Significance of Serum Hepatocyte Growth Factor Level in Diagnosis of Hepatocellular Carcinoma

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Background and study aim : Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and one of the most common cancers worldwide. New serum tumor markers are required for diagnosis of HCC as alpha-fetoprotein (AFP), which is the most widely used marker, has poor diagnostic accuracy. Hepatocyte growth factor (HGF) initially identified and molecularly cloned as a potent mitogen of primary cultured hepatocytes, has multiple activities in a variety of tissues during the course of development and also in various disease states. This study was conducted to assess the diagnostic value of serum HGF as a biomarker for diagnosis HCC.

Patients and Methods: This study was conducted on 30 patients with documented HCC and 30 cirrhotic patients with no evidence of HCC; as well as 25 healthy subjects who served as control group. The levels of AFP and HGF were measured for all cases together with full clinical assessment, liver biochemical profile, viral markers, ultrasound, and abdominal triphasic computerized tomography (CT) scan.

Results: The mean value of serum HGF was highly significantly elevated in HCC group compared to the control group ($P<0.001$), and its level was higher in HCC than cirrhotic group (795.8 ± 312.04 Vs 322.7 ± 45.2 pg/ml respectively) with significant difference ($P<0.001$). There was significant correlation between serum HGF level and both ALT, MELD score in HCC group ($P = 0.005$, 0.02 respectively). At cut off level equal or more than 426.1 pg/ml, HGF could diagnose HCC with 96.7% sensitivity and 98.2% specificity and AUROC was 0.99.

Conclusion: Serum level of HGF could be considered useful marker for diagnosis of HCV related HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver **[1].** Over a decade (1993-2002), there was nearly a two fold increase of the proportion of HCC among chronic liver disease (CLD) patients in Egypt with a significant decline of hepatitis B virus (HBV) and slight increase of hepatitis C virus (HCV) as risk factors **[2].** The incidence and mortality rates for HCC are nearly identical, indicating the overall poor survival of patients with this tumor. Therefore, the most effective treatment relies on the early diagnosis of HCC **[3].** Alpha-fetoprotein (AFP) is a serological marker currently available for the detection of hepatocellular carcinoma. Its poor sensitivity renders it unsatisfactory for this purpose and suggests an urgent need for novel biomarkers for early stage HCC detection **[4].** HGF is a pleiotropic growth factor originally isolated from rat platelets. HGF-like factor known as macrophage-stimulating protein (MSP) is an 82 kDa, 674 amino acid residue hetero dimeric glucoprotein [5]. It has a wide range of effects from embryonic development and liver regeneration to protection and/or repair of various organs including kidneys, lungs, and cardiovascular system **[6].** HGF (scatter factor) is the most potent growth factor for hepatocytes and its receptor, the N-methyl-N′-nitroso-guanidine human osteosarcoma transforming gene trans-membrane tyrosine kinase (c-Met) is implicated in HCC carcinogenesis and progression through activation of multiple signaling pathways that direct cell growth, proliferation, survival and motility **[7]**. Aberrant Met/HGF activation has been observed in many

tumor types **[8].** Met/HGF inhibition has emerged as targeted anticancer therapies **[9]**. Ongoing clinical development with tivantinib, cabozantinib, onartuzumab, crizotinib, rilotumumab and ficlatuzumab has shown encouraging results **[8]**. The aim of this work was to investigate the diagnostic significance of hepatocyte growth factor in HCV related HCC cirrhotic patients and to assess its sensitivity and specificity as compared to AFP.

PATIENTS AND METHODS

Study groups:
This prospective study was conducted on 60 HCV cirrhotic patients admitted to Hepatology, Gastroenterology and Infectious Diseases Department. Benha Faculty of Medicine, Benha University in period between March 2014 and December 2014. In addition to 25 apparently healthy subjects served as control group. They were divided into three groups:

Group I (liver cirrhosis group): included 30 patients with post hepatitic liver cirrhosis without HCC, the majority of them were males [17 patients (56.7%)].

Group II (HCC group): included 30 cirrhotic patients with HCC, the majority of them were males [23 patients (76.7%)].

Group III (control group): included 25 apparently healthy subjects, 13 of them were males [13 patients (52%)].

