Serum MicroRNA-122 and MicroRNA-155: Markers of Disease Progression in Hepatitis C viral infection

Abeer Sheneef [1], Asmaa M. Gouda [1], Asmaa N. Mohammad [2]Laila M. Yousef [3], Amal K. Noureldin[4]

Departments of [1]Medical Microbiology & Immunology, [2]Tropical Medicine, [3]Clinical Pathology, [4]Internal Medicine, Faculty of Medicine, Sohag University, Sohag, Egypt.

Chronic HCV with its longstanding complications of cirrhosis and HCC is a highly prevalent and challenging problem in Egypt. Recently, microRNAs are ranked as potential biomarkers for early diagnosis of HCV related complications. The aim of the present study was to evaluate the role of miRNA-122 and miRNA-155 for prediction of progression of HCV infection and for diagnosis of HCC. A total of 92 chronic HCV patients [chronic HCV (group 1, n =32); chronic HCV with cirrhosis (group 2, n=31); chronic HCV with HCC (group 3, n=29)] were enrolled into the study. Expression of serum miRNA-122 and miRNA-155 was assayed by real-time PCR in all participants. The serum level of miR-122 was significantly higher in chronic HCV patients than in healthy controls and both of cirrhotic and HCC patients (*P*<0.001). Serum miR-155 was significantly elevated in HCC than in controls and non-HCC patients (*P*<0.001). MiR-155 at the cut-off value of >6.11 for HCC diagnosis, had sensitivity and specificity of 72.4% and 95.2%, respectively. In conclusion; microRNA-122 is a potential marker of progression of hepatocytes injury in patients infected with HCV but not a reliable marker for diagnosis of HCC. MicroRNA-155 is a relatively reliable marker for HCC detection.

Hepatitis C virus (HCV) infection is a major health problem particularly in the developing countries. According to the Egyptian demographic health survey, the prevalence of HCV infection in Egypt is 14.7%, which constitutes a great burden to the health service providers [1]. The vast majority of HCV infections culminate into chronic liver disease with its remote sequelae of liver cirrhosis and hepatocellular carcinoma (HCC) [2]. HCV infection is reordered as the most common cause of both chronic liver disease and HCC in Egypt [3]. Hepatocellular carcinoma is nominated as the commonest primary liver cancer and the third leading cause of cancer-related death worldwide [4].

Although many markers have been introduced for evaluation of the severity of hepatic cell injury in patients with HCV infection, yet the liver specific alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT), remain the most commonly used markers. Paradoxically, chronic HCV infection has been described in the absence of remarkable alternations of ALT and GGT [5]. In addition to that, HCC is usually detected at advanced stage which worsens its prognosis. This is attributed mainly to the lack of reliable biomarkers for its early detection. Alfa-fetoprotein (AFP), Lens culinaris agglutinin- reactive AFP (AFP-L3), and des-γ-carboxy-prothrombin (DCP) which are the widely used serological tumor markers for HCC, lack specificity and sensitivity for early detection of HCC [6]. According to the previously mentioned premises, the introduction of de novo markers which could be utilized for early and accurate detection of both liver cell

injury and HCC would be invaluable tools in the management of patients with HCV infection.

Various alternations of the molecular pathways leading to carcinogenesis have been described in HCC [7]. Some of these pathways have been recently discovered as being regulated by microRNAs which are intracellular and extracellular small (~22 nucleotide), non-coding RNAs that inhibit messenger RNAs (mRNAs) by binding to their 3' un-translated regions (UTRs) [8, 9]. Although the intracellular miRNAs regulate numerous crucial functions such as cell metabolism, proliferation, and apoptosis, the function of circulating (extracellular) miRNAs is not well clarified [10]. However, their extracellular existence in the serum and other body fluids could be either due to tissue damage or an inherent cellular mechanism for transporting specific miRNAs during the process of cell-cell communication [11].

MicroRNAs, especially miR-122 which is the most abundant miRNA in the liver have been implicated in various aspects of HCV infection and HCC [12]. Alterations in the cellular miRNA upon viral infection may either regulate viral infection, play a role in the virus-induced hepatic damage or might be a mere stigma of host defense against such infection. In addition to its anti-inflammatory and anti-carcinogenic activities, MiR-122 is a well known regulator of HCV translation and replication through double binding to two target sites in the 5'-UTR [13, 14]. Moreover, HCV infection has been also shown to induce miR-155 over-expression that via activation of Wnt/β-catenin signaling- inhibits apoptosis of hepatocytes and induces carcinogenesis [15]. Herein, up-regulation of miR-155 in serum, peripheral blood mononuclear cells (PBMC) and liver tissues of patients with chronic HCV could be a negative prognostic marker, suggesting incomplete virus elimination, enhanced liver damage and increased risk of HCC [16, 17].

It has been previously reported that some specific miRNAs including miR-122 and miR-155 are differentially regulated in the serum of HCV infected patients [18-20]. The circulating miRNAs are stable molecules and readily detectable by real time PCR. These merits have rendered them attractive markers not only as diagnostic and prognostic markers but also for monitoring of therapy in different diseases including HCV infection and HCC. The aforementioned backgrounds could be considered as a logic foundation for studying the miRNA-mediated effects of HCV infection, particularly in a country like Egypt, which has the highest prevalence of HCV infection worldwide. Herein the aim of the present study was to evaluate the role of circulating miRNAs; miRNA-122 and miRNA-155 in chronic HCV infected patients and correlate them with the progression of the virus - induced pathogenesis as well as diagnosis of HCC.

