Advances in Microbiology, 2016, 6, 320-331
Published Online April 2016 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2016.64031

The Role of Circulating MicroRNAs as Markers of Disease Progression in Hepatitis C Virus Infected Egyptian Patients

Reem Abdel Hamid Harfoush[1], Marwa Ahmed Meheissen[1*], Reham Abel Haleem Abo Elwafa[2], Doaa Ahmed Elwazzan[3]

[1]Medical Microbiology and Immunology Department, Faculty of Medicine, University of Alexandria, Alexandria, Egypt
[2]Clinical and Chemical Pathology Department, Faculty of Medicine, University of Alexandria, Alexandria, Egypt
[3]Tropical Medicine Department, Faculty of Medicine, University of Alexandria, Alexandria, Egypt
Email: *marwa.meheissen@alexmed.edu.eg

Received 29 January 2016; accepted 17 April 2016; published 20 April 2016

Abstract

Background: 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. Aim: The aim of the study was to evaluate the expression levels of circulating plasma miRNAs (miRNA-21 & miRNA-122) in Egyptian patients with chronic uncomplicated and complicated HCV. Patients & Methods: This study was conducted on 60 Chronic HCV infected patients. Patients were divided into three groups (20 patients each): uncomplicated HCV, cirrhosis, and hepatocellular carcinoma (HCC). All patients were subjected to laboratory investigations including complete blood picture, liver function tests. Expression levels of miRNA-21 and -122 in plasma using RT-PCR were determined. Results: MiRNA-21showed significant fold increase in chronic uncomplicated HCV while significant fold decrease in cirrhotic and HCC groups (P = 0.036). On the other hand, miRNA-122 showed significant fold elevation in both chronic uncomplicated and cirrhotic groups and significant fold decrease in HCC group (P = 0.005). ROC curve analysis for miRNA-122 yielded 68.4% sensitivity and 100% specificity for the differentiation of HCC patients from non-HCC at a cutoff 0.184. Neither miRNA-21 nor miRNA-122 was a successful predictor for HCC diagnosis. Conclusion: MiRNA-122 can be used as novel non-invasive biomarker for monitoring HCV related disease progression.

Keywords

MicroRNA, Hepatocellular Carcinoma, Hepatitis C Virus

*Corresponding author.

How to cite this paper: Harfoush, R.A.H., Meheissen, M.A., Elwafa, R.A.H.A. and Elwazzan, D.A. (2016) The Role of Circulating MicroRNAs as Markers of Disease Progression in Hepatitis C Virus Infected Egyptian Patients. *Advances in Microbiology*, **6**, 320-331. http://dx.doi.org/10.4236/aim.2016.64031

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with an estimated prevalence of 170 million cases worldwide. The severity of the disease varies from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma [1].

Egypt has the highest prevalence of HCV infection worldwide (15%) and the highest prevalence of HCV-4; HCV-4 is responsible for 90% of the total HCV infections in Egypt [2]. This extraordinarily high prevalence has resulted in an increasing incidence of hepatocellular carcinoma in Egypt, which is now the second most frequent cause of cancer and cancer mortality among men [3].

Currently, there are no biomarkers for the early detection of HCC, and most patients are diagnosed at advanced stages, which are associated with very poor prognosis. Alpha-Fetoprotein (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) has rejected its use, since 2010, whether for the surveillance or the diagnosis of HCC [4].

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 [5]. An integral role of miRNAs in cancer pathogenesis has begun to emerge. MiRNA expression profiling reveals characteristic signatures for many tumor types [6]. Importantly, 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 [7].

MiRNA-21 is linked to human liver pathogenesis, ranging from normal liver integrity to cirrhosis to HCC. The expression of this miRNA has been used as an example of the relevance of specific miRNAs to disease progression, starting from the induction of hepatitis to liver cirrhosis and finally to HCC [8]. A close link between miRNA-21 and hepatic fibrosis is supported by the findings that transforming growth factor b (TGF-b), a critical mediator of hepatic fibrogenesis, promotes the expression of miRNA-21, and that miRNA-21 decreases the expression of SMAD7, a negative regulator of TGF-b signaling [9].

MiRNA-122 has a positive role in HCV replication and it stimulates HCV translation by enhancing the association of ribosomes with the viral RNA at an early initiation stage. It has also been implicated in the regulation of different metabolic pathways in liver cells (*i.e.*, cholesterol metabolism), and has become the most studied miRNA involved in HCV infection. Its expression was found to be significantly down regulated in tumors compared with non-malignant liver tissues [10].

To date, very limited data exist concerning alterations of the miRNA-21, 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-21 & miRNA-122) expression levels in Egyptian patients with chronic HCV as well as in patients with HCV-related complications (cirrhosis and HCC) to investigate their usefulness as non-invasive diagnostic markers for HCV disease progression.

2. Patients and Methods

2.1. Study Design

Sixty HCV infected patients (positive for anti-HCV antibodies and HCV-RNA for at least six months) attending The Tropical medicine department in Alexandria Main University Hospital were included in this prospective study. They were divided into three groups: group 1 = Patients with uncomplicated chronic hepatitis C infection (n = 20), group 2 = Patients with chronic hepatitis C infection and cirrhosis (n = 20), group 3 = Patients with chronic hepatitis C infection and HCC (n = 20). A control group of ten age and sex-matched healthy volunteers (with normal liver enzymes, normal hepatic ultrasound and negative for HBV, HCV and HIV) was included in the miRNAs expression levels analysis by RT-PCR.

Exclusion criteria included patients with: Decompensated liver disease, Malignancy other than HCC, Organ

transplantation, Co-infection with HIV or HBV, Immunosuppression and autoimmune co-morbidities.

All patients were anti-HCV positive with detectable plasma HCV RNA by PCR (COBAS® TaqMan® HCV Test, v2. Kit, Roche Molecular Systems' COBAS® AmpliPrep). Child-Pugh score was used to assess the severity of liver cirrhosis. Grading and staging of chronic hepatitis were evaluated histologically by percutaneous needle liver biopsy according to METAVIR grading and staging system [11]. All HCC patients were on top of HCV cirrhosis and HCC diagnosis was made upon the presence of hepatic focal lesions diagnosed by abdominal ultrasound and confirmed by triphasic computed tomography (CT) and/or magnetic resonance imaging according to American Association for the Study of Liver Diseases (AASLD) 2011 guidelines [12].

