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ORIGINAL ARTICLE

Clinical Significance of AFP, HGF & Micro RNA-122 Levels in Patients with Liver Cirrhosis and Hepatocellular Carcinoma

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ABSTRACT

Background: *Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and is advanced by severe viral hepatitis B or C (HBV or HCV). HCC is frequently diagnosed late requiring costly surgical resection or transplantation. Alpha fetoprotein (AFP) measurements, together with ultrasound are the most non-invasive methods for detection of HCC. Also, serum levels of hepatocyte growth factor (HGF) vary in liver diseases, reflecting hepatic damage and hepatocellular dysfunction. The discovery of miRNAs circulating in the peripheral blood has opened new directions of research to identify new non-invasive markers for diagnosis of diseases.* ***Objectives:*** *To assess the serum level of HGF and AFP in patients with liver cirrhosis with HCV infection and those of HCC .Also, to evaluate the expression levels of circulating plasma miRNA-122 in Egyptian patients.* ***Methodology:*** *Anti-HCV and HCV RNA were detected by an enzyme immunoassay and the viral RNA mini kit respectively among patients with cirrhosis and HCC. Also, serum AFP and HGF concentration was measured by sandwich ELISA for patients with HCC, patients with cirrhosis and normal controls. Correlation between HGF levels AFP and biochemical (transaminases, prothrombin activity, albumin, bilirubin), or virological (hepatitis C virus load) parameters was analyzed. . Expression level of miRNA-122 in plasma using RT-PCR were determined.* ***Results:*** *The serum AFP level was significantly elevated in cirrhotic and HCC patients in comparison to the control, with the highest level in the HCC group. On the other hand, the HGF level was significantly higher in HCC group than patients with cirrhosis. For HGF, the sensitivity and specificity were better than AFP (95% and 80% respectively). MiRNA-122 showed significant elevation in patients with cirrhosis groups and HCC group (P = 0.001) in comparison to the control group. Receiver operator characteristic (ROC) curve analysis for miRNA-122 yielded 73% sensitivity and 96 % specificity for the differentiation of HCC patients from non-HCC at a cutoff 0.165.* ***Conclusion:*** *HGF was better than AFP as noninvasive biomarkers for the detection of HCC in HCV cirrhotic patients. As its specificity was 96 %. MiRNA-122 can be used as novel non-invasive biomarker for monitoring HCV related disease progression.*

INTRODUCTION

Liver cirrhosis represents the final stage of several chronic hepatic diseases **.** It is a diffuse process of architectural disorganization characterized by fibrosis and the formation of structurally abnormal parenchymal nodules [1]**.** This results in portal hypertension, Porto-systemic shunting, and a decrease in the effective parenchymal mass [2]**.** Progressive accumulation of collagen as well as other proteins in the extracellular matrix eventually results in disrupted liver morphology, parenchymal function impairment, and ultimately portal hypertension and its related sequels [3].

Hepatocellular carcinoma is one of the most common malignant tumors with a high rate of morbidity and mortality [4]**.** It represents the fifth most common cancer worldwide and the third most frequent cause of cancer-related death. It is one of the fastest tumors resulting from chronic infection by hepatitis B and C viruses**.** Patients with advanced liver disease, particularly cirrhosis, are those at risk for HCC should be screened every 6 months for its development [5]**.**

This emphasizes the need to investigate the contribution of different biomarkers pathways to tumor development in different HCC, selected according to their clinical and pathological features, to identify novel

biomarkers targets for early diagnosis, chemoprevention, and treatment [6].

AFP is a glycoprotein with a molecular weight of 70 kDa, which is synthesized in the endodermal cells of the yolk sac during early fetal development and subsequently in embryonic hepatocytes [7]. AFP has mainly been used for diagnosis of HCC; however, its sensitivity and specificity are not satisfying that the Practice Guidelines of the American Association for the Study of Liver Diseases (AASLD) as it rejected its use, since 2010, whether for the surveillance or the diagnosis of HCC [3].

The diagnostic value of AFP is more and more questioned, as it elevates in patients with HCC, cirrhosis, chronic hepatitis, liver necrosis, pregnancy, or gonadal tumors [8]. Therefore, new and more specific markers for early detection of HCC are critically needed to improve the survival of affected patients [9].