Patients and Methods

Study design and patients

This is a case-control study which was conducted during the period between 1st March 2016 and 1st October 2016, in the central research laboratory, faculty of medicine, Sohag university. All patients with untreated chronic HCV infection (n = 92) who attended the outpatient clinic of the Tropical and Internal medicine departments of Sohag University hospital, were asked to participate in the study. The participants were allocated into 3 groups; uncomplicated chronic HCV patients (group 1; n = 32), chronic HCV patients with cirrhosis (group 2;

n=31) and chronic HCV patients with hepatocellular carcinoma (group 3; n= 29). The exclusion criteria of the participants were the presence of co-infection with HIV or HBV, malignancy other than HCC, or autoimmune co-morbidities, and those receiving immune-suppressive drugs. A total of 10 volunteers with normal liver enzymes, normal hepatic ultrasound and without serologic evidence of HCV, HBV or HIV infections were included, as internal reference, in the miRNAs expression level analysis. The study was approved by the official recommendations of the ethics committee of Sohag faculty of medicine, Sohag University and written informed consents were obtained from all participants.

All patients underwent detailed history taking and thorough clinical examination with particular focusing to the presence of jaundice, ascites, lower limbs edema, hepatomegaly, splenomegaly and encephalopathy. Complete blood count (CBC), liver function tests, specific HCV testing (anti-HCV antibodies by enzyme-linked immunosorbent assay (ELISA) and HCV RNA and viral load by real time PCR were then done for all patients. Chronic HCV infection was defined by the persistence of HCV antibodies and HCV RNA in the patients' serum for at least 6 months. Abdominal sonographic examination was then done in order to detect the presence of hepatic fibrosis, HCC, splenomegaly, and to measure the portal vein diameter. Child-Pugh score was used to assess the severity of liver cirrhosis [21]. The degree of liver fibrosis-if present- was then accurately assessed with fibro-scanning. The diagnosis of HCC, if suspected, was then ascertained with measurement of serum level of AFP, and confirmed by triphasic computed tomography (CT) and/or magnetic resonance imaging according to American Association for the Study of Liver Diseases (AASLD) guidelines [22].

MicroRNAs Gene Expression Analysis

- Serum preparation and miRNA extraction

Peripheral venous blood samples from all participants were collected into plain vacutainer tubes and centrifuged at 1000 ×g for 15 min at room temperature. An additional centrifugation at 2000 ×g for 10 min was done to completely remove any remaining cells. The supernatant was transferred to Eppendorf tubes for immediate miRNA extraction using mirVana™ PARIS ™ miRNA Isolation Kit (Ambion, USA) following the enrichment procedure for small RNAs purification according to the manufacturer's protocol. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm using NanoDrop Spectrophotometer (Quawell Q5000, USA). An A_{260}:A_{280} ratio of 1.8–2.1 is the measure of highly pure RNA.

- Reverse Transcription (RT)

The studied microRNAs; miR-122, miR-155 and an endogenous control RNU6B was used as the reference gene, were reverse transcribed into single stranded complimentary DNA (cDNA) using TaqMan® microRNA reverse transcription kit (PN 4366596) (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocol. Reverse transcription was performed in a 15 µl reaction volume; 5 µl of RNA, 3 µl RT primer and 7 µl RT master mix (0.15 µl 100 mM dNTP mix, 1,00 µl multiscribe ™ RT enzyme, 1,5 µl 10× buffer, 0,19 µl RNase inhibitor and 4,16 µl nuclease free water). Thermal cycler tubes were incubated on ice for 5 min and loaded into the thermal cycler (Biometra, Germany) using the following conditions:16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and then held at 4 °C.

- Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Real time- qPCR was done in a 20 µl reaction volume using StepOne real time PCR system (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contains 1.33 µl RT product, 10 µl TaqMan® Universal PCR Master Mix II, free of UNG, 1 µl TaqMan® MicroRNA Assay (20×) (miR-122, miR-155 and RNU6B) and 7.67 µl nuclease free water. Real-time PCR amplification was performed according to the following conditions: 95 °C for 10 min to activate the AmpliTaq Gold® enzyme followed by 40 amplification cycles. Each cycle consists of denaturation at 95°C for 15 s, primer annealing and primer extension at 60°C for 60 s.

Relative expression of the studied miRNAs was calculated by StepOne Software v2.0 (Applied Biosystems, Foster City, CA, USA) using the $2^{-\Delta\Delta CT}$ equation where the data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control [23]. Comparative cycle threshold (ΔCT) was calculated by subtracting the Ct values of RNU6B from the Ct values of the target miRNAs

(122 or 155). ΔΔCt was then calculated by subtracting mean ΔCT of the control samples from ΔCT of tested samples. Fold change of each candidate miRNA within each group was then calculated using the equation $2^{-\Delta\Delta CT}$.

Statistical Analysis

Data were analyzed using STATA intercooled version 12.1. Normally distributed quantitative data were represented as mean, standard deviation and were analyzed using ANOVA for comparison of the means of three groups while student t-test used to compare means of two groups. Quantitative data with uneven distribution were represented as mean and range (Min-Max) and Kruskal Wallis test was used for comparison of three or more groups while Mann-Whitney test was used to compare between two groups. Qualitative data were represented as number and percentage and compared using either Chi square test or fisher exact test. Spearman's correlation coefficient analyses were done when indicated. Data were analyzed by sensitivity, specificity, positive, and negative predictive value derived from the receiver operating characteristic (ROC) curve. The diagnostic accuracy of miR-122 & miR-155 for diagnosis of HCC were expressed as the area under the ROC curve (AUC). Best cut off point was determined by using Medcalc for Windows (version 11.0). Graphs were produced by using STATA program. *P* value < 0.05 was considered significant.