A written informed consent for specimen use was obtained from all study subjects and the study protocol was approved by the ethics review committee of Alexandria University Hospitals.

2.2. Data and Sample Collection

1) Thorough history taking and full clinical assessment.

2) Ultrasound data and histopathological data for all study patients were collected from their medical files (grading and staging of liver disease, viral load, HCC U/S data).

3) Blood Sampling: Ten mL of peripheral blood was collected from each patient. Five mL were put on potassium ethylenediamine 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). Plasma samples for PCR were stored at −80˚C until further processing.

2.3. Biochemical Investigations

Routine workup including liver function tests (ALT, AST, alkaline phosphatase (ALP)), total bilirubin, albumin, creatinine, prothrombin activity and prothrombin international normalized ratio (INR), as well as complete blood counts (CBC) including haemoglobin (Hb), platelet and total leukocyte counts (TLC) were performed.

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

Real-time quantitative RT-PCR for miRNA was performed to detect the expression levels of miRNA-21 and -122. RNU6B was used as internal control.

2.4.1. RNA Extraction

Total RNA with preserved miRNAs was extracted from 200 µl plasma with the miRNAN easy extraction kit (Qiagen, Valencia, CA, USA) using 1 ml QIAzol lysis reagent and incubated for 5 min at RT. 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 miRNAN 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 buffer RWT 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 8000gat room temperature for 15 sec. After this, another 500 µl of buffer RPE was added to the column prior to centrifugation at 8000gat 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.

2.4.2. Reverse Transcription

The specific cDNA of miRNA-122, -21 and RNU6B 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 mM dNTPs, 50 U/µl MultiScribe Reverse transcriptase, 10× Reverse transcriptase buffer, 20 U/µl Rnase 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.

2.4.3. MiRNA Amplification

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

2.4.4. Relative Quantitation of Target MiRNA Expression

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

2.5. Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, which revealed that the data are not normally distributed. Quantitative data were described using median, range. Qualitative data were described using number and percent. The correlation between quantitative variables was done using Spearman rank correlation test. Comparing quantitative variables between 3 groups or more of cases was conducted using Kruskal Wallis test. Comparing quantitative variables between 2 groups or more of cases was conducted using Mann Whitney U test. Adjusted Odds ratio was calculated using multiple logistic regressions. ROC curve analysis was done to discriminate between HCC from non-HCC and to determine the cutoff point, which has the highest sensitivity and specificity. In all statistical tests, level of significance of 0.05 was used, below which the results were considered to be statistically significant.

3. Results

3.1. Demographic and Laboratory Features of Patients

Studied patients showed a significant trend of elder age with progression of liver disease from chronic HCV to HCC ($P = 0.037$). There was a male predominance in HCV-related liver disease patients in the two groups representing 62.5% & 100% in non-HCC, and HCC groups, respectively. Serum creatinine, alkaline phosphatase and total bilirubin levels ($P = 0.001$ for each) and INR ($P < 0.001$) were significantly lower in chronic uncomplicated HCV versus the 2 other groups. Whereas Serum ALT was significantly higher in cirrhotic group versus the 2 other groups ($P < 0.001$), and serum AST level was only significantly different between HCC and chronic HCV patients ($P = 0.001$). Hepatic synthetic functions; albumin concentration and prothrombin activity tended to decrease significantly ($P < 0.0001$ for each) during liver disease progression among studied groups. Regarding blood picture, HCC and cirrhotic patients showed significantly lower platelet count versus uncomplicated HCV patients ($P = 0.004$). Total leucocyte count was significantly higher in HCC group versus the cirrhotic group, while, the hemoglobin level was significantly higher in cirrhotic group versus the 2 other groups ($P = 0.001$). Only the HCV RNA level (viral load) was not significantly different between studied groups ($P = 0.244$) (**Table 1**).

3.2. Differential Expression of Circulating MiRNA Levels

3.2.1. MiRNA-21

Analysis of median fold change in expression level of circulating miRNA-21 in HCV patients in comparison to normal controls ($n = 10$), showed that miRNA-21 displayed the signature fold decrease in expression in both cirrhotic HCV and HCC groups (0.64 and 0.69 respectively) and the signature fold increasing in expression level in chronic uncomplicated HCV (1.93). Comparing miRNA-21 expression level between different studied groups displayed an increasing tendency towards statistical significance fold elevation in expression of miRNA-21 in plasma of chronic HCV patients (1.93) in comparison to cirrhotic HCV (0.64) and in comparison to HCC (0.69) groups with P value (0.036), with no significant difference between cirrhotic and HCC groups ($P = 1.00$) (**Table 2**, **Figure 1**).

3.2.2. MiRNA-122

Analysis of median fold change in expression level of circulating miRNA-122 in HCV patients in comparison to

Table 1. Demographic and clinical data of uncomplicated, cirrhotic and HCC chronic HCV patients.