HGF is a cytokine with a glycoprotein nature produced by mesenchymal cells of different organs, among the liver it is implicated in hepatic regeneration [10], and considered a prognostic factor in liver diseases. This is due to its correlation with liver damage and dysfunction, as well as its usefulness as tumors marker for diagnosis of hepatocellular carcinoma [11].

The invasiveness of liver biopsy procedure and the absence of a reliable biomarker for diagnosis of HCV related disease progression (Cirrhosis and HCC) emphasize the need for an alternative sensitive, reliable and non-invasive biomarker tool. MicroRNAs (miRNAs) are small non-coding RNAs (18 - 24 nucleotides) that interact with their target mRNAs to inhibit translation by promoting mRNA degradation or to block translation by binding to complementary sequences in the 39-untranslated region of mRNAs [12]. An integral role of miRNAs in cancer pathogenesis has begun to emerge.

MiRNA expression profile reveals characteristic signatures for many tumor types . MiRNAs have also been detected in human serum and plasma, where they are remarkably stable, raising the possibility that unique miRNA patterns in serum and plasma might be used as non-invasive disease markers [13].

Micro RNAs in large part derived from cells with damaged plasma membrane. In liver, miRNAs play fundamental functional roles in the regulation of physiological and pathological processes. In fact, the liver specific miRNA–122 is the most abundant miRNA in the liver, accounting for up to 72% of hepatic miRNAs . On a functional level, it was demonstrated that miRNA–122 is essential for liver homeostasis plays an important role in regulating hepatocyte development, differentiation, apoptosis and modulates hepatic lipid metabolism [14]. Loss of miRNA–122 promotes steatosis, inflammation, fibrosis and liver cancer by regulating hepatic networks of genes involved in cell cycle regulation, lipid metabolism, inflammation and oncogenesis, in humans miRNA–122 expression was associated with hepatocarcinogenesis and miRNA–122 loss was associated with a poor prognosis of patients with hepatocellular carcinoma, while miRNA – 122 overexpression sensitized HCC cells to chemotherapy and was associated with long survival times [15]. The stability of hepatitis C virus (HCV) is dependent on a functional interaction between the HCV genome and miRNA-122. By blocking this interaction with a specific antisense oligonucleotide, it was possible to inhibit viral replication in HCV patients, thus demonstrating the potential of miRNAs as therapeutic targets in liver disease [16]. To date, a limited data exist concerning alterations of the miRNA-122 concentrations in serum or plasma of patients with HCV-induced uncomplicated or complicated chronic hepatitis C. In light of this deficiency, the aim of the present study was to evaluate circulating plasma miRNAs miRNA-122 expression level in Egyptian patients with HCV-related complications (cirrhosis and HCC) to investigate their usefulness as non-invasive diagnostic markers for HCV disease progression.

METHODOLOGY

This study was conducted at the Microbiology unit, in collaboration with the Internal Medicine Department in Shebin El-Koum Teaching Hospital during the period from December 2014 to April 2016.The participants were divided into three groups:

- **Group 1** included 20 healthy volunteers as a control group (15 male & 5 female), their ages ranged from 25 to 64 years old.
- **Group 2** included 30 patients with liver cirrhosis (24 male & 6 female), their ages ranged from 46 to 63 years old.
- **Group 3** included 20 patients with HCC (18 male & 2 female), and their ages ranged from 46 to 61 years old.

All patients were diagnosed clinically and confirmed by biochemical investigations, abdominal ultrasonography, and abdominal triphasic compute tomography.

Inclusion criteria:

HCV infections (defined as a positive serology result by a second or third generation enzyme-linked immunosorbent assay and a positive result from a polymerase chain reaction test) and an increase in the serum aminotransferase levels for at least 6 months

Exclusion criteria:

Autoimmune hepatitis, drug-induced hepatitis, other autoimmune diseases, those with renal, cardiovascular, or respiratory diseases, patients with any malignancy other than HCC and pregnant females.

Informed consent was obtained from patients and controls. The protocol conformed to the ethical guide lines of the research committee.