Patients less than 18 years old, patients with liver cirrhosis due to other causes than HCV infection (HBV, Autoimmune hepatitis, Alcoholic,… etc), patients with hepatic focal lesions other than HCC (Hemangiona, Hepatoblastoma,…etc), and patients with metastatic focal lesions or with vascular invasion were excluded from this study.

Methodology:
Full history taking and clinical examination were done to all patients and the following routine laboratory investigations:

- Complete blood picture.
- Liver profile tests: alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), serum bilirubin, serum albumin, prothromhin time and concentration (PT and PC) and international normalized ratio (INR).
- Serum creatinine.
- Hepatitis markers: hepatitis B surface antigen (HBsAg) and anti-HCV antibody by 3rd generation ELISA [enzyme linked immune-sorbant assay].
- Evaluation of the severity of liver cirrhosis was assessed in each cirrhotic patient with the Modified Child score **[10]** and MELD score **[11]**.

Imaging studies:

- Abdominal ultrasonography was performed for all patients and controls included in the study using LOGIQ P6/P6 PRO QUICK GUIDE machine with a convex-sector probe (PVF-375 MT-2.57 MHz). Liver was assessed for size, smoothness of the surface, texture, portal vein diameter. The presence of focal lesions and their detailed description as regards number, size, site, echogenicity were reported. Doppler ultrasound was used to assess the patency of the portal vein. It was also used to detect any Doppler signals inside and around the lesions as the presence of intra-lesional arterial signals is highly suggestive of malignancy **[12]**. A complete abdominal scanning was done to detect any other abnormality including the presence of ascites, lymph nodes or abdominal masses.
- Triphasic abdominal CT scanning: Spiral tri-phasic CT abdomen was done to all patients in HCC group for the diagnosis of hepatic focal lesions with specific features of HCC as previously described **[13, 14, 15]**.

Tumor Markers :
A 2 ml blood sample was drawn from each subject after being diagnosed. Blood samples were centrifuged and serum aliquoted and stored at (- 20°C) until tested for AFP and HGF.

1- Measurement of serum (AFP) (ng/dl):
Serum AFP was measured by enzyme-linked immunosorbent assay (ELISA) technique using commercially available immunometric assays (by Monobind Inc. Lake Forest, CA92630, USA) with lower limit of detection (1.8 ng/ml) and normal reference range (2 – 10.9 ng/ml) obtained by manufacturer instruction.

2- Measurement of serum (HGF):
Serum HGF was measured by enzyme-linked immunosorbent assay (ELISA) technique (by Sunred bio company- Lot NO.=201411) with lower limit of detection (4.336 pg/ml) and normal reference range (59.7-157.3 pg/ml) obtained by manufacture instructions.

Principle:
An anti-human HGF coating antibody is adsorbed onto micro wells. Human HGF present in the sample or standard binds to antibodies adsorbed to the micro wells; a biotin-conjugated anti-human HGF antibody binds to human HGF captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-human HGF.

Assay protocol-:
The following steps were done as recommended by (Sunred Bio Biomedical Company): All reagents were brought to room temperature before use and each sample, standard, blank and optional control sample was assayed . First each test sample was diluted before use (50 µL of diluted sample was added into pre-designated wells).Then calibrator solutions were prepared as recommended by manufacture.

The Micro titer Plate was incubated at 22°Croom temperature for thirty (30 ± 2) minutes (Plate was kept covered during incubation).The contents of the wells were aspirated and each well was filled completely with appropriately diluted wash solution and aspirate. This step was repeated three times, Then 50 µL of appropriately diluted Enzyme-Antibody was conjugated to each well and incubated at 22°C room temperature for thirty (30± 2) minutes(Plate was kept covered in the dark during incubation).

The wells were washed then 50 µL of tetra methylbenzidine (TMB) Substrate Solution was added to each well.(The plate was incubated in the dark at RT for precisely 10 minutes). After ten minutes, 50 µL of Stop Solution was added to each well.and the absorbance was determined at 450 nm of the contents of each well.