Result

A total of 92 chronic HCV patients [chronic HCV (group 1, n =32); chronic HCV with cirrhosis (group 2, n=31); chronic HCV with HCC (group 3, n=29)] were enrolled into the study; their ages ranged from 20-65 years. The gender distribution (52 males and 40 females) was comparable in the three groups of studied patients. There was a significant difference between patients with liver cirrhosis (LC) and HCC versus chronic HCV patients in terms of age distribution (20-65 years), patients with chronic hepatitis (CH) were younger than those with liver cirrhosis and HCC (both $P<0.001$). All hallmarks of hepatic decompensation including jaundice, GIT tract bleeding, splenomegaly, and ascites were significantly higher in group 2 & 3 compared to group 1. The median level of α-fetoprotein was significantly higher in patients with HCC. The clinical and the laboratory data of the patients are shown in Table 1 and 2.

Analysis of the median fold change in expression level of miR-122 of the patients compared to the normal control group showed that miR-122 displayed a significant fold increase in CH group (6.79) ($P= 0.0001$) and a significant fold decrease in both groups with cirrhosis (0.79) and HCC (0.72) (Table 2). Comparing the serum miR-122 expression level between different studied groups displayed an increasing tendency towards a significant fold elevation in expression in CH patients (6.79) in comparison to liver cirrhosis (0.79) and HCC patients (0.72) ($P<0.001$). No significant fold change in miR-122 expression was found between HCC and liver cirrhosis groups ($P= 0.17$) (Figure 1).

Analysis of the median fold change in the expression level of miR-155 in patients' sera in comparison to the normal control group showed significant fold increase in all the studied groups; CH (3,13), LC (3,13) and HCC (7.33) (P=0,0001) (Table 2). Comparing miR-155 expression level between the different studied groups displayed an increasing tendency towards significant fold elevation in expression in HCC patients (7.33) in comparison to LC (3.13) and CH patients (3.13) ($P<0.001$). No significant fold change in miR-155 expression was found between CH and liver cirrhosis groups (P=0.48) (Figure 2).

Table 1. Demographic and clinical data of the studied patients

Variable	Group 1 (n=32) CH	Group 2 (n=31) LC	Group 3 (n=29) HCC	*P* value
Age (years) Mean ± SD	35.63±8.71	54.45±7.86	51.07±11.06	<0.0001[a], <0.0001[b] <0.0001[c], NS[d]
Gender				
Male	17 (53.13%)	18 (58.06%)	17 (58.62%)	NS
Female	15 (46.88%)	13 (41.38%)	12 (41.38%)	
Jaundice				
No	25 (78.13%)	18 (58.06%)	17 (58.62%)	NS
Yes	7 (21.88%)	13 (41.94%)	12 (41.38%)	
Bleeding tendency				
No	27 (84.38%)	19 (61.29%)	10 (34.48%)	<0.0001[a], 0.04[b]
Yes	5 (15.63%)	12 (38.71%)	19 (65.52%)	<0.0001[c], 0.04[d]
Upper GIT bleeding				
No	32 (100%)	23 (74.19%)	20 (68.97%)	0.003[a], 0.002[b]
Yes	0	8 (25.81%)	9 (31.03%)	0.001[c], NS[d]
Ascites				
No	32 (100%)	13 (41.94%)	7 (24.14%)	<0.0001[a], <0.0001[b]
Yes	0	18 (58.06%)	22 (75.86%)	<0.0001[c], NS[d]
Hepatomegaly				
No	15 (46.88%)	26 (83.87%)	22 (75.86%)	0.004[a], 0.002[b]
Yes	17 (53.13%)	5 (16.13%)	7 (24.14%)	0.02[c], NS[d]
Splenomegaly				
No	5 (15.63%)	5 (16.13%)	6 (20.69%)	NS
Yes	27 (84.38%)	26 (83.87%)	23 (79.31%)	
Focal lesions				
Single	----	-----	14 (48.28%)	-----
Multiple			15 (51.72%)	
Portal vein thrombosis				
No	30 (93.75%)	28 (90.32%)	20 (68.97%)	0.02[a], NS[b]
Yes	2 (6.25%)	3 (9.68%)	9 (31.03%)	0.01[c], 0.04[d]
Portal hypertension				
No	29 (90.63%)	16 (51.61%)	9 (31.03%)	<0.0001[a], 0.001[b]
Yes	3 (9.38%)	15 (48.39%)	20 (68.97%)	<0.0001[c], 0.NS[d]
Child- Pugh				
A		11 (35.48%)	4 (13.79%)	
B	------	10 (32.26%)	7 (24.14%)	0.049[d]
C		10 (32.26%)	18 (62.07%)	
Fibroscan				
F1-2	12 (37.50%)	-----	-------	------
F3-4	20 (62.50%)			

CH; chronic HCV, LC; liver cirrhosis, HCC; hepatocellular carcinoma. *P*>0.05 is not significant (NS).
[a] *P* value compared the three group, [b] *P* value compared CH & cirrhosis, [c] *P* value compared CH & HCC, and [d] *P* value compared cirrhosis & HCC