Specimen collection

Blood Sampling: Ten mL of peripheral blood was collected from each patient. Five mL were put on potassium ethylene diamine tetra-acetic acid (K3 EDTA) tubes to separate plasma samples (used for RT-PCR, and CBC) while five mL were collected into plain tubes to separate serum samples (used in biochemical analysis) and AFP, HGF assay. Plasma samples for RT-PCR were stored at −80˚C until further processing.

Serum liver function tests were done to each participate in the study; Alanine- aminotransferase by continuous monitoring method[17]. Aspartate aminotransferase by continuous monitoring method [17]. Serum bilirubin: by Diazo method of Malloy and Evelyne [18]. Serum total protein: by bromocresolgreen (BCG) method [18].

Detection of HCV antibody and RNA.

Anti HCV antibodies were detected by Murex anti HCV (version 4) according to the manufacturer instructions. An ELISA which utilizes microplates coated with HCV specific antigens derived from "core" and "ns" regions encoding core peptide, recombinant NS3, NS4 and NS5 peptides [19].

HCV RNA was extracted using the viral RNA mini kit (Qiagen, Hilden, Germany), according to the protocol provided. The first strand complementary DNA (cDNA) was synthesized. Initial denaturation was performed at 95℃ for 5 min. Polymerase chain reaction amplification was carried out at 94℃ for 1 min, 57℃ for 1 min, and 72℃ for 1 min for a total of 40 cycles and final extension at 72℃ for 7 min. The primer sequences were as follow:

forward—5′CGCGCGACTAGGAAGACTTC3′ and reverse—5′ACCCTCGTTTCCGTACAGAG 3′.

Detection of AFP by ELISA Method:

Serum AFP was measured using DRG® AFP ELISA Kit (Catalog No. AF237T) by Kit supplied from Calbiotech Inc. Cordell Ct., El Cajon, CA 92020

AFP is expressed in ng/ml. AFU activity is measured by enzyme degradation of an artificial substrate (p-nitrophenyl alpha -L-fucopyranoside). The liberated p-nitrophenol was detected by colorimetric reaction and measured at 450nm.

The enzyme activity is measured as nanomoles of p-nitrophenyl cleaved per 1 ml of serum per hour (nmol/ml/hr) [20].

Detection of HGF by ELISA Method:

The HGF ELISA (eBioscience Europe/International. Bender MedSystem GmbH campus Vienna Biocenter 21030 vienna, Austria) is a “sandwich” enzyme immunoassay employing monoclonal antibodies. The procedures were done according to the manufacturer instructions. The results of HGF were expressed as pg/ml [21].

Detection of MiRNA Expression Levels by Quantitative Real-Time Reverse-Transcription (RT)-PCR[22].

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miRNA-122.RNU6B was used as internal control.

RNA Extraction

Total RNA with preserved miRNAs was extracted from 200 µl plasma with the miRNA easy extraction kit (Qiagen, Valencia, CA, USA) using 1 ml QIAzollysis reagent and incubated for 5 min at room temprature. Then, 200 µL of chloroform was added, and the samples were vortexed for 15 sec, and incubated for 2 - 3 min at room temperature. This was followed by centrifugation at 14,000 g at 4˚C for 15 min. The upper watery phase was removed, and an equal volume of 100% ethanol was added. Each 700 µl of this mixture were placed in miRNA easy Mini spin column in a 2 ml collection tube and centrifuged at 8000 g at room temperature for 15 sec. After the mixture completely passed through the column, 700 µl of RWT buffer was added to each column prior to centrifugation at 8000 g at room temperature for 15 sec. 500 µl of buffer RPE was added to the column prior to centrifugation at 8000g at room temperature for 15 sec. After this, another 500 µl of buffer RPE was added to the column prior to centrifugation at 8000g at room temperature for 2 min. The column was placed in a new collection tube and centrifuged at full speed for 2 min. Then, the column was transferred to a new 1.5 ml collection tube, 50 µl of RNase-free water was pipetted directly onto the column and the column was centrifuged for 1 min at 8000 g to elute RNA.