Parameter	Non-malignant		Group 3 = HCC (N = 20)	Kruskal Wallis (P value)
	Group 1 = Uncomplicated HCV (N = 20)	Group 2 = HCV + Cirrhosis (N = 20)		
Age Median (min-max)	41[c] (25 - 52)	50 (39 - 55)	50[a] (35 - 85)	0.037*
Sex Male Female	8 (40%) 12 (60%)	17 (85%) 3 (15%)	20 (100%) 0	0.001^
WBCS count (10^3/mm^3) Median (min-max)	4.55 (3.5 - 8.16)	4.1[c] (3 - 4.5)	5.7[b] (3.1 - 15.52)	0.008*
Hemoglobin level (g/dl) Median (min-max)	12.5[b] (11.2 - 14.7)	9.2[a, c] (9 - 10)	11.2[b] (6.7 - 15.6)	0.001*
Platelet count (10^3/mm^3) Median (min-max)	215[b, c] (142 - 242)	110[a] (52 - 120)	92[a] (75 - 391)	0.004*
Creatinine (mg/dl) Median (min-max)	0.7[b, c] (0.6 - 1)	1.1[a] (1 - 1.7)	1.5[a] (0.6 - 9)	0.001*
INR Median (min-max)	1.04[b, c] (1 - 1.08)	1.5[a] (1.08 - 2)	1.4[a] (1.28 - 2.31)	<0.001*
Total bilirubin (mg/dl) Median (min-max)	0.5[b, c] (0.4 - 1.1)	1.9[a] (1.2 - 3.5)	2.1[a] (0.6 -6.2)	0.001*
Alkaline phosphatase (IU/L) Median (min-max)	61[b, c] (42 - 132)	134[a] (98 - 172)	127[a] (113 - 413)	0.001*
ALT (IU/L) Median (min-max)	41[b] (20 - 79)	92[a, c] (80 - 121)	50[b] (19 - 160)	0.002*
AST (IU/L) Median (min-max)	47.5[c] (23 - 86)	96 (80 - 154)	119[a] (28 - 740)	0.001*
Albumin (g/dl) Median (min-max)	4.2[c] (3.6 - 4.5)	3.1[c] (2.6 - 3.2)	2.5[a, b] (2.2 - 2.5)	<0.001*
Prothrombin activity % Median (min-max)	85[b, c] (82 - 100)	62[a] (43 - 72)	55.5[a] (29 - 63.1)	<0.001*
HCV RNA level (IU/ml) Median (min-max)	2.04×10^6 ($14383 - 6.69 \times 10^7$)	1.34×10^6 ($1.22 \times 10^5 - 2.54 \times 10^6$)	2.59×10^6 ($2.94 \times 10^5 - 1.45 \times 10^7$)	0.244

*: Significant by Kruskal Wallis test; $P < 0.05$; [a]: Significant compared to HCV uncomplicated group by pairwise comparison test; [b]: Significant compared to HCV cirrhosis group by pairwise comparison test; [c]: Significant compared to HCC group by pairwise comparison test; ^: Significant by Monte-Carlo test; $P < 0.05$.

normal controls (n = 10), revealed that miRNA-122 displayed the signature fold decrease in expression in HCC group (0.14) and the signature fold increasing in expression level in both chronic HCV (0.49) and cirrhotic HCV (0.64). Comparing miRNA-122 expression level between different studied groups displayed an increasing tendency towards statistical significance fold elevation in expression of miRNA-122 in plasma of chronic uncomplicated HCV patients (0.49) in comparison to HCC group (0.14) with P value (0.005) (**Table 2**, **Figure 2**).

Table 2. MiRNA-21 & -122 levels in the three different groups in comparison to controls.

Parameter	Controls (N = 10)	Non-HCC		Group 3 = HCC (N = 20)	Kruskal Wallis (P value)
		Group 1 = Uncomplicated HCV (N = 20)	Group 2 = HCV + Cirrhosis (N = 20)		
miRNA 21 (RQ) Median (min-max)	1.306[c, d] (0.01 - 13.89)	1.93[c, d] (0.97 - 7.09)	0.64[a, b] (0.28 - 0.9)	0.69[a, b] (0.06 - 6.44)	0.036*
miRNA 122 (RQ) Median (min-max)	0.405[d] (0.25 - 14.65)	0.49[d] (0.25 - 3.95)	0.64 (0.21 - 2.29)	0.14[a, b] (0.01 - 1.87)	0.005*

*: Significant by Kruskal Wallis test; P < 0.05; [a]: Significant compared to control group by pairwise comparison test; [b]: Significant compared to uncomplicated HCV group by pairwise comparison test; [c]: Significant compared to HCV cirrhosis group by pairwise comparison test; [d]: Significant compared to HCC group by pairwise comparison test.

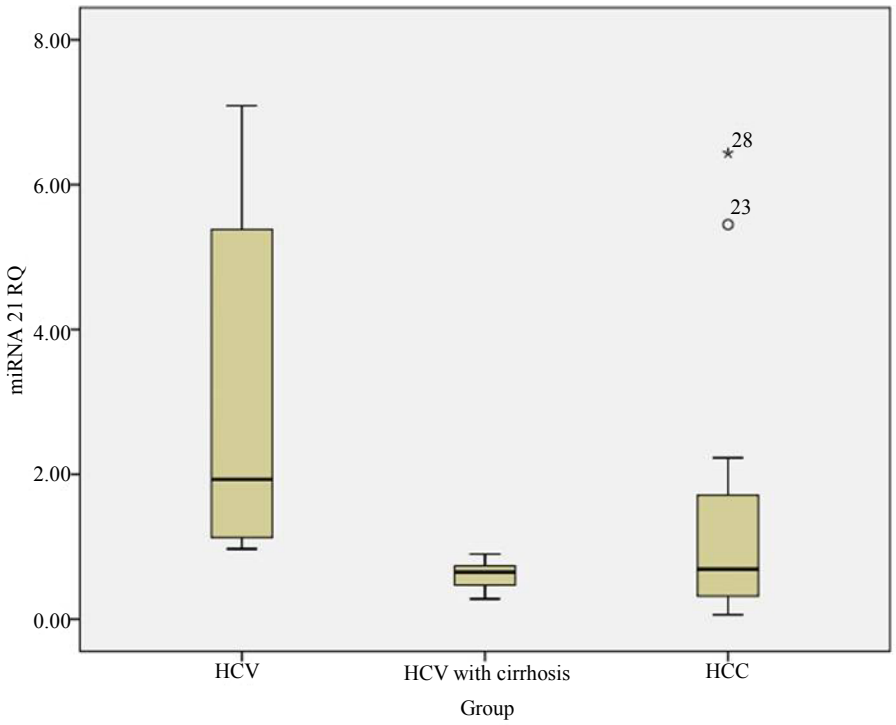

Figure 1. Differential microRNA-21 expression in the plasma of the three studied groups of patients as compared to normal controls.