Table 2. Laboratory findings of the studied patients

Variable	Group 1 (n=32) CH	Group 2 (n=31) LC	Group 3 (n=29) HCC	*P* value
ALT (IU/L) Median (Min-Max)	84.5 (30-265)	51 (9-145)	26 (14-143)	0.0001[a], 0.002[b] 0.0001[c], NS[d]
AST (IU/L) Median (Min-Max)	62.5 (30-170)	62 (12-110)	78 (23-186)	NS[a]
Bilirubin (mg/dl) Median (Min-Max)	1.1 (0.6-2.9)	1.8 (0.6-6)	2.1 (0.8-8.1)	0.001[a], 0.03[b] 0.0002[c], NS[d]
Albumin (g/dl) Median (Min-Max)	3.5 (2.9-4.1)	2.9 (0.5-4.1)	2.6 (0.5-3.7)	0.0001[a],0.0001[b] 0.0001[c], NS[d]
PT (second) Median (Min-Max)	12.7 (11.3-13.9)	14.7 (11.5-29)	18.9 (11.4-29)	0.0001[a],0.0001[b] 0.0001[c], 0.07[d]
PC (%) Median (Min-Max)	95 (69-100)	67 (24-100)	50 (24-100)	0.0001[a],0.0001[b] 0.0001[c], NS[d]
Platelets ($\times 10^3/mm^3$) Median (Min-Max)	210 (98-380)	12 (18-340)	100 (15-450)	0.001[a], 0.04[b] 0.002[c], NS[d]
AFP (ng/ml) Median (Min-Max)	19.5 (2-98)	116 (14-250)	411 (30-2543)	0.0001[a],0.0001[b] 0.0001[c] 0.0001[d]
HCV RNA level (IU/ml) Median (Min-Max)	4708.5 (0-45600000)	890 (0-654000000)	--------	NS[b]
miR-122 Median (Min-Max)	6.79 (0.36-12.74)	0.79 (0.02-4.36)	0.72 (0.02-2.81)	0.0001[a],0.0001[b] 0.0001[c], NS[d]
miR-155 Median (Min-Max)	3.13 (0.13-12.23)	3.13 (0.83-5.99)	7.33 (0.43-14.13)	0.0001[a], NS[b] 0.0003[c], 0.0001[d]

CH; chronic HCV, LC; liver cirrhosis, HCC; hepatocellular carcinoma, PT& PC; prothrombin time& concentration, ALT& AST; alanine & aspartate aminotransferase, AFP; α-Fetoprotein. *P*>0.05 is not significant (NS).

[a] *P* value compared the three group, [b] *P* value compared CH & cirrhosis, [c] *P* value compared CH & HCC, and [d] *P* value compared cirrhosis & HCC

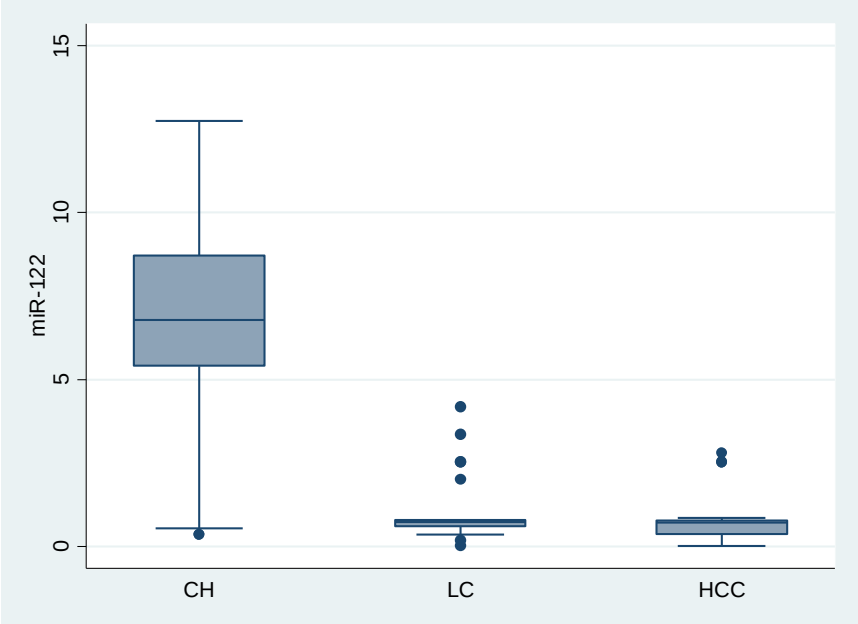

Figure 1. Comparison of miR-122 expression level among the three studied groups**.**

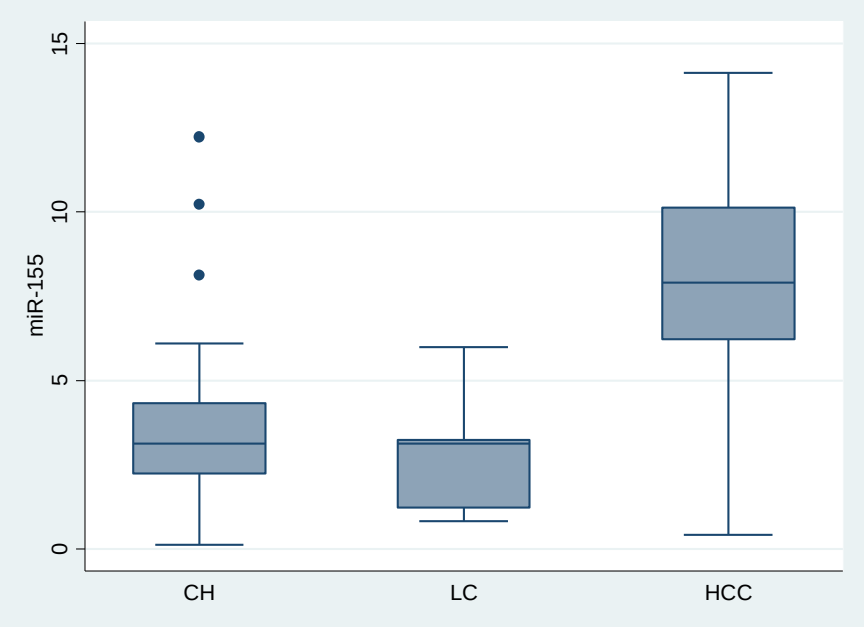

Figure 2. Comparison of miR-155 expression level among the three studied groups.