Statistical Analysis:
Median, range, mean, and standard deviation were used for descriptive statistics, as appropriate. Categorical variables were tested with Fisher's exact test or $\chi 2$ test. Continuous variables were tested with Student t-test or analysis of variance (ANOVA). Comparison of plasma HGF levels and clinical characteristics among the three groups of subjects were analyzed using ANOVA test, post hoc tests, and Mann-Whitney U test. Correlation between plasma levels of HGF and AFP were analyzed using Spearman's correlation coefficient. Receiver operating characteristics (ROC) analysis was used to evaluate the diagnostic value of HGF and AFP and to identify the optimal threshold values. Sensitivity and specificity, positive and negative predictive values of HGF and AFP were profiled by curves. Calculations were done with the Statistical Package for the Social Sciences version 22 (IBM, SPSS, Statistics, V.22, 2012, IBM crop., New York. USA).

RESULTS

Demographic criteria of studied groups were shown in table (1). Mean age of patients with HCC was highly significantly elevated than other groups (P<0.001). The number of males within HCC, cirrhosis and control groups were higher than females.

Three patients (10%) in cirrhotic group were Child class A compared to one patient (3.3%) in HCC group. Seventeen patients (56.7%) in cirrhosis group and 10 patients (33.3%) of the HCC group were Child class B, while 10 patients (33.3%) in the cirrhosis group were Child class C compared to 19 patients (63.3%) in the HCC group with no statistically significant difference between both groups (P value = 0.25).

Table (2) showed highly statistically significant difference between studied groups as regard all laboratory parameters with higher values of liver profile tests and low serum albumin level in HCC group when compared to other groups. The mean value of serum AFP and HGF was highly significantly elevated in HCC and cirrhotic groups than control groups, and the mean level of HGF was higher in HCC group (795.8 ± 312.04 pg/ml) than cirrhotic group (322.7 ± 45.2 pg/ml) with highly significant difference (P<0.001) (Table 3).

Correlation studies revealed significant correlation between serum level of HGF and ALT (P value = 0.005) (Table 4 and Fig. 1) and MELD score (P value = 0.02) (Table 5 and Fig. 2) in HCC group. Table (6) showed no statistically significant correlation between HGF level and tumor number, size, site and shape.

Table (7) and Fig. (3) showed performance of biomarkers (HGF and AFP). At cut off level ≥426.1 pg/ml, HGF had higher sensitivity, specificity, positive predictive value, negative predictive value than AFP in diagnosis of HCC (96.7%, 98.2%, 96.7%, 98.2% vs. 83.3%, 92.7%, 86.2%, 91.1%) respectively with AUROC was 0.99 (P<0.001).

The ultrasonographic features of the focal hepatic lesions in HCC patients showed that 22 patients (73.3%) had single focal lesion, 8 patients (26.7%) had multiple focal lesions. Mainly in Rt lobe [17 patients (56.7%)] while in Lt lobe [8

patients (26.7%)] and 5 patients (16.7%) had focal lesions detected in both lobes. 13 patients (43.3%) had focal lesion <3 cm while 17 patients (56.7%) had focal lesion (3-5 Cm). 26 lesions were hypoechoic (86.7%), 3 lesions were hyperechoic (10%) and only one lesion (3.3%) was isoechoic.

As regard CT pattern of HCC in triphasic CT scan confirmed HCC diagnosis as all lesions showed typical enhancement in arterial phase followed by venous washout in the delayed portal/venous phase.

Table (1): Demographic criteria of studied groups

Variables	Cirrhotic group (N=30)	HCC group (N=30)	Control group (N=25)	P- value
Age (years) (Mean ± SD)	47.03±5.2	49.5±8.9	34.9±8.5	<0.001**
Gender N (%)				
Male	17(56.7%)	23(76.7%)	13(52%)	0.12
Female	13(43.3%)	7(23.3%)	12(48%)	