Reverse Transcription

The specific cDNA of miRNA-122 and RNU6B (U6B small nuclear RNA gene) were synthesized from RNA using gene-specific primers according to the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Each Reverse transcriptase reaction (15 µl reaction volume) consisted of: 7 µl Master Mix (100 mMdNTPs, 50 U/µlMultiScribe Reverse transcriptase, 10× Reverse transcriptase buffer, 20 U/µlRnase inhibitor), 3 µl primer and 5 µl RNA sample. The 15-µl reaction volumes were incubated in Applied Biosystems Cycler (Bio-Rad Laboratories, Hercules, CA, USA) for 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C, and then held at 4˚C.

MiRNA Amplification

Real-time PCR was performed using an Applied Biosystems One Step real- time PCR system. Each PCR reaction mixture included 10 µl 1× TaqMan universal PCR Master Mix, 1µl of primers and probe mix of the TaqMan MicroRNA Assays (Applied Biosystems), 1.33 µlRTase product and nuclease free water to a final volume of 20 µl.

Relative Quantitation of Target MiRNA Expression

By using the comparative cycle threshold (CT) method. The raw data were presented as the relative quantity of target miRNA, normalized with respect to RNU6B.

Statistical analysis

Statistical analysis was performed using statistical package for social science version 14.0 (Spss, Inc., Chicago, III, USA) for windows. Continuous variables were analyzed as mean ± standard deviation (SD). Differences among different groups regarding continuous variables with normal distribution were analyzed with univariate ANOVA and Bon Ferroni post hoc test and those not normally distributed were analyzed by Kruskal Wallis test then pairwise comparison was done to detect differences between groups by Mann – whitney U–test. P value of <0.05 was considered statistically significant; the correlation coefficients (r) were calculated by using the spearman correlation.

RESULTS

Statistical analysis of age and gender showed no significant difference among the studied groups. About 60% of both patients with cirrhosis and HCC patients received antiviral therapy; 83.3% and 75% of patients with cirrhosis and HCC patients had anti-HCV respectively. Thirteen out of 20 HCC patients (65%) had HCV-RNA (Table 1). Eighty six% of HCV antibody positive patients had HCV-RNA (Table 2). The hemoglobin level, total RBCs, ALP, GGT, total bilirubin and serum albumin were significantly reduced ($P < 0.05$) in patients with cirrhosis and HCC patients (Table 3). The serum AFP level was significantly elevated in patients with cirrhosis and HCC patients in comparison to the control, with the highest level in the HCC group. On the other hand, the HGF level was significantly higher in HCC group than patients with cirrhosis (range from580 to 5170 pg/mL) (Table 4).

The highest expression plasma level of miRNA–122 was in HCC group (820±255 copies / ml), followed by patients with cirrhosis with mean plasma level (323±76 copies / ml). There are highly statistical significant differences in miRNA–122 levels among the studied groups ($P < 0.001$) (Table 5).

The diagnostic performance of AFP, HGF and miRNA-122 were examined that were differentially expressed in HCC and non HCC (cirrhotic) groups, to discriminate between the two groups. ROC analysis was used to determine the optimum cut-off value for the studied diagnostic markers.

Table 6 showed the sensitivity, specificity, diagnostic accuracy, positive and negative predictive values of AFP & HGF and at the optimal selected cut-off values.

Table 1: Patients and Characters of Diseases

Characteristics	Total number of patients (n=50)		Test of significance	P value
	Patients with Cirrhosis group (n=30)	HCC group (n=20)		
Age (year) Mean±SD	54.3±7.9	53.7±7.3	**t**=0.28	0.78
Gender	**No. %**	**No. %**		
Male	24 80	18 90	χ^2= 0.89	0.35
Female	6 20	2 10		
Smoking	**No. %**	**No. %**		
Yes	22 73.3	10 50	χ^2=2.84	0.09
No	8 26.7	10 50		
Antiviral therapy	**No. %**	**No. %**		
Yes	18 60	12 60	χ^2=0.00	1.00
No	12 40	8 40		
Anti-HCV positive	**No. %**	**No. %**		
Yes	25 83.3	15 75	χ^2=0.52	0.47
No	5 16.7	5 25		
	No %	**No %**		
HCV RNA	**30 100**	**13 65**	**χ^2=0.93**	**0.74**