3.3. Diagnostic Performance of Mi-RNAs

We examined the diagnostic performance of plasma miRNA-21 and miRNA-122 that were differentially expressed in HCC and non-HCC groups, to discriminate between the two groups. Receiver operator characteristic (ROC) analysis was used to determine the optimum cut-off value for the studied diagnostic markers. ROC analysis (**Figure 3**) revealed AUC = 0.811, and P = 0.002 for miRNA-122 thus discriminating between HCC and non-HCC patients, while miRNA-21 failed to do so, with AUC = 0.61 and P = 0.275, a sensitivity of only 42.1% and specificity of 13.3% at a cutoff of 0.51. The calculated cut-off of miRNA-122 that showed the highest sensitivity (68.4%) and specificity (100%) was 0.184. Finally, the sensitivity and specificity of combined miRNA-21 and miRNA-122 quantitative expression were 73.7% and 80%, respectively, with a cutoff of 0.64. When comparing ROC curves of miRNA-122 and that of combined miRNA-122 and -21, there was no statistically significant difference in diagnostic accuracy between miRNA-122 (AUC = 0.811) and combined mi-RNA-122 and -21 (AUC = 0.78), (P = 0.819).

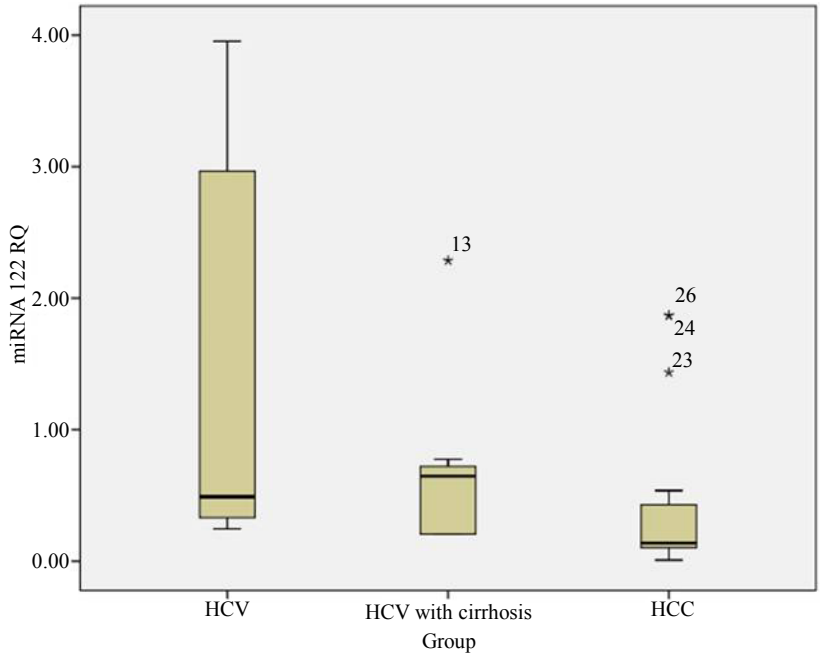

Figure 2. Differential microRNA-122 expression in the plasma of the three studied groups of patients as compared to normal controls.

3.4. Relation of Circulating MiRNAs and All the Studied Parameters

To verify the correlation between the expression levels of miRNA-21 and miRNA-122 with all studied parameters (**Table 3**), spearman correlation was performed. It was found that miRNA-122 expression levels were significantly positively correlated with AST levels ($r_s = 0.846$, $P \leq 0.001$), while negatively correlated with HCV RNA level ($r_s = -0.056$, $P = 0.012$) in the HCC group. All other tested parameters in both HCC and non-HCC groups failed to show any correlation with miRNA-122 expression levels. Concerning miRNA-21; in the non-HCC group, there was a significant positive correlation between miRNA-21 levels and serum albumin, prothrombin activity, hemoglobin level and platelets count ($P < 0.05$). On the other hand, a significant negative correlation of miRNA-21 with ALT, AST, total bilirubin, creatinine, INR and ALP levels was found ($P < 0.05$). While only ALP and AST in HCC group could correlate negatively and positively with miRNA-21 respectively.

Regarding the grading and staging (median = 1.5 in HCV uncomplicated group, and 4 in HCV cirrhotic group) of liver disease in non-HCC group, a significant negative correlation was found between the expression level of miRNA-21 and Metavir staging and grading system ($P = 0.001$).

When we investigated the relation between the expression levels of miRNA-21 and miRNA-122 and U/S characteristics of HCC patients (number of nodules, intrahepatic extension, presence or absence of capsule, portal vein thrombosis and lymph node metastasis) (data not shown), we couldn't establish any significant differences between any of these features and circulating miRNAs.

3.5. Measurement of the Power of Circulating MiRNAs to Predict HCC Diagnosis

Univariate logistic regression analysis was performed to select the predictive factor associated with HCV-related HCC diagnosis. Neither miRNA-21 nor miRNA-122 was a successful predictor for HCC diagnosis, while serum AST level and INR were showed to be significant in predicting HCC diagnosis (OR = 1.019, P = 0.018 AND OR = 29.886, P = 0.032 respectively) (**Table 4**).

4. Discussion

Prior results show that dysregulation of miRNA expression is a frequent occurrence in diverse types of cancer [13]. In this study, we identified two miRNAs: miRNA-21 and miRNA-122 that were dysregulated in HCV-infected patient samples.