The level of miR-122 within the uncomplicated chronic HCV patients showed a statistically significant negative correlation with the degree of fibrosis of the liver (fibroscan) (r=−0.58, *P*=0.01). On the other hand, its level was positively correlated with ALT level (r=0.52, *P*=0.02). Moreover, It was found that miR-122 level within the cirrhotic group was significantly negatively correlated with the degree of clinical impairment of the liver (Child-Pugh score) (r=−0.57, *P*=0.01) and HCV RNA level (r=−0.49, *P*=0.03). However, all the other tested parameters in both chronic HCV and cirrhotic groups failed to show any correlation with miRNA-122 or miR-155 expression levels, (Table 3).

Table 3. Correlation analysis between miR-122 & miR-155 expression levels and clinical and laboratory parameters of chronic HCV patients with and without liver cirrhosis.

Variable	miR-122				miR-155			
	Group 1 (n=32) CH		Group 2 (n=31) LC		Group 1 (n=32) CH		Group 2 (n=31) LC	
	r	*P* value	r	*P* value	r	*P* value	r	*P* value
Child Pugh score A B C	-	-	-0.57	0.01*	-	-	0.31	NS
Fibroscan F1-F2 F3-F4	-0.58	0.01*	-	-	0	NS	-	-
Bilirubin	-0.14	NS	-0.29	NS	-0.23	NS	0.15	NS
ALT (IU/L)	0.52	0.02*	-0.28	NS	0.10	NS	-0.35	NS
AST (IU/L)	-0.18	NS	-0.10	NS	0.11	NS	-0.05	NS
HCV RNA level (IU/ml)	-0.002	NS	-0.49	0.03*	-0.30	NS	-0.19	NS
AFP	0.08	NS	-0.19	NS	-0.17	NS	0.02	NS
miR-122	-	-	-	-	0.34	NS	-0.09	NS
miR-155	0.34	NS	-0.09	NS	-	-	-	-

CH; chronic HCV, LC; liver cirrhosis, ALT& AST; alanine & aspartate aminotransferase, AFP; α-Fetoprotein, Correlation coefficient (r), * *P*>0.05 is not significant (NS).

Univariate analysis showed that the age, bilirubin level, low prothrombin concentration, AFP, miR-122 and miR-155 are important risk factors in patients with HCC. After adjustment for age and liver functions, and miR-155 in the multivariate model, miR-155 remained as a significant risk factor in patients with HCC. MiR-155 was a successful predictor for HCC diagnosis (OR =1.5, *P* = 0.04) (Table 4).

Table 4. Univariate and multivariate analysis showing the predictive power of different factors for HCC diagnosis

Variable	Odds ratio (95% confidence interval)	*P* value	Adjusted Odds ratio (95% confidence interval)	*P* value
Age	1.05 (1.00-1.09)	0.03	0.91 (0.78-1.06)	NS
Gender	1.13 (0.47-2.76)	NS	0.47 (0.04-5.16)	NS
ALT	0.98 (0.97-99)	0.005	0.94 (0.87-1.02)	NS
AST	1.01 (1.00-1.02)	0.02	1.04 (0.99-1.09)	NS
Bilirubin	1.61 (1.15-2.25)	0.006	1.07 (0.33-3.42)	NS
PC	0.92 (0.93-0.98)	<0.0001	0.96 (0.90-1.03)	NS
Albumin	0.79 (0.22-0.64)	<0.0001	1.36 (0.30-6.25)	NS
AFP	1.01 (1.00-1.02)	0.001	1.01 (0.99-1.02)	NS
Platelets	0.99 (0.99-1.00)	NS	0.99 (0.98-1.07)	NS
miR-122	0.53 (0.34-0.83)	0.005	0.13 (0.004-4.32)	NS
miR-155	1.47 (1.23-1.79%)	<0.0001	1.50 (1.02-2.22)	*0.04

PC; prothrombin concentration, ALT& AST; alanine & aspartate aminotransferase, AFP; α-Fetoprotein,
**P*>0.05 is not significant (NS).

ROC curve analysis of circulating miR-122 revealed that at a cut-off value of ≤2.81 could differentiate HCC from non-HCC patients with a sensitivity of 100%, specificity of 46%; area under curve (AUC) 0.75 (*P*<0.001, 95% CI (0.65–0.84). However, ROC curve analysis of miR-155 revealed that at a cut-off value of >6.11 could differentiate HCC from non-HCC patients with a sensitivity of 72.4%, specificity of 95.2%; AUC 0.78 (P< 0.001, 95% CI (0.68- 0.86) (Table 5 & Figure 3, 4).

Table 5. Diagnostic performance of miR-122 and miR-155 for discriminating HCC from Non-HCC

	AUC	*P* value	Best cut off point	Sensitivity	Specificity	PPV	NPV	Diagnostic accuracy
miR-122	0.75 (0.65-0.84)	<0.0001	≤2.81	100%	46%	46%	100%	73%
miR-155	0.78(0.68-0.86)	<0.0001	>6.11	72.4%	95.2%	87.%	88.%	83.8%

AUC; area under the curve, PPV; positive predictive value, NPV; negative predictive value. *P*<0.05 is significant

