

Assessment of Alpha-1-Acid Glycoprotein as a new Biomarker for Hepatocellular Carcinoma

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Background and study aim: The outcome of patients with hepatocellular carcinoma (HCC) remains poor because of late diagnosis. We aimed to evaluate the performances of serum alpha -1-acid glycoprotein (AAG) for the diagnosis of HCC, especially for HCC with low alpha-fetoprotein (AFP).

Patients and Methods: Ninety patients included in this study, 60 had HCC, and 30 (50%) of these were AFP low HCC (AFP \leq 20 ng/mL). The remaining 30 patients were chronic hepatitis C and cirrhosis without HCC as control group. Plasma AAG was analyzed using quantitative enzyme immunoassay technique.

Results: Serum level of AAG was significantly elevated in low AFP HCC group compared with high AFP HCC and

cirrhotic without HCC group, 1307.20 ± 9627 vs (850.82 ± 795.14 and 309.77 ± 220.17 respectively). Receiver operating characteristic (ROC) curve showed that the best cut off for AAG and AFP was 740 μ g/ml and 20 ng/mL respectively. The area under the curve of AAG was significantly higher than that for AFP (0.95 vs 0.92) respectively. AAG at a cut-off value of 740 μ g/ml provides higher sensitivity (73.3% vs 62%, respectively) and specificity (74.0%, and 71%, respectively) in low AFP HCC than high AFP HCC

Conclusion: The role of AFP in the diagnosis of HCC is limited; AAG had better performance in diagnosing HCC patients with low AFP. So Serum level of AAG might be used as a potential diagnostic marker for hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [1]. Alfa-fetoprotein (AFP) has been the most widely used plasma marker for diagnosis, surveillance and as a prognostic indicator of HCC patients' survival [2].

Several studies indicated that high plasma levels of AFP are related to poor prognosis, as well as histologic grade of malignancy [3]. Those with high plasma AFP level at the time of HCC diagnosis have more unfavourable outcomes compared to patients with low AFP level [4], however, AFP has a low sensitivity in detection of HCC even it often increase in the absence of HCC [5].

Alpha-1-acid glycoprotein (AAG) is an acute phase protein, synthesized

predominantly in the liver. Cytokines can cause plasma AAG level to increase as a part of an inflammatory response [6]. The concentration of AAG significantly increased under the pathologic state of infection, inflammation and tumor and its level may change in various liver diseases as in patients with acute hepatitis and patients with liver cancer [7]. The plasma level of AAG has been suggested to be a potential marker for diagnosing cirrhosis and HCC [8]. It was shown that combination of AAG and AFP improves the accuracy of HCC diagnosis [9].

In this study we aimed to evaluate the role of AAG in the diagnosis of hepatocellular carcinoma (HCC), and its clinical significance in HCC patients with low AFP (\leq 20 ng/ml) and in HCC patients with high AFP ($>$ 20 ng/ml).

PATIENTS AND METHODS

Patients:

This observational study was conducted on ninety patients admitted to Hepatology and Gastroenterology Department, Beni-seuf general Hospital during the period from March 2016 to September 2016 and a written informed consent was obtained from all participants prior to recruitment and divided into three groups:

- Group A: included 30 HCC patients with low AFP (≤ 20 ng/ml).
- Group B: included 30 HCC patients with high AFP (> 20 ng/ml).
- Group C: included 30 patients with HCV cirrhotics without HCC served as control group.

Liver cirrhosis was documented by clinical evaluation, laboratory investigation and evidence of cirrhosis by abdominal U/S. The diagnosis of HCC was confirmed by triphasic CT according to American association of study of liver diseases [10]. The diagnosis of HCV was dependent on detection of HCV-Ab and confirmed by HCV RNA positivity

Patients below 18 years and more 70 years, extremely ill patients, patients with malignancies other than HCC, patients with hepatic metastatic lesions and patients with a previous history of HCC treatment were excluded from the study

Methodology :

The enrolled patients were subjected to full history taking, thorough clinical examination and laboratory investigation including complete blood count (CBC), liver and kidney profile tests Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV) markers and AFP. Severity of liver cirrhosis assessed by using Modified Child-Pugh score.