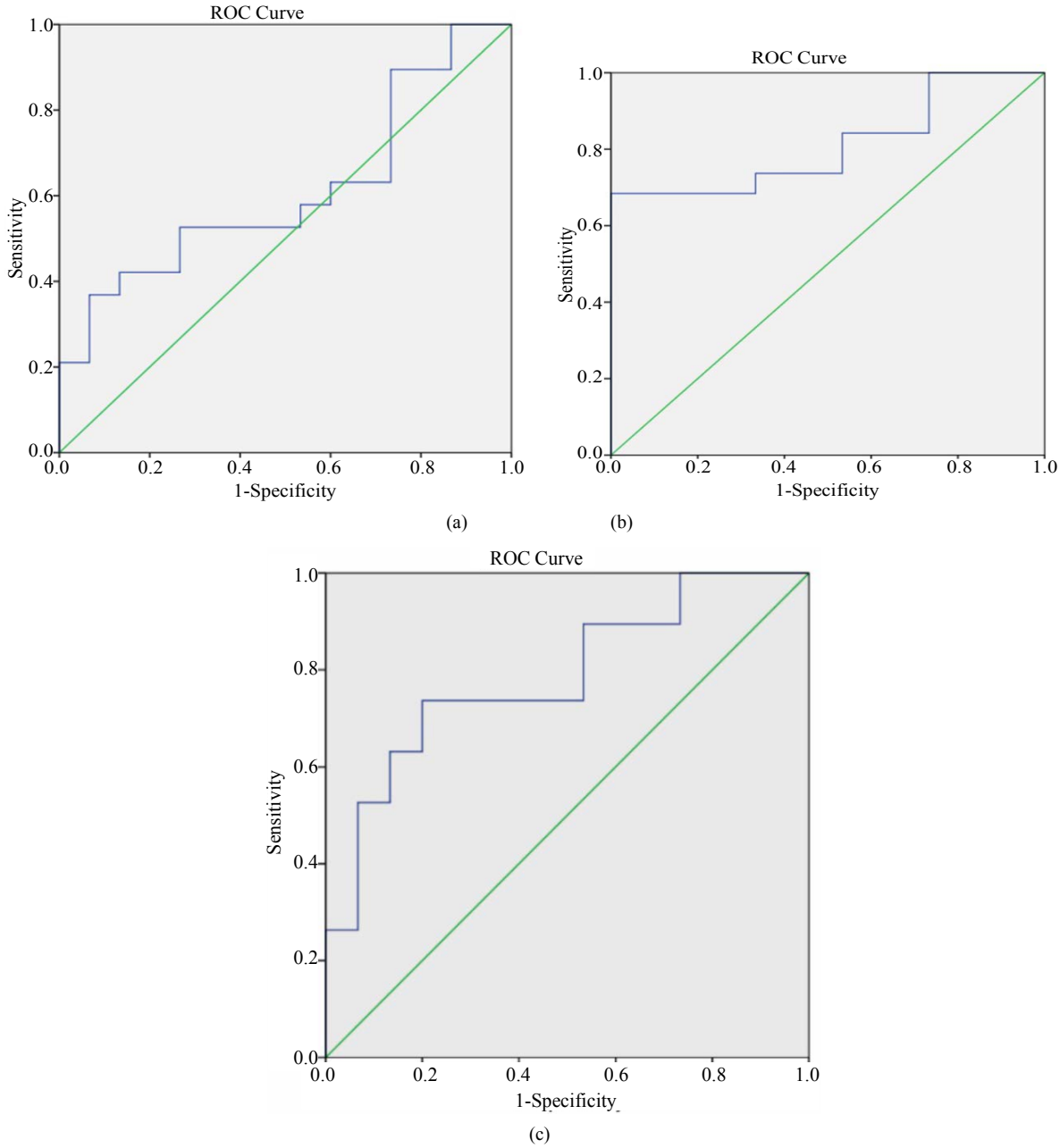

Figure 3. (a) Receiver operator characteristic curve for miRNA-122 as a discriminant between hepatocellular carcinoma vs non-hepatocellular carcinoma; (b) Receiver operator characteristic curve for miRNA-122 as a discriminant between hepatocellular carcinoma vs non-hepatocellular carcinoma; (c) Receiver operator characteristic curve for combined miRNA-21 & miRNA-122 as a discriminant between hepatocellular carcinoma vs non-hepatocellular carcinoma.

Our miRNA-21 results are not matching with many other studies that report an up-regulation of miRNA-21 in many cancers including HCC [14]-[19]. Bihrer *et al.* [20] demonstrated that the level of miRNA-21 in sera from patients with chronic hepatitis C associated HCC was elevated compared to healthy controls. But they also detected increased levels of miRNA-21 in sera from patients with chronic hepatitis C without HCC, suggesting that this elevation of serum miRNA-21 levels appears to be mainly associated with chronic hepatitis rather than HCC. They found that miRNA-21 serum levels strongly correlated with parameters of ongoing liver damage (ALT and AST). Unexpectedly, ALT and AST levels in our study negatively correlated significantly with miRNA-21 level in non-HCC group; albumin and prothrombin levels showed a significant positive correlation

Table 3. Correlation between miRNA-21 and miRNA-122 expression levels and the studied parameters.

Variable	MiRNA-21				MiRNA-122			
	Non-HCC		HCC		Non-HCC		HCC	
	r_S	P value	r_S	P value	r_S	P value	r_S	P value
Age	−0.578	0.024*	0.081	0.740	−0.173	0.538	0.188	0.442
WBCs count (×1000/ cmm)	0.479	0.071	−0.192	0.430	0.207	0.458	−0.192	0.430
Hemoglobin level (g/dl)	0.792	<0.001*	0.118	0.631	0.472	0.075	0.063	0.799
Platelet count (x1000/ cmm)	0.745	0.001*	−0.427	0.680	0.453	0.090	0.027	0.912
Albumin (g/dl)	0.783	0.001*	0.228	0.348	0.489	0.064	0.103	0.674
Creatinine (mg/dl)	−0.718	0.003*	0.093	0.704	−0.409	0.130	0.004	0.986
INR	−0.60	0.018*	0.254	0.294	−0.251	0.367	0.216	0.375
Total bilirubin (mg/dl)	−0.576	0.025*	0.154	0.529	−0.71	0.801	0.223	0.358
Alkaline phosphatase (IU/L)	−0.605	0.017*	−0.532	0.019*	−0.182	0.517	−0.229	0.346
ALT (IU/L)	−0.691	0.004*	0.146	0.55	−0.046	0.871	0.224	0.356
AST (IU/L)	−0.771	0.001*	0.474	0.040*	−0.344	0.210	0.846	<0.001*
Prothrombin activity%	0.610	0.016*	−0.296	0.219	0.233	0.403	−0.228	0.349
HCV RNA level (IU/ml)	0.369	0.176	−0.189	0.437	0.223	0.424	−0.564	0.012*
Grading	−0.776	0.001*	-	-	−0.201	0.473	-	-
Staging	−0.776	0.001*	-	-	−0.201	0.473	-	-
Diameter of lesion	-	-	0.069	0.780	-	-	0.140	0.567
AFP	-	-	0.293	0.223	-	-	0.368	0.121
LDH	-	-	−0.097	0.692	-	-	−0.078	0.751
MiRNA-21	-	-	-	-	0.490	0.064	0.556	0.014*
MiRNA-122	0.490	0.064	0.556	0.014*	-	-	-	-

r_s: Spearman correlation.