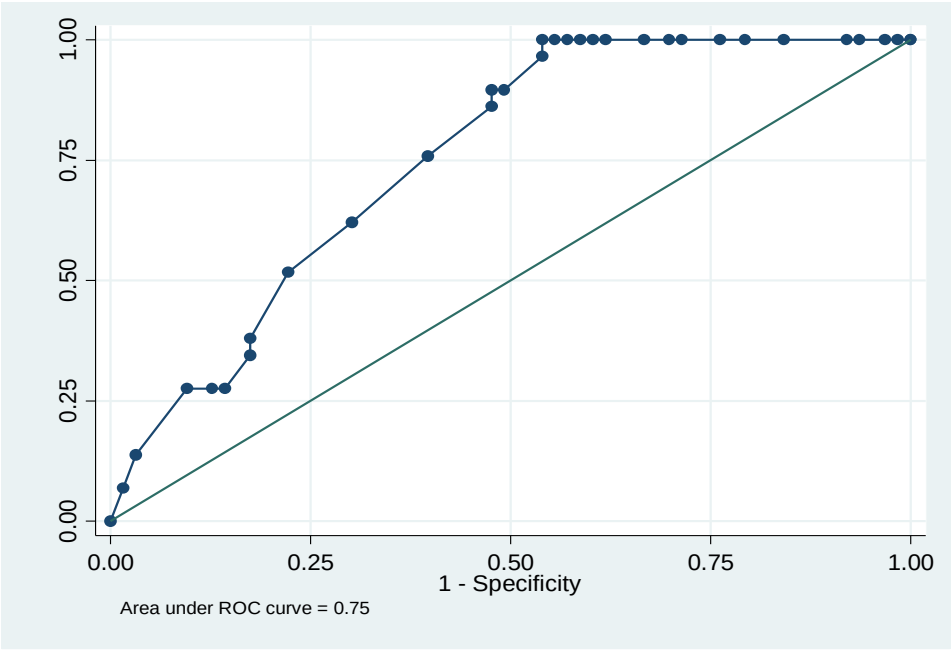

Figure 3. Receiver operator characteristic curve for miRNA-122 as a discriminator between HCC versus non-HCC patients.

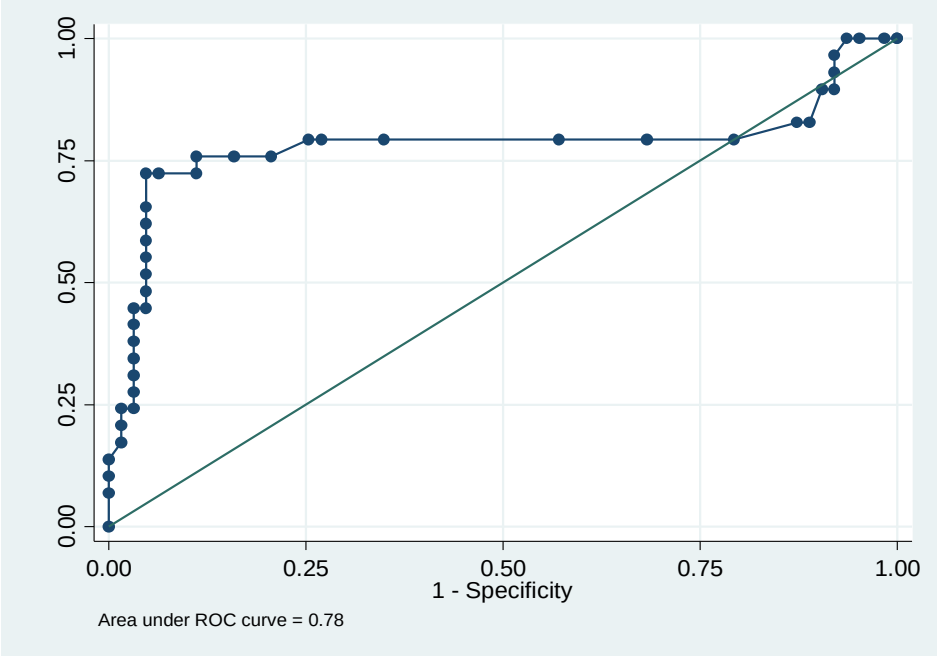

Figure 4. Receiver operator characteristic curve for miR-155 as a discriminator between HCC versus non-HCC patients.

Discussion

The present study showed that the serum level of miR-122 was significantly higher in patients with chronic HCV infection and significantly lower in patients with liver cirrhosis and HCC compared to the healthy individuals. This variability in the level of miR-122 could be explained through a postulation with 3 consecutive phases. Firstly, under normal circumstances, the level of miR-122 is almost higher in the hepatocytes than in the peripheral blood. Secondly, upon viral infection, replication and triggering of the immune system and hence stimulation of the inflammatory process in the hepaocytes, miR-122 is released from the hepatocytes into the circulation in a high amount; which might explain the high level of miR-122 in the particular group of patients with chronic HCV infection. Lastly, with progression of the process of liver injury, damage, and finally cirrhosis with or without the development of HCC, with total or almost total loss of the hepatocytes, the level of miR-122 drops to a level lower than the healthy individuals [24].

An alternative plausible mechanism is that the low level of miR-122 in patients with HCC could be regarded as an evidence for the direct role of miR-122 in the development of hepatic carcinogenesis. It was previously reported that miR-122 is a molecule which may function as a tumor suppressor gene and its down regulation may be implicated in the pathogenesis of HCC [25]. However, analysis of the subpopulations of the participants of the present study has disproved this speculation. The nearly similar level of miR-122 in patients with cirrhosis and HCC would support the previous notion which considered miR-122 as a potential marker of hepatic cell damage rather than a marker of HCC.