Blood sampling and biochemical assays:

Fasting venous blood samples (5ml) were collected by well-trained laboratory technicians under complete aseptic conditions then distributed as follows:

- a- 1 mL of whole blood was collected in an EDTA vacutainer and mixed gently for complete blood count measurement that was performed by automated hematology system (Sysmex XE 5000; Sysmex America, Inc., Mundelein, IL, USA).
- b- 4 mL of venous blood samples were collected in plain test tubes containing no anticoagulant, allowed to clot for 30 mins at room temperature, then centrifuged for 15 mins at

1000× g. The serum was removed, aliquoted then stored at $\leq -20^{\circ}\text{C}$ until assayed and thawed immediately before the measurement, the separated serum was used for the following assays :

- Biochemical tests using Beckman CX4 chemistry analyzer (NY, USA, supplied by the Eastern Co. For Eng, Egypt), these tests including :
 - Fasting blood glucose level.
 - Liver function tests: Serum albumin, total and direct bilirubin, Liver enzymes including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (GGT).
 - Kidney function tests : including serum creatinine.
- Viral infection status (HCVAb and HBS Ag) were assayed using an enzyme immunoassay (EIA) Kit (Abbott, Axyam USA).
- Serum AFP level (ng/ml)was assyed using an enzyme immunoassay (EIA) Kit (Roche Mannheim, Germany) .
- Serum AAG levels were measured using human ELISA (sandwich technique) kits provided by Human AGP1/alpha 1 acid glycoprotein PicoKine™ ELISA Kit (Boster Biological Technology, Pleasanton CA, USA, Catalog # EK1486) for research use only with assay range 1.56-100 $\mu\text{g/ml}$.

Assay procedure of AAG :

100 of Standard or diluted sample was added to the bottom of micro ELISA plate well, covered with a new plate sealer, then incubated for 90 minutes at 37 degree, then 100 μL of biotinylated anti human AGP1 antibody was added, covered with a new adhesive strip and incubated at 37 degree for another 60minutes. Aspiration and wash was performed three times followed by addition of 200 μL of Avidin biotin Peroxidase complex (ABC) and incubated at room temperature for another 30 minutes, wash plate 5 times again. 90 μL of substrate was added to each well and incubated in the dark for 15-20 minutes. Finally, 100 μL of Stop Solution was added to each well, where the color turned to yellow immediately and the optical density (OD) was read at 450 nm within 30 minutes.

Calculation of results:

The duplicate readings for standard and samples was averaged and subtracted the average zero

standard optical density. A standard curve was created by plotting the mean OD value on the Y-axis against the concentration on the X-axis and a fit curve was drawn by some professional software and a best fitting equation of standard curve was calculated using OD values and concentrations of standard samples.

Statistical Analysis:

The statistical analysis was conducted using STATA/SE version 11.2 for Windows (STATA corporation, College Station, Texas). Data are reported as means \pm SD, Non-normally distributed data were analyzed by the chi-square test. Spearman pearman correlation test was used to analyse the relation between categorical data. The ROC curve used to determine the most sensitive and specific cut-off value for AAG for diagnosing HCC. Corresponding distribution tables were consulted to get the "P" (probability value). Statistical significance was accepted at P value <0.05 .

RESULTS

This study was conducted on 90 cases. The epidemiologic characteristics of the three patients' groups were summarized in table 1, divided into three groups: Group A included 30 HCC patients with low AFP (73.3% males and 26.7% females), with a mean (SD) of 59.83 \pm 6.16years.

Group B: included 30 HCC patients with high AFP (83.3% males and 16.7% females), with a mean (SD) of 62.17 \pm 5.416years. Group C: included 30 cirrhotic patients with chronic hepatitis C (served as control group). 46.6% males and 53.4% females, their mean ages was 62.17 \pm 5.416 years. There were highly statistically significant differences in age and gender among the studied groups(p=0.003 and 0.001 respectively).

As regard biochemical and molecular parameters :

There was a highly statistical significant difference between studied groups as regard ALT, AST, serum albumin, serum bilirubin, PC, INR, serum AFP, and serum AAG levels. AAG was high in HCC patients with low AFP than HCC patients with high AFP and cirrhotic patients without HCC (1307.20 \pm 962.77 Vs 850.82 \pm 795 and 309.77 \pm 220 respectively) table 2.

Majority of studied patients in group A were Child Class B (43.3) in group B and group C were Child Class A (40 and 63.3 respectively) table 3.

Tables 5 showed that AAG at a cut-off value 740 μ g/ml had high sensitivity, specificity, PPV and NPV in diagnosis of HCC with low level of AFP than HCC with high level of AFP (73.3 vs 68.1, 74 vs 71, 95.5 Vs 91 and 82.3 vs 88) respectively with area under the curve 0.95 vs 0.81 respectively.