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

Factor	Odds Ratio	95% confidence interval	P value
Age	1.090	0.988 - 1.202	0.084
Sex	0.926	0.537 - 1.596	0.782
HCV RNA level	1	-	0.748
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***
MiRNA-21	0.975	0.748 - 1.271	0.852
MiRNA-122	0.681	0.360 - 1.291	0.239

with miRNA-21 in the same group, and AST correlated positively with miRNA-21 in HCC group. This discrepancy may be attributed to the small sample size especially that miRNA-21 level also correlated negatively with fibrosis grade.

Although there is clear evidence for a close relation between miRNA-21 and hepatic fibrosis, the hepatic level of miRNA-21 correlates with the stage of liver fibrosis [21], and circulating miRNAs are expected to be affected by fibrosis progression [22]. Serum miRNA levels may initially rise following release from inflamed hepatocytes in HCV followed by a drop in the levels with fibrosis progression due to hepatocyte loss and accumulation of extracellular matrix [23]. A significant negative correlation between miRNA-21 levels and staging of liver fi-

brosis in non-HCC group was reported in the present study.

There was no correlation between the concentrations of miRNA-21 and HCV viral load in our study, in accordance with Bihrer *et al*. [20] and Su *et al*. [24]. This can be reconciled with the lack of correlation of HCV serum level and disease activity in patients with CHC [25].

Our data showed an elevated circulating miRNA-122 expression level in chronic HCV patients in comparison to HCC group. Similarly, previous studies data revealed a decreased miRNA-122 expression level in HCCs and that was poorly relevant for survival [26]-[28]. In another study [29] miRNA-122 expression did not differ significantly between patients with HCC and liver cirrhosis alone. However, several studies have found that the level of serum miRNA-122 was significantly elevated in patients with HCC [23] [30] [31].

The discrepancies 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) [32] or may be due to the small patient population [33].

The current work demonstrated that by ROC analysis, miRNA-122 could discriminate between HCC and non-HCC patients, while miRNA-21 failed to discriminate. This might be related to what was previously reported that miRNA-122 was highly selective for the liver [20], whereas miRNA-21 showed significant expression in other cells and tissues such as in lymphocytes [34]. Thus, release of miRNA-21 from several different cell types may contribute to higher serum miRNA-21 in chronic hepatitis C patients. The combination of miRNA-21 and miRNA-122 did not show any significant difference in diagnostic accuracy when compared to miRNA-122 alone. So we suggest using miRNA-122 alone as this will be cost effective than using both markers and much easier to interpret.

Similar to other studies [35] [36], we couldn't demonstrate a statistically significant correlation between serum miRNA-122 expression level and liver synthetic functions tests (Albumin, bilirubin and prothrombin), necroinflammatory markers (ALT, ALP), in HCC vs non-HCC group. However, in the HCC group miRNA-122 level was significantly positively correlated with AST levels. On the other hand, Köberle *et al*. [27] reported that serum miRNA-122 correlated with clinical chemistry parameters of hepatic necroinflammation, liver function and synthetic capacity. While Ezzat *et al*. [29] showed that miRNA-122 expression levels were positively correlated with AST, ALT, and ALP and negatively correlated with prothrombin concentration from all groups (cirrhosis and HCC).

In contrast to the inhibitory role on HBV replication, miRNA-122 is essential for HCV RNA replication [34]. However, we found a negative correlation of miRNA-122 with serum HCV RNA, matching with Marquez *et al*. [21] who found that miRNA-122 expression was altered in HCV-infected liver, and miRNA-122 level was inversely correlated, with viral load, fibrosis and serum liver transaminase levels. A possible explanation for this inverse relation is that, counter-intuitively, the beneficial role of miRNA-122 for the virus *in vitro* does not translate into a positive correlation between its expression and HCV load in patients [33].

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 could predict HCC diagnosis. Neither miRNA-21 nor miRNA-122, AFP was a successful predictor for HCC diagnosis. High levels of AFP accompany liver diseases other than HCC. In addition, a significant proportion of HCC patients did not have an elevated AFP. Non-tumors liver cells also abundantly express AFP mRNA. AFP represents liver cell-specific, not tumor specific markers [37].

In the light of this study, we could conclude that expression of miRNA-21 and 122 was down regulated in a group of Egyptian patients with HCC as compared to those with chronic HCV infection and cirrhosis. MiRNA-122 could discriminate between HCC and non-HCC patients, alone and when combined with miRNA-21. Other factors such as serum AST level and INR, but not AFP, could predict diagnosis of HCC. Further studies on large scale are recommended to evaluate miRNA-21 and 122 as diagnostic and prognostic tools in Egyptian patients with HCV related liver diseases.

Conflict of Interests

No competing financial interests exist.

References

[1] Rehermann, B. (2009) Hepatitis C: Virus versus Innate and Adaptive Immune Responses—A Tale of Coevolution and Coexistence. *Journal of Clinical Investigations*, **119**, 1745-1754. http://dx.doi.org/10.1172/JCI39133

[2] Ray, S.C., Arthur, R.R., Carella, A., Bukh, J. and Thomas, D.L. (2000) Genetic Epidemiology of Hepatitis C Virus throughout Egypt. *Journal of Infectious Diseases*, **182**, 698-700. http://dx.doi.org/10.1086/315786

[3] El-Zayadi, A.R., Badran, H.M., Barakat, E.M., Mel-D, A., Shawky, S., Mohamed, M.K., Selim, O. and Saeid, A. (2005) Hepatocellular Carcinoma in Egypt: A Single Center Study over a Decade. *World Journal of Gastroenterology*, **11**, 5193-5198.

[4] Zinkin, N.T., Grall, F., Bhaskar, K., Otu, H.H. and Spentzos, D.D. (2008) Serum Proteomics and Biomarkers in Hepatocellular Carcinoma and Chronic Liver Disease. *Clinical Cancer Research*, **14**, 470-477. http://dx.doi.org/10.1158/1078-0432.CCR-07-0586

[5] Du, T. and Zamore, P.D. (2005) Microprimer: The Biogenesis and Function of Micro-RNA. *Development*, **132**, 4645-4652. http://dx.doi.org/10.1242/dev.02070

[6] Volinia, S., Calin, G.A., Liu, C.G., Ambs, S. and Cimmino, A. (2006) A Micro-RNA Expression Signature of Human Solid Tumors Defines Cancer Gene Targets. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 2257-2261. http://dx.doi.org/10.1073/pnas.0510565103

[7] Mitchell, P.S., Parkin, R.K., Kroh, E.M., Fritz, B.R. and Wyman, S.K. (2008) Circulating Micro-RNAs as Stable Blood-Based Markers for Cancer Detection. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 10513-10518. http://dx.doi.org/10.1073/pnas.0804549105

[8] Johannes, H., Nicolas, Z. and Mathias, H. (2011) The Parallel Universe: Micro-RNAs and Their Role in Chronic Hepatitis, Liver Tissue Damage and Hepatocarcinogenesis. *European Journal of Medical Sciences*, **141**, Article ID: w13287.