The current study showed that a miR-122 cut off level of ≤2.81 had 100% sensitivity for detection of HCC; a sensitivity which is much higher than that provided with the use of alpha fetoprotein (39%-64%) [26-27]. This high sensitivity would render miR-122 an attractive and sensitive marker for diagnosis of HCC and could replace other invasive diagnostic procedures. However, when this finding was copulated with the previously mentioned findings, this high sensitivity may be deceiving, as it may reflect a mere end stage of total or almost total loss of the hepatocyes function. This notion was further strengthened by the remarkably low specificity (46%) and relatively low diagnostic accuracy (73%) of miR-122 for diagnosing HCC. A last supporting evidence for this postulation was obtained after logistic analysis of the risk factors for HCC. After adjustment for the age, gender and liver impairment, miR-122 was not found to be a risk factor for HCC.

The level of miR-122 was negatively correlated to the degree of clinical impairment of the liver (Child-Pugh score) and the degree of liver fibrosis (Fibroscan). Although these findings seem appealing, its practical value might be readily limited. This is because the negative correlation provided by the present study is crude and did not stratify the different grades of liver impairment and fibrosis according certain cut off values of miR-122. Whatever, these findings collectively suggest that miR-122 is a reliable marker of liver cell damage and

might be a valuable adjunct to other clinical, biochemical, and imaging techniques which evaluate the liver cell damage in patients with HCV infection.

The data in the literature is highly contradicting; while many studies support our results [28-31], others show inconsistent findings [32-34]. These contradictory findings could be explained by many reasons such as differences in the sample size of the different studies, variability of the technique for miR-122 extraction and quantitation, diversity in HCV genotypes, and the coexistence of other risk factors for HCC other than HCV like HBV infection and alcoholic induced liver injury.

In contrast to miR-122, the present study reported a significant elevation of miR-155 in patients with HCV, liver cirrhosis and HCC compared to the control. Moreover its level was significantly higher in patients with HCC compared to that in HCV and cirrhotic patients. Up regulation of miR-155 in patients with HCV has been previously reported suggesting its role in the pathogenesis and progression of both chronic HCV infection and HCC [15-16]. The findings of the present study provided many evidences which supported these postulations. Firstly, the selective elevation of miR-155 in the particular patients with HCC could support the previous assumption that miR-155 may act as an oncogene [15-35]. Secondly, a cut off >6.11 has a reasonably high sensitivity (72.4%) and diagnostic accuracy (83.85) and very high specificity (95.2%) for diagnosing HCC. This diagnostic accuracy may be further improved if coupled with other non-invasive diagnostic tools like alpha fetoprotein. Thirdly, after controlling the variables affecting the risk of HCC, miR-155 remained a significant factor; a finding reinforcing the hypothesis of the oncogenic role of miR-155 in HCC. Collectively, these findings denote that the miR-155 is released from patients with HCC in a specific and not a haphazard manner.

The present study has many limitations; the most evident of which is the small sample size. This was attributed to the financial constrains and the high costs of the kits necessary for microRNA assays. Lack of statistical analysis of alpha fetoprotein particularly in patients with HCC was another shortcoming. However, we intentionally rejected this analysis because the marker was previously extensively studied in the literature. Moreover, the American association for the study of liver diseases has questioned its diagnostic accuracy as a specific marker for patients with HCC [22]. Despite these limitations, the current study highlighted the role of miR-122 as a marker for progression of the process of liver cell injury and miR-155 as a relatively reliable marker for detection of HCC.

In conclusions, MicroRNA-122 is a potential marker for hepatocytes damage and a marker of progression of the process of liver cell injury in patients with HCV infection. MicroRNA-122 is not a reliable marker for diagnosis of HCC. MicroRNA-155 is a relatively reliable marker for detection of patients with HCC. These markers may be valuable add-ons to other clinical, biochemical, and imaging techniques in patients with HCV infection, liver cirrhosis, and HCC. Their diagnostic accuracy could be improved if coupled with other tests. However, these conclusions remain to be reappraised in further studies with larger sample size.

References

1. El-Zanaty F, Way A. Egypt Demographic and Health Survey 2008. Egyptian: Ministry of Health. Cairo: El-Zanaty and Associates, and Macro International; 2009.

2. Strickland GT, Elhefni H, Salman T, Waked I, Abdel Hamid H, Mikhail NN Esmat G, and Fix A. Role of hepatitis C infection chronic liver disease in Egypt. Am J Trop Med Hyg 2002; 67 (4), 436-442.

3. El-Zayadi AR, Badran HM, Barakat EM, Attia Mel-D, Shawky S, Mohamed MK, Selim O, Saeid A. Hepatocellular carcinoma in Egypt: a single center study over a decade. World J Gastroenterol 2005; 11: 5193-5198.

4. Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. Gastroenterol. 2004; 127: S5-S16.

5. Pradat P, Alberti A, Poynard T, Esteban JI, Weiland O, Marcellin P, Badalamenti S, Trépo C. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. Hepatol. 2002; 36:973-7.

6. Forner A, Vilana R, Ayuso C, Bianchi L, Solé M, Ayuso JR, Boix L, Sala M, Varela M, Llovet JM, Brú C, Bruix J. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. Hepatol. 2008; 47: 97–104.

7. Lee JS, Thorgeirsson SS. Comparative and integrative functional genomics of HCC. Oncogene. 2006; 25: 3801- 3809.

8. Carthew RW and Sontheimer EJ. (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 2009; 136, 642–655.

9. Fabian MR, Sonenberg N and Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Annu. Rev. Biochem. 2010; 79, 351–379.

10. Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatol. 2009; 49:1098–1112.

11. Shwetha S, Gouthamchandra K, Chandra M, Ravishankar B, Khaja MN, Das S. Circulating miRNA profile in HCV infected serum: novel insight into pathogenesis. Sci Rep. 2013; 3:1555.

12. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 2005; 309: 1577-1581.

13. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S and Orum H. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 2010; 327, 198–201.