Table (1): Demographic characteristics of the patients

Data	Group (A) (HCC with low AFP) n=30	Group (B) (HCC with high AFP) n=30	Group (C) (without HCC) n=30	P value
Age (year)				
• Range	46-72	44-69	35- 56	0.003
• Mean \pm SD	59.83 \pm 6.16	62.17 5.41	45.57 \pm 6.20	
Sex				
• Male (n.%)	22/30(73.3)	25/30(83.3)	14/30(46.6)	0.001
• Female (n.%)	8/30(26.7)	5/30(16.7)	16/30(53.4)	
Residence				
• Urban (n.%)	12(40)	14(46.6)	13(43.3)	0.52
• Rural (n.%)	18(60)	16(53.3)	17(56.60)	

Table (2): Biochemical parameters of studied groups

Labs	Group (A) (HCC with low AFP) n=30	Group (B) (HCC with high AFP) n=30	Group (C) (without HCC) n=30	P value
Hb mg/dl	11.26±1.34	11.85±1.03	11.08±1.08	0.397
WBCs x10 ³ /mm ³	7.01±2.55	6.10±2.93	7.28±2.58	0.215
Platelets x10 ³ /mm ³	139.54±35.35	153.97±74.80	122.03±30.67	0.201
ALT u/l	80.77±49.9	94.03±51.3	42.73±37.3	0.008*
AST u/l	92.13±60.1	89.93±58.3	43.60±31.3	0.006*
Albumin g/dl	3.12±0.5	2.93±0.6	3.57±0.6	0.02*
Bilirubin mg/dl	2.97±3.4	2.99±3.5	1.84±0.7	0.003*
PC %	57.83±8.5	59.49±9.3	71.67±7.8	0.012*
INR	1.55±0.21	1.42±0.23	1.13±0.11	0.04*
Creatinine mg/dl	1.48±0.73	1.16±0.56	0.8±0.6	0.003*
HCV RNA by PCR x10 ³	950.8±752.7	761.26±670.6	829.7±782.8	0.14
AFP	9.89±4.27	460±98.84	14.78±9.35	0.001*
AAG N: 50-120 µg/ml	1307.20±962.77	850.82±795.14	309.77±220.12	<0.001*

Table (3): Severity of liver cirrhosis assessed by Child-Pugh classification between studied groups

Child grade	Group (A) (HCC with low AFP) n=30	Group (B) (HCC with high AFP) n=30	Group (C) (Cirrhotic without HCC) n=30	P-value
	n (%)	n (%)	n (%)	
Child A	11(36.6)	12(40)	21(63.3)	0.008*
Child B	13(43.3)	11(36.6)	8(33.3)	0.13
Child C	6(20)	7(23.3)	1(3.33)	0.02*

Table (4): Correlation between AAG and different parameters among HCC groups

Variable/AFP	Spearman correlation	P value
Child-Pugh classification	0.41	0.02
Tumor number	0.32	0.01
Tumor size	0.16	0.09
AFP	0.54	0.03

Table (5): Diagnostic performance of AAG in diagnosis of HCC

AAG (µg/ml)	Cut-off Value	Sensitivity %	Specificity %	PPV %	NPV %	Area under curve (AUC)
Group (A) n=30	740	73.3	74	95.5	82.3	0.95
Group (B) n=30		68.1	71	91	88	0.81

This table shows that AAG at a cut-off value 740 µg/ml had high sensitivity, specificity, PPV and NPV in diagnosis of HCC with low level of AFP than HCC with high level of AFP.

Table (6): Diagnostic performance of AFP in diagnosis of HCC

AFP (ng/ml):	Cut-off Value	Sensitivity %	Specificity %	PPV %	NPV %	Area under curve (AUC)
HCC patients	20	63.2	79	84.9	82.7	0.92

This table shows that AFP level of (20 ng/dl) had low sensitivity (63.2%), good specificity (79%) and high PPV, NPV (84.9 and 82.7) respectively.

DISCUSSION

Prognosis and survival of patients with HCC is affected by the HCC stage at the time of diagnosis. So reliable markers would greatly improve the chances of early detection Of HCC [7].