Table 2: Demographic and hematological data of the studied patients and the control groups

Data	Patients with Cirrhosis	HCC patients	Controls (n=20)	P value
Hemoglobin (12g/ dl):	8.85±1.97	9.5±1.95	13.52±1.75	0.001
WBC count (×10^3 cell/ul):	7.39±1.46	5.64±3.1	8.17±3.77	0.938
RBCs (×106 cell/µl	4.77±0.45	3.66± 0.58	8.84±1.97	0.001
Platelets	128 ± 3.5	119 ± 7.3	380 ± 6.2	0.001
ALT(45U2-4):	43.5(26.5-54)	55(31-71)	20.5(17-36)	0.043
AST(U/L):	96(60.5-136)	150(108-210)	70(57.2-82.5)	0.001
GGT(50U/L):	2(1.27-3.27)	1.6(1.29-4.07)	0.8(0.6-1.05)	0.001
TOTAL BILIRUBIN: mg/dL	7.5 ±7.2	10.5±9.96	0.48 ± 0.20	0.001
ALBUMIN (g/dl):	1.58±0.38	1.49±0.24	0.95±0.20	0.001
INR (1.2):	1.59±0.39	1.49±-.24	0.96±0.21	0.001

Table 3: Serum levels of AFP, HGF in studied groups

	Control group (No.20)	Patients with cirrhosis group (No.=20)	HCC group (No.=30)	Kruskal Wallis Test	P value
AFP(ng/ml)					
Median	**3.15**	3.2	**118**	**23.2**	**<0.001****
Range	**(1.97-4.55)ng /ml**	(2.65-7.23)ng/ml	**(70.5-215)ng/ml**		
HGF(pg/ml)					
Median	365.0	870.0	1425.0	26.79	<0.001**
Range	110 - 1100	110 - 2190	580 - 5170		

*significant difference **highly significant difference

Table 4: Validity of serum levels of HGF (pg/ml), AFP) (ng/ml) and both for diagnosis of HCC

Validity	HGF (pg/ml)	AFP (ng/ml)	Both HGF and AFP
AUC	**0.955**	**0.656**	**-**
Cutoff	**880**	**3.25**	**-**
Sensitivity	**95%**	**70%**	**70%**
Specificity	**80%**	**65%**	**100%**
PPV	**82.6%**	**66.7%**	**100%**
NPV	**94.1%**	**68.4%**	**76.9%**
Accuracy	**87.5%**	**67.5%**	**85%**

PPV: positive predictive value NPV: Negative predictive value

Table 5: Micro RNA-122 expression in the studied groups

	Control N = 25	Cirrhosis No=	HCC No=	P value
mi RNA – 122 Copies / ml	22±13	323±76	820±255	<0.001*

*significant difference

Table 6: Validity of serum levels of HGF (pg/ml), AFP) (ng/ml) and miRNA 122 for diagnosis of HCC

Validity	HGF (pg/ml)	AFP (ng/ml)	miRNA 122
AUC	0.955	0.656	0.800
Cutoff	880	3.25	0.165
Sensitivity	95%	70%	73
Specificity	80%	65%	96
PPV	82.6%	66.7%	78%
NPV	94.1%	68.4%	70%
Accuracy	87.5%	67.5%	83%

PPV: positive predictive value NPV: Negative predictive value

Serum AFP (Fig. 1) recorded a moderate sensitivity (70%) and specificity (65%) with a moderate diagnostic accuracy of 67.5%. The positive and negative predictive values were 66.7% and 68.4% respectively. For HGF, the sensitivity and specificity were better than AFP (95% and 80% respectively).The diagnostic accuracy was 87.5%. The positive and negative predictive values were 82.6%, 94.1% respectively (Fig. 2). The specificity of both AFP and HGF for detection of HCC reached 100%. ROC curves of the markers are shown in (Fig. 3). The areas under the **r**eceiver **o**perating **c**haracteristic ROC curve for AFP, HGF indicating the validity of using all the markers in diagnosis of HCC in patients with cirrhosis.