Table (2): Laboratory characteristics among studied groups

Variables	Cirrhotic group (N=30)	HCC group (N=30)	Control group (N=25)	P- value	Post Hoc Test
	(Mean±SD)	(Mean±SD)	(Mean±SD)		
Hb (g/dl)	11.9±1.9	10.6±2.3	13.01±1.8	<0.001**	P1=0.02* P2=<0.001** P3=0.04*
Platelet (cell/cm)×10^3	127.6±65.8	125.2±55.8	290.9±80.1	<0.001**	P1=0.89 P2=<0.001** P3=<0.001**
WBC (cell/cm)×10^3	7.3±3.9	5.5±2.4	5.8±1.7	0.04*	P1=0.02* P2=0.03* P3=0.95
ALT (IU/L)	47.2±35.4	54.3±30.7	29.4±15.1	0.004*	P1=0.79 P2=0.04* P3=0.001*
AST (IU/L)	57.0±29.5	67.5±46.8	27.7±15.03	<0.001**	P1=0.66 P2=<0.001** P3=<0.001**
Total bilirubin (mg/dl)	1.28 ±0.47	2.69±2.3	0.86±0.19	<0.001**	P1=0.007* P2=0.001** P3=0.001**
Albumin (gm /dl)	3.2±0.93	2.9±0.61	4.3±0.79	<0.001**	P1=0.89 P2=0.002* P3=0.004*
PT (seconds)	16.1±5.8	15.6±3.5	12.7±0.77	<0.001**	P1=0.96 P2=0.008* P3=<0.001**
INR	1.4±0.51	1.4±0.73	1.00±0.01	<0.001**	P1=1.00 P2=<0.001** P3=0.01*
ALP (IU/L)	126.5±28.9	160.1±36.1	52.5±19.2	<0.001**	P1=0.001* P2=<0.001** P3=<0.001**
Creatinine (mg / dl)	0.97±0.79	1.1±0.98	0.51±0.16	<0.001**	P1=0.92 P2=0.01* P3=0.008*

P1. Comparison between cirrhotic and HCC groups P2. Comparison between cirrhotic and control groups
P3. Comparison between HCC and control groups

Table (3): Serum levels of AFP and HGF among studied groups

Variables	Cirrhotic group (N=30)	HCC Group (N=30)	Control group (N=25)	P- value	Mann Whitney test
AFP (ng/dl)	25.01±2.6	264.5±589.7	4.2±4.5	<0.001**	P1=<0.001** P2=<0.001** P3=<0.001**
HGF (pg/dl)	322.7±45.2	795.8±312.04	108.5±48.8	<0.001**	P1=<0.001** P2=<0.001** P3=<0.001**

P1. Comparison between cirrhotic and HCC groups
P2. Comparison between cirrhotic and control groups
P3. Comparison between HCC and control groups

Table (4): Spearman's correlation between serum level of HGF and others parameters among cirrhotic and HCC groups

Variables	Serum HGF level			
	Cirrhotic group (N=30)		HCC group (N=30)	
	r	P value	r	P value
Age (years)	0.240	0.20	0.321	0.08
Hb (g/dl)	-0.001	0.99	-0.212	0.26
Platelet (cell/cm)×10^3	-0.160	0.39	-0.250	0.18
WBC (cell/cm)×10^3	-0.016	0.93	-0.004	0.99
ALT (IU/L)	-0.035	0.85	0.497	0.005*
AST (IU/L)	0.256	0.17	-0.121	0.53
Total bilirubin (mg/dl)	0.140	0.46	0.244	0.19
Albumin	-0.161	0.39	0.206	0.27
PT (seconds)	0.270	0.15	0.405	0.06
INR	0.139	0.46	0.418	0.09
ALP (IU/L)	-0.071	0.71	0.103	0.59
Creatinine	-0.117	0.54	-0.081	0.67
AFP (ng/dl)	0.229	0.23	0.179	0.35

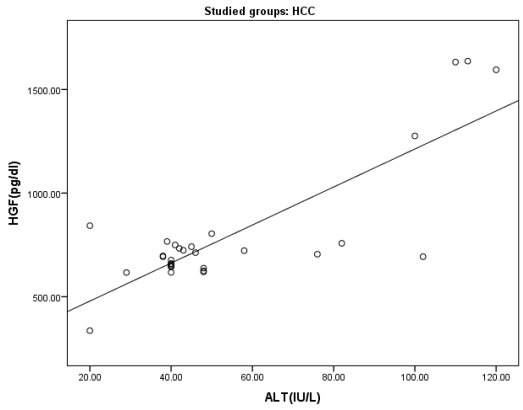

Fig. (1): Scatter Plot curve is showing correlation between serum level of HGF and ALT in HCC Group

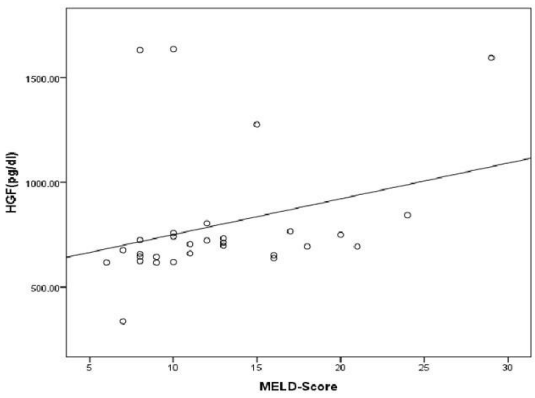

Fig. (2): Scatter Plot curve is showing correlation between serum level of HGF and MELD scores in HCC Group