Surveillance for HCC usually depends on AFP and abdominal ultrasonography. yet, the low sensitivity and specificity of AFP make it as a poor biomarker with abdominal ultrasonography for early detection of HCC [11]. so, a good new biomarker is required for early detection of HCC. Plasma level of AAG has been suggested as a new biomarker for HCC and cirrhosis [7]. The aim of this study was to assess the role of AAG in the diagnosis of HCC, especially with low alfa-fetoprotein (AFP) (≤ 20 ng/ml) and in HCC with high alfa-fetoprotein (AFP) (> 20 ng/ml). In the current study HCC patients were older than cirrhotic HCV patients, (as the mean age in the HCC group A was 59.83 ± 6.16 , group B was 62.17 ± 5.41 while in chronic liver disease group C was 45.57 ± 6.20). This finding was in agreement with Shaker who reported that the peak age group for HCC was from 50-70 years with a mean age of 58.7 years [12]. Also in agreement with El-Zayadi et al. who found that patients of the age group 40-59 years were at 3.7 times, and of age group 60 years were at 11 times more risk to develop HCC [13]. Also, there was a male predominance among the HCC groups, (as the number of males were 22 male to 8 female in group A and the number of males were 25 male to 5 female in group B). This finding was in agreement with Sharaf-Eldin et al. [14] and Holah et al. [15] who reported that males predominated with male to female ratio of 4.7:1 and 5:1 among HCC patients respectively.

Also, El-Serag and Rudolph [16] reported that males have higher liver cancer rates than females, with male: female ratios usually averaging between 2:1 and 4:1 the reasons for higher rates of liver cancer in males may relate to gender specific differences in exposure to risk factors.

The higher incidence of HCC in males might be due to the stimulatory effects of androgen and

the protective effect of estrogen. The biological activity of natural progesterone on HCC is controversial and lacks clear investigations [17]. Men are more likely to be infected with HBV and HCV, consume alcohol, smoke cigarettes and have increased iron stores. Higher levels of androgenic hormones, body mass index, and increased genetic susceptibility may also adversely affect male risk [16]. In the present study, HCC cases were more from rural areas, this result was in agreement with Shaker et al. who stated that the incidence of HCC is more common in rural areas than urban [12]. In the present study, AST, ALT and serum bilirubin were higher in HCC patients. These results were in agreement with Abu El Makarem et al. who reported that AST and serum bilirubin are higher in HCC patients than cirrhotics [18].

Other liver biochemical profile in this study as serum albumin, prothrombin concentration and INR were lower in HCC patients than in cirrhotic patients. This result was in agreement with Wong et al. and Baghdady et al. who reported the same results [19,20].

In this study, most of the patients with HCC were Child A and B, followed by Child C, These results were in agreement with Alves et al. who reported that (53.1%) of HCC cases were Child B followed by Child A (31.3%) then Child C (12.5%) [21]. And El-Sawy who reported that (40%) Of advanced HCC cases were Child B, followed by Child A (32.7%) then Child C (27.3%) [22].

In the current study there was significant positive correlation between AAG and severity of liver disease (Child-Pugh grade), tumor number and AFP but there was no significant correlation between AAG and the size of the tumor, this goes in agreement with a study by Kanget et al. that found AAG had similar sensitivity value in differentiating HCC regardless of the tumor size [13].

AFP used as a diagnostic test with a cut-off value 20 ng/ml provides sensitivity is only 63.2% and the specificity is 73%, This goes in agreement

with Gonzalez and Keeffe defining an elevated AFP level >20 ng/ml, confers a sensitivity of 60% and specificity of 80% [24]. While Jiang et al. reported that sensitivity of AFP was about 79.7% and specificity was about 80.3% in HCC cases [25].

In this study, ROC analysis of AAG used as a diagnostic test suggested that a cut-off value of 740 µg/ml provides sensitivity, specificity, PPV and NPV for group A of (73.3%, 74.0%, 95.5%, 82.3%) respectively, and for group B (68%, 71%, 91%, 88%) respectively. This shows increase in sensitivity and specificity of AAG in low AFP HCC (group A) more than that in high AFP HCC (group B), so these results allows us to purpose that determining AAG concentration could be especially powerful in patients with HCC especially with non-diagnostic AFP concentrations.

The results of our study are supported by Bachtiar et al. who reported that the level of AAG was low in patients with AFP high HCC but was higher in patients with AFP low HCC. the clinical performance of AAG increased in low AFP HCC (<20 ng/ml) as Sensitivity, accuracy, PPV and NPV of AAG in patients with AFP-low HCC were (82%, 90%, 91%, and 89%, respectively) while in patients with AFP-high HCC Sensitivity, accuracy, PPV, and NPV of AAG were (62%, 82%, 89%, and 79%, respectively). At cut-off value of 800 µg/ml for AAG and 20 ng/ml for AFP [9].

CONCLUSION

Serum Alpha 1 Acid Glycoprotein (AAG) concentration could be used as a potential marker for hepatocellular carcinoma.

Consent

All authors declared that written informed consent was obtained from the patients for publication of this paper.

Ethical Approval

Ethical clearance was obtained from Beni-seuf general Hospital's ethics committee.

Competing Interests

Authors have declared that no competing interests exist.

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