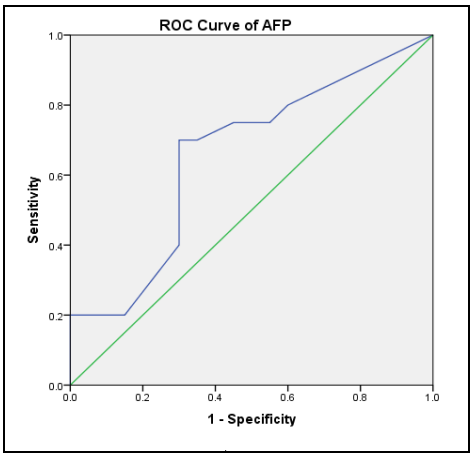

Fig 1: ROC curve Of AFP.

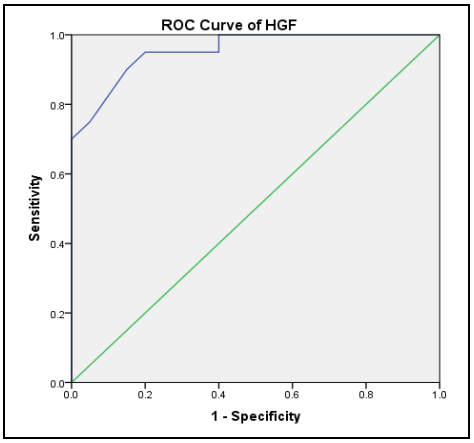

Fig. 2: ROC curve Of HGF.

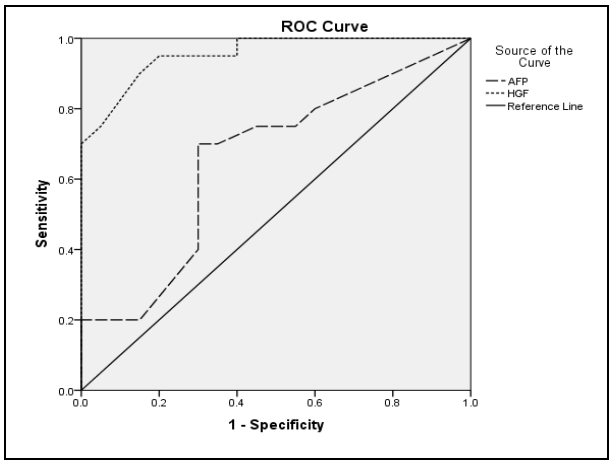

Fig. 3: ROC curve of both AFP & HGF.

ROC analysis for miRNA-122 revealed AUC = 0.800 thus discriminating between HCC and non-HCC patients. The calculated cut-off of miRNA-122 that showed the highest sensitivity (73%) and specificity (96%) was 0.165

In correlations between miRNA–122 and other laboratory parameters, there were significant correlation between miRNA–122 and AST. There were non-significant correlation between miRNA–122 and the other studied parameters (Table 7).

In the current study, we used univariate logistic regression analysis to select the predictive factor associated with HCV-related HCC diagnosis, and we found that serum AST level and INR, HGF and miRNA-122 could predict HCC diagnosis. AFP was not a successful predictor for HCC diagnosis (Table 8).

Table 7: MiRNA-122 expression level and the studied parameters.

	MiRNA-122			
	Cirrhosis		HCC	
	r_s	P value	r_s	P value
Hemoglobin (12g/ dl):	0.387	0.067	0.060	0.653
WBC count (×10^3 cell/ul):	0.472	0.471	−0.192	0.430
RBCs (×106 cell/µl	0.280	0.445	0.073	0.186
Platelets	0.473	0.097	0.032	0.812
ALT:	−0.046	0.871	0.224	0.356
AST:	−0.344	0.210	0.846	<0.001*
GGT:	0.490	0.064	0.556	0.265
TOTAL BILIRUBIN: mg/dL	−0.71	0.801	0.223	0.358
ALBUMIN (g/dl):	0.489	0.064	0.103	0.674
INR (1.2):	−0.267	0.398	0.116	0.275

rs: Spearman correlation.