[9] Matsuzaki, K., Murata, M., Yoshida, K., Sekimoto, G., Uemura, Y., Sakaida, N., Kaibori, M., Kamiyama, Y., Nishizawa, M., Fujisawa, J., Okazaki, K. and Seki, T. (2007) Chronic Inflammation Associated with Hepatitis C Virus Infection Perturbs Hepatic Transforming Growth Factor Beta Signaling, Promoting Cirrhosis and Hepatocellular Carcinoma. *Hepatology*, **46**, 48-57. http://dx.doi.org/10.1002/hep.21672

[10] Walter, H.T., Duverlie, G. and Bengrine, A. (2012) Micro-RNAs and Hepatitis C Virus: Toward the End of MiRNA-122 Supremacy. *Journal of Virology*, **9**, 109. http://dx.doi.org/10.1186/1743-422X-9-109

[11] Shiha, G. and Zalata, K. (2001) Ishak versus METAVIR: Terminology, Convertibility and Correlation with Laboratory Changes in Chronic Hepatitis C. In: Takahashi, H., Ed., *Liver Biopsy*, InTech, Rijeka, 155-170.

[12] Bruix, J. and Sherman, M. (2011) American Association for the Study of Liver Diseases (AASLD) Practice Guidelines. Management of Hepatocellular Carcinoma: An Update. *Hepatology*, **53**, 1020-1022. http://dx.doi.org/10.1002/hep.24199

[13] Varnholt, H., Drebber, U., Schulze, F., Wedemeyer, I., Schirmacher, P., Dienes, H.P. and Odenthal, M. (2008) MicroRNA Gene Expression Profile of Hepatitis C Virus-Associated Hepatocellular Carcinoma. *Hepatology*, **47**, 12123-12132.

[14] Ladeiro, Y., Couchy, G., Balabaud, C., Bioulac-Sage, P., Pelletier, L., Rebouissou, S. and Zucman-Rossi, J. (2008) MicroRNA Profiling in Hepatocellular Tumors Is Associated with Clinical Features and Oncogene/Tumor Suppressor Gene Mutations. *Hepatology*, **47**, 1955-1963. http://dx.doi.org/10.1002/hep.22256

[15] Chan, J.A., Krichevsky, A.M. and Kosik, K.S. (2005) MicroRNA-21 Is an Antiapoptotic Factor in Human Glioblastoma Cells. *Cancer Research*, **65**, 6029-6033. http://dx.doi.org/10.1158/0008-5472.CAN-05-0137

[16] Fulci, V., Chiaretti, S., Goldoni, M., Azzalin, G., Carucci, N., Tavolaro, S., Castellano, L., Magrelli, A., Citarella, F., Messina, M., Maggio, R., Peragine, N., Santangelo, S., Mauro, F.R., Landgraf, P., Tuschl, T., Weir, D.B., Chien, M., Russo, J.J., Ju, J., Sheridan, R., Sander, C., Zavolan, M., Guarini, A., Foà, R. and Macino, G. (2007) Quantitative Technologies Establish a Novel microRNA Profile of Chronic Lymphocytic Leukemia. *Blood*, **109**, 4944-4951. http://dx.doi.org/10.1182/blood-2006-12-062398

[17] Iorio, M.V., Ferracin, M., Liu, C.G., Veronese, A., Spizzo, A., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M., Ménard, S., Palazzo, J.P., Rosenberg, A., Musiani, P., Volinia, S., Nenci, I., Calin, G.A., Querzoli, P., Negrini, M. and Croce, C.M. (2005) MicroRNA Gene Expression Deregulation in Human Breast Cancer. *Cancer Research*, **65**, 7065-7070. http://dx.doi.org/10.1158/0008-5472.CAN-05-1783

[18] Lee, E.J., Gusev, Y., Jiang, J., Nuovo, G.J., Lerner, M.R., Frankel, W.L., Morgan, D.L., Postier, R.G., Brackett, D.G. and Schmittgen, T.D. (2007) Expression profiling Identifies microRNA Signature in Pancreatic Cancer. *International Journal of Cancer*, **120**, 1046-1054. http://dx.doi.org/10.1002/ijc.22394

[19] Huang, Y.S., Dai, Y., Yu, X.F., Bao, S.Y., Yin, Y.B., Tang, M. and Hu, C.X. (2008) Microarray Analysis of microRNA Expression in Hepatocellular Carcinoma and Non-Tumorous Tissues without Viral Hepatitis. *Journal of Gastroenterology and Hepatology*, **23**, 87-94. http://dx.doi.org/10.1111/j.1440-1746.2007.05223.x

[20] Bihrer, V., Waidmann, O., Friedrich-Rust, M., Forestier, N., Susser, S. and Haupenthal, J. (2011) Serum microRNA-21 as Marker for Necroinflammation in Hepatitis C Patients with and without Hepatocellular Carcinoma. *PLoS ONE*, **6**,

e26971. http://dx.doi.org/10.1371/journal.pone.0026971

[21] Marquez, R.T., Bandyopadhyay, S., Wendlandt, E.B., Keck, K., Hoffer, B.A., Icardi, M.S., Christensen, R.N., Schmidt, W.N. and McCaffrey, A.P. (2010) Correlation between microRNA Expression Levels and Clinical Parameters Associated with Chronic Hepatitis C Viral Infection in Humans. *Laboratory Investigations*, **90**, 1727-1736. http://dx.doi.org/10.1038/labinvest.2010.126