14. Hsu SH, Wang B, Kota J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, Mao H, Wei M, Clark KR, Mendell JR, Caligiuri MA, Jacob ST, Mendell JT, Ghoshal K. Essential metabolic anti-inflammatory, and antitumorogenic functions of miR-122 in liver. J Clin Invest 2012; 122: 2871–2883.

15. Zhang Y, Wei W, Cheng N, Wang K, Li B, Jiang X, Sun S. Hepatitis C virus-induced upregulation of microRNA-155 promotes hepatocarcinogenesis by activating Wnt signaling. Hepatol. 2012; 56(5):1631–40.

16- Bala S, Tilahun Y, Taha O, Alao H, Kodys K, Catalano D, Szabo G. Increased microRNA-155 expression in the serum and peripheral monocytes in chronic HCV infection. J Transl Med 2012; 10: 151.

17. Riad SE, El-Ekiaby N, Mekky RY, Ahmed R, El Din MA, El- Sayed M, Abouelkhair MM, Salah A, Zekri AR, Esmat G, Abdelaziz AI. Expression signature of microRNA-155 in hepatitis C virus genotype 4 infection. Biomed Rep 2015; 3: 93-97.

18. Bihrer V, Friedrich-Rust M, Kronenberger B, Forestier N, Haupenthal J, Shi Y, Peveling-Oberhag J, Radeke HH, Sarrazin C, Herrmann E, Zeuzem S, Waidmann O, Piiper A. Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. Am J Gastroenterol 2011; 106 (9), 1663–1669.

19. Bihrer V, Waidmann O, Friedrich-Rust M, Forestier N, Susser S, Haupenthal J, Welker M, Shi Y, Peveling-Oberhag J, Polta A, Von Wagner M, Radeke HH, Sarrazin C, Trojan J, Zeuzem S, Kronenberger B, Piiper A. Serum microRNA-21 as marker for necroinflammation in hepatitis C patients

with and without hepatocellular carcinoma. PLoS One 2011; 6(10):e26971.

20. Cermelli S, Ruggieri A, Marrero JA, Ioannou GN & Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. PLoS One 6, e23937, 2011.

21. Pugh R, Murray-Lyon I, Dawson J, Pietroni M, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg 1973; 60(8):646–649.

22. Bruix J and Sherman M. American Association for the Study of Liver Diseases (AASLD) Practice Guidelines. Management of Hepatocellular Carcinoma: An Update. Hepatology. 2011: 53, 1020-1022.

23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods. 2001; 25:402–8.

24. Trebicka J, Anadol E, Elfimova N, Strack I, Roggendorf M, Viazov S, Wedemeyer I, Drebber U, Rockstroh J, Sauerbruch T, Dienes HP, Odenthal M. Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. J Hepatol 2013; 58: 234-239.

25. Saito Y, Friedman JM, Chihara Y, Egger G, Chuang JC, Liang G. Epigenetic therapy upregulates the tumor suppressor microRNA-126 and its host gene EGFL7 in human cancer cells. Biochem Biophys Res Commun. 2009; 13; 379(3):726-31.

26. Okuda H, Nakanishi T, Takatsu K, Saito A, Hayashi N, Takasaki K, Takenami K, Yamamoto M, Nakano M. Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma. Cancer 2000; 88: 544-549.

27. Marrero JA, Lok AS. Newer markers for hepatocellular carcinoma. Gastroenterology 2004; 127: S113-S119

28. Harfoush RAH, Meheissen MA, Abo Elwafa RAH, Elwazzan DA. The Role of Circulating MicroRNAs as Markers of Disease Progression in Hepatitis C Virus Infected Egyptian Patients. Advances in Microbiology 2016; 6, 320-331.

29. Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin G.A, Giovannini C, Ferrazzi E, Grazi GL, Croce CM, Bolondi L and Negrini M. Cyclin G1 Is a Target of miR-122a, a microRNA Frequently Down-Regulated in Human Hepatocellular Carcinoma. Cancer Research 2007; 67, 6092-6099.

30. Köberle V, Kronenberger B, Pleli T, Trojan J, Imelmann E, Peveling-Oberhag J, Welker MW, Elhendawy M, Zeuzem S, Piiper A and Waidmann O. Serum microRNA-1 and microRNA-122 Are Prognostic Markers in Patients with Hepatocellular Carcinoma. Eur J Cancer 2013; 49, 3442-3449.

31. EL-Abd NE, Fawzy AN, EL-Sheikh SM and Soliman ME. Circulating miRNA-122, miRNA-199a, and miRNA-16 as Biomarkers for Early Detection of Hepatocellular Carcinoma in Egyptian Patients with Chronic Hepatitis C Virus Infection. Molecular Diagnostic Therapy 2015; 19, 213-220.

32. El-Garem H, Ammer A, Shehab H, Shaker O, Anwer M, El-Akel W and Omar H. Circulating micro- RNA, miR-122 and miR-221 Signature in Egyptian Patients with Chronic Hepatitis C Related Hepatocellular Carcinoma. World Journal of Hepatology 2014; 27, 818-824.

33. Qi J, Wang J, Katamaya H, Sen S, Liu SM. Circulating micro- RNAs (cmiRNAs) as novel potential biomarkers for hepatocellular carcinoma. Neoplasma. 2013;60(2):35–142.

34. Xu J, Wu C, Che X, Wang L, Yu D, Zhang T, Huang L, Li H, Tan W, Wang C, Linet D. Circulating MicroRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. Mol Carcinog. 2011; 50:136–42.

35. Wang XW, Heegaard NH, Qrum H. MicroRNAs in liver disease. Gastroenterology 2012; 142: 1431–1443.