Table 8: Univariate analysis showing the predictive power of different factors for HCC diagnosis

Factor	Odds Ratio	95% confidence interval	P value
AST	1.019	1.003 - 1.035	0.018*
ALT	0.998	0.987 - 1.009	0.745
INR	29.886	1.329 - 672.283	0.032*
AFP	0.823	1.007 - 1.271	0.678
HGF	1.090	0.988 - 1.202	0.084*
MiRNA-122	1.081	1.360 - 1.081	0.018*

*significant difference

DISCUSSION

HCC generally develops following an orderly progression from cirrhosis to dysplastic nodules to early cancer development, which can be reliably cured if discovered before the development of vascular invasion. Early detection of HCC provides the best chance for a curative treatment [23].

The aim of this study was to assess the serum level of AFP and HGF in patients with liver cirrhosis due to HCV infection and those of HCC, and also to evaluate the expression levels of circulating plasma miRNA-122 among the studied groups.

This study revealed that, there was no statistically significant difference in age between HCC patients and other studied groups, The same results were obtained by Fouad et al. [24]. On the other hand, Zekri et al.[25] reported a significantly higher mean of patients' age with HCC than that of the other groups. This might be due to the studied cases were in early stage of cirrhosis, which had started at younger age.

In this study, the serum heamoglobulin (Hb) level was significantly lower in liver cirrhosis and HCC patients than controls. This difference could be explained by acute and chronic gastrointestinal blood loss, hypersplenism, bone marrow suppression, and the anemia in chronic diseases**.** This was in agreement with Sakisaka et al.[26] who reported that although raising in serum erythropoietin levels up to 23% in HCC patients, elevations in Hb concentration or packed cell volume were uncommon and most patients were anemic because of other side effects of the tumor**.**

In the present study, the total leucocytic and platelet counts were not significantly reduced in patients with cirrhosis and those with HCC in comparison with the other groups. This was in agreement with the results of Pratt and Kaplan[27], which might be due to hypersplenism with splenic margination and portal hypertension with attendant congestive splenomegaly. An enlarged spleen can result in temporary sequestration of up to 90% of the circulating platelet mass. Decreased thrombopoietin levels might also contribute to thrombocytopenia**.**

Elevated aminotransferase levels are sensitive for liver injury**.** ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis [28]**.** Also in the present study, serum bilirubin concentration was significantly elevated in both patients with cirrhosis and HCC groups than control group. This was in agreement with Karabulut et al. who demonstrated that the serum bilirubin concentration was a well-established marker of the hepatic synthetic function [29]**.**

The AFP level was highly significantly increased in the HCC group [median 118 (70.5-215) ng/ml] than both the patients with cirrhosis group [median 3.2 (2.65-7.23) ng/ml] and the control group [median 3.15 (1.97-4.55) ng/ml] (P< 0.001). Other studies demonstrated the same results Jia *et al.*[30] and Gadelhak *et al.*[31]. El Badrawy *et al*.[32] reported that tissue expression of AFP

was positive in patients with cirrhosis and high expression was reported in HCC patients.

In our study, the serum HGF was highly significantly elevated in the HCC group [median 1425 (580 – 5170) pg/ml] and the patients with cirrhosis group [median 870.0 (110 – 2190) pg/ml] compared with that of the control group [median 365.0 (110 – 1100) pg/ml] (P<0.001). Other researchers confirmed the results of increasing HGF levels in patients with liver cirrhosis and HCC [32, 33]. Yamagamim *et al.*[34], in his study found that the mean serum HGF concentration was significantly higher in patients with HCC than in patients with chronic hepatitis or cirrhosis. Also, *Karabulut et al*.[29] reported that the baseline serum HGF levels were significantly higher in patients with HCC than in the control group (P< 0.001).

Serum AFP (Fig. 1) recorded a moderate sensitivity (70%) and specificity (65%) with a moderate diagnostic accuracy of 67.5%. The positive and negative predictive values were 66.7% and 68.4% respectively. For HGF, the sensitivity and specificity were better than AFP (95% and 80% respectively). The diagnostic accuracy was 87.5%. The positive and negative predictive values were 82.6%, 94.1% respectively (Fig. 2). The specificity of both AFP and HGF for detection of HCC reached 100%. The combination of both markers in this study improved overall accuracy (85%), sensitivity (70%), specificity (100%), PPV (100 %), and NPV (77.9 %) in prediction of HCC.