[22] Bihrer, V., Friedrich-Rust, M., Kronenberger, B., Forestier, N., Haupenthal, J., Shi, Y., Peveling-Oberhag, J., Radeke, H.H., Sarrazin, C., Herrmann, E., Zeuzem, S., Waidmann, O. and Piiper, A. (2011) Serum miR-122 as a Biomarker of Necroinflammation in Patients with Chronic Hepatitis C Virus Infection. *American Journal of Gastroenterology*, **106**, 1663-1669. http://dx.doi.org/10.1038/ajg.2011.161

[23] Trebicka, J., Anadol, E., Elfimova, N., Strack, I., Roggendorf, M., Viazov, S., Wedemeyer, I., Drebber, U., Rockstroh, J., Sauerbruch, T., Dienes, H.P. and Odenthal, M. (2013) Hepatic and Serum Levels of miR-122 after Chronic HCV-Induced Fibrosis. *Journal of Hepatology*, **58**, 234-239. http://dx.doi.org/10.1016/j.jhep.2012.10.015

[24] Su, T.H., Liu, C.H., Liu, C.G., Chen, C.L., Ting, T.T., Tseng, T.C., Chen, P.J., Kao, L.H. and Chen, D.S. (2013) Serum microRNA-122 Level Correlates with Virologic Responses to Pegylated Interferon Therapy in Chronic Hepatitis C. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 7844-7849. http://dx.doi.org/10.1073/pnas.1306138110

[25] Poynard, T., Bedossa, P. and Opolon, P. (1997) Natural History of Liver Fibrosis Progression in Patients with Chronic Hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC Groups. *Lancet*, **349**, 825-832. http://dx.doi.org/10.1016/S0140-6736(96)07642-8

[26] Gramantieri, L., Ferracin, M., Fornari, F., Veronese, A., Sabbioni, S., Liu, C.G., Calin, G.A., Giovannini, C., Ferrazzi, E., Grazi, G.L., Croce, C.M., Bolondi, L. and Negrini, M. (2007) Cyclin G1 Is a Target of miR-122a, a microRNA Frequently Down-Regulated in Human Hepatocellular Carcinoma. *Cancer Research*, **67**, 6092-6099. http://dx.doi.org/10.1158/0008-5472.CAN-06-4607

[27] Köberle, V., Kronenberger, B., Pleli, T., Trojan, J., Imelmann, E., Peveling-Oberhag, J., Welker, M.W., Elhendawy, M., Zeuzem, S., Piiper, A. and Waidmann, O. (2013) Serum microRNA-1 and microRNA-122 Are Prognostic Markers in Patients with Hepatocellular Carcinoma. *European Journal of Cancer*, **49**, 3442-3449. http://dx.doi.org/10.1016/j.ejca.2013.06.002

[28] EL-Abd, N.E., Fawzy, A.N., EL-Sheikh, S.M. and Soliman, M.E. (2015) 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*, **19**, 213-220. http://dx.doi.org/10.1007/s40291-015-0148-1

[29] Ezzat, H., Lotfy, A.M., Alalfy, M.N., El-Taher, S.M., Mokhtar, A., Mohamed, S.A. and EL-Senosy, F.M. (2014) The Significance of Circulating MicroRNA-122 as a Non Invasive Diagnostic Marker of Liver Injury in Egyptian Chronic Hepatitis C Virus Infected and Cirrhotic Patients with and without Hepatocellular Carcinoma. *Clinical Medicine and Diagnostics*, **4**, 1-8.

[30] Fattovich, G., Giustina, G., Sanchez-Tapias, J., Quero, C., Mas, A., Olivotto, P.G., Solinas, A., Almasio, P., Hadziyannis, S., Degos, F., de Moura, M.C., Krogsgaard, K., Pantalena, M., Realdi, G., Corrocher, R. and Schalm, S.W. (1998) Delayed Clearance of Serum HBsAg in Compensated Cirrhosis B: Relation to Interferon Alpha Therapy and Disease Prognosis. European Concerted Action on Viral Hepatitis (EUROHEP). *American Journal of Gastroenterology*, **93**, 896-900. http://dx.doi.org/10.1111/j.1572-0241.1998.00272.x

[31] Parkin, D.M., Bray, F.I. and Deves, S.S. (2001) Cancer Burden in the Year 2000. The Global Picture. *European Journal of Cancer*, **37**, S4-S66. http://dx.doi.org/10.1016/S0959-8049(01)00267-2

[32] Borel, F., Konstantinova, P. and Jansen, P.L. (2012) Diagnostic and Therapeutic Potential of miRNA Signatures in Patients with Hepatocellular Carcinoma. *Journal of Hepatology*, **56**, 1371-1383. http://dx.doi.org/10.1016/j.jhep.2011.11.026

[33] Bandiera, S., Pfeffer, S., Thomas, F. and Zeisel, M.B. (2015) MiR-122—A Key Factor and Therapeutic Target in Liver Disease. *Journal of Hepatology*, **62**, 448-457. http://dx.doi.org/10.1016/j.jhep.2014.10.004

[34] Fan, H.-X. and Tang, H. (2014) Complex Interactions between microRNAs and Hepatitis B/C Viruses. *World Journal of Gastroenterology*, **20**, 13477-13492. http://dx.doi.org/10.3748/wjg.v20.i37.13477

[35] El-Garem, H., Ammer, A., Shehab, H., Shaker, O., Anwer, M., El-Akel, W. and Omar, H. (2014) Circulating microRNA, miR-122 and miR-221 Signature in Egyptian Patients with Chronic Hepatitis C Related Hepatocellular Carcinoma. *World Journal of Hepatology*, **27**, 818-824. http://dx.doi.org/10.4254/wjh.v6.i11.818

[36] Motawi, T.K., Shaker, O.G., El-Maraghy, S.A. and Senousy, M.A. (2015) Serum microRNAs as Potential Biomarkers for Early Diagnosis of Hepatitis C Virus-Related Hepatocellular Carcinoma in Egyptian Patients. *PLoS ONE*, **10**, e0137706. http://dx.doi.org/10.1371/journal.pone.0137706

[37] Yi, R., Qin, Y., Macara, I.G. and Cullen, B.R. (2003) Exportin-5 Mediates the Nuclear Export of Pre-MicroRNAs and Short Hairpin RNAs. *Genes & Development*, **17**, 3011-3016. http://dx.doi.org/10.1101/gad.1158803