Table (5): Spearman's correlation between serum level of HGF and severity of liver disease assessed by (Child-Pugh score, MELD scores)

Variables	HGF	
	r	P value
Child score	0.209	0.46
MELD score	0.442	**0.02***

Table (6): Spearman's correlation between serum level of HGF and characters of hepatic focal lesions among HCC group

Variables	HGF	
	r	P- value
Tumor number	-0.141	0.46
Tumor size	0.102	0.59
Tumor site	0.116	0.54
Tumor echogenicity	-0.059	0.76

Table (7): Diagnostic performance of serum level of AFP and serum level of HGF in HCC group

Variables	Cutoff point	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC	P - value
AFP ng/ml	≥ 9.5	83.3%	92.7%	86.2%	91.1%	89.4%	0.900	<0.001
HGF pg/ml	≥ 426.1	96.7%	98.2%	96.7%	98.2%	97.6%	0.995	<0.001

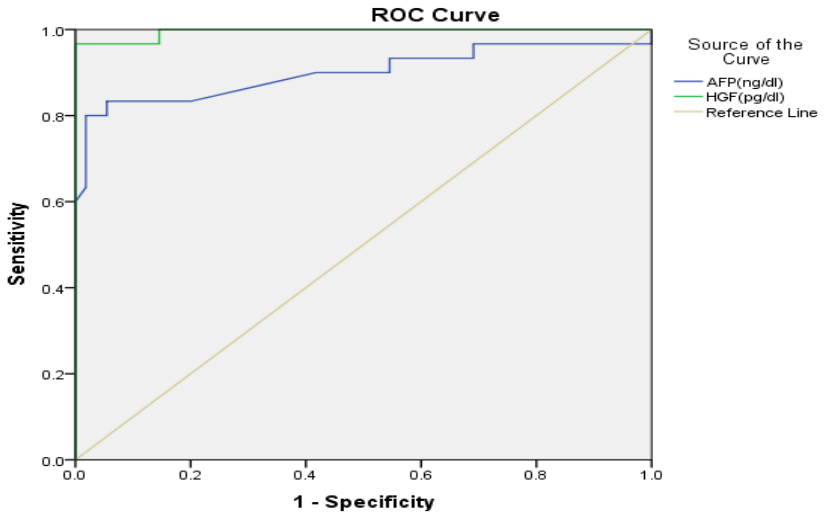

Fig. (3): Receiver Operating Characteristics (ROC) curve which show AFP and HGF sensitivity and specificity in diagnosis of HCC

DISCUSSION

HCC is the primary type of liver cancer, and both the age adjusted incidence and mortality of HCC have steadily increased in recent years **[7].** In Egypt HCC incidence rates have been doubled over the last ten years **[2,16,17].** And that is attributed to the growing HCV incidence **[18,19].** Currently, early diagnosis of HCC is the most important step in HCC management. Most imaging techniques help to discover HCC after considerable time of onset of tumor. In most instances, oncologists depend on AFP as the commonest and feasible marker for assessing HCC in addition to imaging. However, AFP is not completely reliable marker in early HCC diagnosis, prevention or therapy due to its low specifiity and sensitivity. Liver biopsy is always considered as an invasive procedure, so chemical findings are still greatly appreciated **[20].** Despite accumulating data regarding the risk factors for HCC, the mechanisms that contribute to HCC tumorigenesis remain poorly understood. There is vast evidence for protumorigenic growth factor signaling dysregulation in human HCCs affecting different signaling systems such as insulin growth factor, HGF, wingless, TGF-α, epidermal growth factor, and TGF–β signaling. There are many studies about HGF with tissue, cell line, and serum in HCC patients **[21].**

