fetoprotein level between 6 and 390 ng/dL in HCC patients with small tumor size. Hah *et al.*[9] stated that one major disadvantage for the use of alpha-fetoprotein is that it can be falsely raised in patients who have active hepatitis but no evidence of HCC. This suggests that higher serum alpha-fetoprotein levels independently could not be used as a reliable marker alone to prove HCC case as approved by Ohhira *et al.*[10].

In our study, Apo A1 level decreased significantly in comparison to normal reference range (> 150 mg/dL). In case of HCC patients, the level ranged between 10 and 105 mg/dL with mean 52.7 mg/dL as agreed by Ehsani Ardakani *et al.*[11], Wang *et al.*[12] and Yang *et al.*[13] who recorded Apo A1 mean level in HCC patients as 30, 13 and 60 mg/dL, respectively. While in cirrhotic patients, Apo A1 mean level was 70.7 mg/dL which was consistent with Mustafa *et al.*[14] whose record was 30 mg/dL while Li *et al.*[15] recorded Apo A1 mean level in cirrhotic patients to reach 23 mg/dL. The above data showed that there was no marked difference in Apo A1 levels that could differentiate between cirrhotic HCV and HCC patients. From the above data, we observed that a decrease in plasma apolipoprotein and lipid levels is positively correlated with the severity of liver

failure as confirmed by Li *et al.*[15].

The results of quantitative analysis revealed that Apo A2 protein was upregulated in the plasma of HCC to reach a range of 52–190 mg/dL with mean 130.9 mg/dL, while in case of cirrhotic patient it reached a range of 50–144 mg/dL with mean 78.9 mg/dL compared to healthy control samples normal level that ranged between 30–50 mg/dL. Oliveira *et al.*[16] and Seki *et al.*[17] agreed with our results and recorded raised Apo A2 level in HCC patients to reach 65 and 98 mg/dL, respectively. Zhang *et al.*[18], Petit *et al.*[19] and Perlemuter *et al.*[20] recorded Apo A2 high level to reach 160, 142 and 180 mg/dL respectively in cirrhotic patients, which agreed with our results.

In our patients, serum concentration of IGF1 showed no differences related to age nor sex as agreed by Kasprzak *et al.*[21] and Adamek *et al.*[22] who stated same results as ours. Upon recording IGF1 level in our HCC patients, it decreased significantly to reach a range of 12–67 mg/dL with a mean level 48.2 mg/dL in comparison to normal reference range (74–300 mg/dL). Kasprzak *et al.*[21] agreed with our results and recorded IGF1 mean level in HCC patients to reach 56 mg/dL. In case of cirrhotic patients, IGF1 level was measured and ranged between 15 and 68 mg/dL with a mean level 49 mg/dL which was lower than normal range.

In our study, IGF1R is reduced in severe liver disease to reach below the normal range in adult which is 93–174 ng/mL. In cases of HCC, it was recorded to be 17–53 ng/dL with mean level 32.6 mg/dL which was agreed by Lin *et al.*[23] who stated IGF1R level in his study to reach 33 ng/dL. In cases of cirrhotic patients, the level was decreased ranging between 17 and 53 ng/dL with a mean level 35.1 mg/dL which was agreed by Yang *et al.*[13] and Kasprzak *et al.*[21] who recorded the level to be 16 and 63 ng/dL, respectively.

In our study, Apo A1 had the ability to differentiate between a case of HCC and cirrhotic HCV patient at cutoff value < 54.5 mg/dL with sensitivity 51.9%, specificity 65.6%, PPV 51.9%, NPV 71.1% and TA 57%. Lin *et al.*[23] stated similar results to us and recorded that Apo A1 sensitivity was 45% and specificity was 56% at cutoff level of 46 mg/dL.

In our study, Apo A2 can discriminate a case of HCC from cirrhotic HCV patient at cutoff level > 127 mg/dL with sensitivity 7.13%, specificity 90.6%, PPV 92.7%, NPV 67.4% and TA 79.7%. Liu *et al.*[24] recorded results similar to us and stated that Apo A2 at cutoff level > 107 mg/dL with sensitivity 13% and specificity 96% to differentiate a case of HCC from cirrhotic HCV patient.

Our study showed that IGF1 and IGF1R were not able to differentiate a case of HCC from cirrhotic HCV which was agreed by Jiang *et al.*[25] who denied the diagnostic value of IGF1 and IGF1R in ruling out a case of HCC.

In our study, alpha-fetoprotein at cutoff value > 48 ng/dL can differentiate a case of HCC from cirrhotic HCV with sensitivity 69.2%, specificity 71.9%, PPV 80%, NPV 95% and TA 70.2%. Ebrahim *et al.*[8] agreed with our results and stated that an alpha-fetoprotein value above 400 ng/dL has been considered to be diagnostic for HCC with sensitivity 70% and specificity 65%.

We thereby concluded that the use of Apo A1 and Apo A2 had the ability to differentiate between a case of HCC and cirrhotic HCV patient while alpha-fetoprotein, IGF1 and IGF1R have no clear role

to differentiate the two cases. Also we observed that all our tested markers had the ability to discriminate a normal healthy person from HCC and cirrhotic HCV patients.

### Conflict of interest statement

We declare that we have no conflict of interest.

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