ROC analysis for miRNA-122 revealed AUC = 0.800 thus discriminating between HCC and non-HCC patients. The calculated cut-off of miRNA-122 that showed the highest sensitivity (73%) and specificity (96%) was 0.165.

Various miRNAs are now being investigated in hepatitis virus infection with the most popular one being miRNA – 122 in the most abundant miRNA in the liver[35].

Cellular miRNAs have been linked with HCC, their availability in the circulation makes them attempting target for early tumor detection [36]. Novel biomarkers for early HCC diagnosis are urgently needed, miRNAs have been very promising as diagnostic markers of HCC, in fact, miRNAs are stable in human serum / plasma as free miRNAs released from cancer cells; many studies have shown that circulating miRNAs are resistant to RNase activity, extreme ph and temperature [37].

In this study the plasma miRNA-122 level is high in all patient groups compared to control group, the highest serum miRNA-122 expression level is in HCC group, the mean serum level is (820±255 copies / ml) which is highly significant compared to control group (22 ± 13 copies / ml) . ROC analysis revealed AUC = 0.800 for miRNA-122 thus discriminating between HCC and non-HCC patients. The calculated cut-off of miRNA-122 that showed the highest sensitivity (73%) and specificity (96%) was 0.165.

Our result is in agreement with the study of *Varnholt et al*.[38] who examined miRNA-122 expression in premalignant dysplastic liver nodules and hepatocellular carcinomas by quantitative PCR, they found that miRNA-122, miRNA–100 and miRNA–10 were overexpressed compared to normal liver parenchyma. In *Trebica et al.*[39] study, hepatic miRNA-122 expression in HCV related HCC in comparison to healthy liver sample; miRNA-122 was strongly up–regulated in malignant liver nodules in comparison to healthy liver. They suggested that miRNA- 122 might down regulate target miRNA of unknown tumor suppressor genes and thus lead to further tumor growth. Also, *Qi et al.*[40] reported that miRNA-122 in serum was significantly higher in HCC patients than healthy controls. They reported that the level of miRNA- 122 was significantly reduced in the postoperative serum samples when compared to the preoperative samples; they suggested that serum miRNA-122 might serve as novel and potential noninvasive biomarker for detection of HCC in healthy subjects. Also, *Elmouttaleb et al.*[41], reported that Serum levels of miRNA-122 were significantly increased in chronic hepatitis virus infected patients, cirrhosis patients, and hepatocellular carcinoma egyptian patients compared to control group.

On the other hand, other authors reported a down regulation in HCC cell line and rodent HCC [41,42] all of which had etiologies other than HCV infection. Because miRNA-122 closely interact with HCV genome and miRNA-122 expression pattern in HCV associated HCCs is directly opposed to non HCV infected HCC, further studies on the role of miRNA-122 in HCCs of non – HCV etiologies are needed to fully understand the function of this unique miRNA in the liver. Ladeiro et al.[60] have established significant down expression of miRNA-122 in 28 HCC liver tissues (mixed etiologies) in comparison to 4 healthy liver tissues by q RT - PCR.[43]

This difference between different studies may arise from variability in technical procedures from sampling, or the use of different normalization controls or control tissues used for normalization (healthy liver or adjacent non-tumor tissue) [44] or may be due to the small patient population [45].

CONCLUSION

HGF was better than AFP as noninvasive biomarkers for the detection of HCC in HCV cirrhotic patients; as its specificity was 80%. Our study concluded that increased expression of serum miRNA-122 in patients with cirrhosis, and hepatocellular carcinoma patients compared to control group (P<0.001).

Thus miRNA-122 can be used as a new biomarker for HCV associated liver disease and can differentiate patients with malignant liver disease from healthy, and patients with cirrhosis groups, so serum miRNA-122 may be able to serve as a promising noninvasive diagnostic marker for HCC. There is positive significant correlation between miRNA-122 and necroinflammatory marker AST in all studied groups. Therefore, serum miRNA-122 is a new potential parameter for liver function.

Future studies for use of miRNA-122 as a diagnostic, predictor of cancer outcome, and monitoring treatment response of HCC could be needed to fully understand the function of this unique miRNA in the liver.

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