The mitogenic, motogenic, morphogenic, and pro neoangiogenic HGF is produced by non-parenchymal cells such as hepatic stellate cells **[22]**, sinusoidal endothelial cells, and kupffer cells **[23,24].** Increased HGF serum levels have been reported in patients with squamous cell carcinoma of the esophagus **[25].** There is a correlation between HGF serum values and a worsening of hepatic chronic disease **[26].** In the presence of liver cirrhosis, the functions of all these cells deteriorate, as do those of parenchymal cells. The mechanism of the high serum HGF level in liver disease could be a disorder of either production or elimination of HGF itself, but the role of HGF in liver disease is complicated and is not yet completely understood **[8].**

In the present study, we evaluated the diagnostic value of HGF in patients with HCV related HCC. In the present study, it was found that the mean age of patients with HCC was higher than other groups with highly significant difference between them (P<0.001). Also, Liver profile tests and AFP were significantly higher and s.albumin was significantly lower in HCC group than the other groups. In agreement with our findings, the study done by Breuhan et al. **[27]** which concluded that the mean age of patients with HCC was significantly higher than that of the other groups (P<0.001) and they had the highest values for various concurrently measured liver profile tests. The present study revealed that the mean of HGF level was higher in HCC group than the other groups with highly statistically significant difference between studied groups (P<0.001) that in agreement with Funakoshi et al. **[28]** who reported that levels of HGF in serum tend to be higher in patients with liver cirrhosis and HCC than with chronic hepatitis. Also the results of the present work supported by study of Yamagamim et al. **[29]** in which plasma samples were taken from 99 patients with chronic viral hepatitis C, cirrhosis, and HCC. The mean HGF level in HCC group (0.533 ± 0.167 ng/ml) was higher than in either chronic hepatitis group (0.383 ± 0.076 ng/ml) with P value = 0.008 or liver cirrhosis group (0.377 ± 0.054 ng/ml) with P value = 0.0016 and concluded that the serum levels of HGF represents the degree of the carcinogenic state (≥0.3 ng/ml, a high risk of developing cancer) in the liver of patients with viral chronic hepatitis C and cirrhosis.

The increase of HGF serum levels (≥0.6 ng/ml for the diagnosis) in cirrhotic patients is an indicator of HCC development. Furthermore, serum HGF levels reveal high carcinogenic states in liver cirrhosis type C **[30].** Induction is due to the increased production of HGF, not only in the liver, but also in distant organs, such as the lung. With the progression of liver damage, clearance of HGF in the liver diminishes. In addition, although patients with liver cirrhosis show a marked increase in serum HGF levels as the molecule is processed from a biologically inactive single-chain precursor from of HGF into the two-chain active form. Levels may be significantly disturbed in the damaged liver, and a single-chain precursor form can become a major form in the serum **[28].**

In the present work, correlation study revealed that there was a highly significant positive correlation between HGF level and ALT level (P = 0.005) and significant positive correlation with MELD score (P=0.02) in HCC group but there was insignificant correlation between HGF level and other studied parameters in both cirrhotic

and HCC group such as age, CBC, other liver profile tests, AFP and Child score. This results in agreement with that reported by Karabulut et el. **[8]** who concluded that serum HGF levels were significant higher in patients with elevated serum ALT levels than others with normal ALT levels (P = 0.05) but in the other hand they found that no correlation between serum levels of HGF and MELD score (P = 0.09), which was in contrast to our results. This difference may be related to difference in patients criteria as the last study included HCV, HBV, alcoholic patients. The present study demonstrated that no correlation between serum HGF level and tumor number, site, size and echogenicity in HCC group. This result were in a concordance with that reported by Karabulut et el. **[8]** as they found no correlation between HGF level and tumor number but there was positive correlation with tumor size (P = 0.01). That can be explained by small number of studied patients in the present work (30 patients) in comparison to the last study (54 patients). Analysis of serum HGF level by ROC curve in the present work revealed satisfactory values regarding sensitivity and specificity at a cut off value (≥ 426.1 pg/ml). Serum HGF had higher sensitivity, specificity, PPV and NPV than AFP for diagnosis of HCC (96.7%, 98.2%, 96.7, 98.2 Vs 83.3%, 92.7%, 86.2%, 91.1% respectively) there for, assessment of serum HGF in HCV cirrhotic patients would be beneficial for diagnosis of HCC.

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Ethical approval: Written informed consent was taken from all patients before participation in this study.The study protocol was approved by the ethical committee of Benha Faculty of Medicine and its University Hospitals.

Conflicts of interest: Non.

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