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Correlation of miR-122 with Bcl-w is a Paradigm for the Role of Micro RNAs in the Liver Injury Development

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Abstract
Circulating miR-122 is commonly deregulated in liver fibrosis and hepatocellular carcinoma (HCC). In this study we tried to explore the potential usefulness of serum miR-122 as noninvasive diagnostic marker for liver injury and HCC in chronic hepatitis C virus infected patients. In addition, tried to examine whether serum level of miR-122 can potentially serve as circulating marker for liver disease stage assessment. Out of 84 participants; 20 patients were HCV positive infected patients, 44 HCC patients infected with HCV, and 20 healthy volunteers were also included. We determined the expression of levels ofmiR-122 and Bcl-w in serum using (qRT-PCR); and compared them with the other clinicopathological parameters. Mean miR-122 expression levels were up-regulated in both patient groups (HCV and HCC) compared to control group. In addition, mean expression levels of miR-122 were significantly higher in HCV group compared to HCC group ($p=0.025$). Conversely, mean Bcl-w expression levels were down-regulated in both patient groups compared to control group but, mean expression levels of Bcl-w were significantly higher in HCC group compared to HCV group($p=<0.001$). Furthermore, expression levels of miR-122 were positively correlated with ALT, AST, ALP and fibrosis stage, and negatively correlated with prothrombin concentration and Albumin in both patient groups.ROC curve analysis for miR-122 yielded 64% sensitivity and 75% specificity for the differentiation of HCC patients from non-HCC at a cutoff 3.85. Conclusion: miR-122 can be used as novel biomarker for liver injury and may be used to discriminate patients with HCC from HCV. Also, miR-122 may represent novel non invasive biomarker for assessment of liver disease severity in patients with chronic hepatitis C virus.

1. Introduction

Hepatitis C virus (HCV) is considered the most common etiology of chronic liver disease in Egypt [1]. Hepatocellular carcinoma (HCC) is the commonest primary liver cancer with increasing incidence to become the 5th commonest malignancy worldwide and the third leading cause of cancer-related death [2]. In Egypt, between 1993 and 2002 there was an almost twofold increase in HCC among chronic liver patients, HCC was reported to account for about 4.7% of chronic liver disease patients [3].

The most common condition associated with hepatocarcinogenesis is cirrhosis which develops 20 to 40 years of chronic liver disease. It has been reported that not only increased cellular proliferation but also, diminished cell death play important roles in hepatocarcinogenesis and tumor progression in HCC [4].

Hepatic fibrosis, defined as excessive accumulation of extracellular matrix components, develops after chronic liver injury mainly due to chronic viral hepatitis B and C, alcoholism and fatty liver disease [5]. Liver fibrosis may progress to liver cirrhosis, the end-stage liver disease, which has been a globally increasing major health problem with high mortality and morbidity in the past twenty years [6].

Liver biopsy is the gold standard for the grading and staging of fibrosis. However, this invasive technique has a mortality rate of 0.1–0.01% and harbors the risk of severe complications [7]. Measurement of liver stiffness using transient elastography can detect severe fibrosis [8], but this technique is hampered by several limitations (e.g., ascites, obesity, cholestasis, hepatic inflammation) [9].

In recent years microRNAs (miRNAs), a family of short (average of 20~25 nucleotide long), naturally occurring, small non-coding RNAs have emerged as important post-transcriptional regulators of gene expression [10]. miRNAs are predicted to control the activity of more than 60% of all protein-coding genes [11]. It has been estimated that miRNAs regulate up to 30% of human genes. Like mRNAs, some miRNAs also show restricted tissue distribution; for example, miRNA-122 is highly enriched in liver, whereas miR-124 is preferentially expressed in neurological tissue [12].

Recent studies have identified several miRNAs as key players in virus-host interactions, during HCV infection it has been demonstrated that a liver-specific miRNA, miR-122, binds to the 5′ nontranslational region of hepatitis C virus (HCV) genomic RNA and is essential for HCV replication in human hepatoma-derived Huh7 cells [13].

MiR-122 down-regulation has been reported in rodent and human HCCs [14], suggesting that its function is associated with hepatocarcinogenesis. In HCC-derived cell lines, miR-122 directly targets cyclin G1 (CCNG1) by binding its 3`-UTR. An inverse correlation between miR-122 and CCNG1 exists in primary liver carcinoma, further emphasizing the importance of miR-122 in HCC pathogenesis [14].In vertebrates, each miRNA has been predicted to target~200 transcripts [15]. A search for other miR-122 regulatory targets that may be involved in the progression of HCC using online prediction algorithms has identified Bcl-w, which is an anti-apoptotic Bcl-2 family member [16].

The present work aimed to assess whether serum levels of miR-122 can be used as a novel noninvasive biomarker for liver injury and to discriminate patients with HCC from those with HCV. The present work was also carried out to examine whether circulating levels of miR-122 can potentially serve as circulating biomarker for liver disease stage assessment.

2. Patients and Methods

This study was carried out on 84 participants. Sixty- four patients were recruited between May 2014 and August 2014 from National liver Institute Menofiya University, Egypt. Twenty healthy volunteers were also included. This study was approved by the research review committee of the Menofiya faculty of Medicine. And all the studied subjects gave their informed consent. The studied subjects were classified into 3 main groups: Group I Included 20 patients with chronic hepatitis C virus infection (HCV). They were 12 males and 8 females; their ages were ranged from 31 to71 years old. Group II Included 44 Patients with hepatocellular carcinoma (HCC); who were associated with HCV. They were 28 males and 16 females; their ages were ranged from 35 to 74 years old. All subjects included in the study were negative for HBs Ag. Exclusion criteria: The patients with known history of autoimmune diseases, diabetes mellitus, chronic inflammatory disease and a history of cancer other than hepatocellular carcinoma, as well as patients with chronic liver disease not HCV related were excluded. Group III is the control group which included 20healthy volunteers with matched age and sex were chosen carefully, the subjects of this group with no evidence of liver disease and who were negative for HCV antibodies.

All patients were subjected to the following:

A-Through history taking and complete clinical examination with stress on; jaundice, hepatomegaly, ascites, splenomegaly, lower limb oedema, and encephalopathy.

B-Abdominal ultrasound; to assess the echo pattern and size of the liver and the presence of periportal fibrosis, the presence of ascites, the size of spleen or any other abnormalities.

C-Abdominal triphasic CT scanning and or MRI were performed for patients as well as liver biopsy and histopathological examination whenever possible.

D-METAVIR score was used to stage liver fibrosis (F0-F4) [17]. Liver fibrosis was scored on a 5-point scale: F0, no fibrosis; F1, portal fibrosis alone; F2, portal fibrosis with rare septae; F3, portal fibrosis with many septae; F4, cirrhosis. The presence of stage F0-F1 was termed minimal or early fibrosis; the presence of stage F2-F4 was termed significant fibrosis; whereas the term advanced fibrosis was reserved for stage F3-F4.

E - Laboratory investigations including:

1 Liver function tests [Total Bilirubin, Albumin, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP) and prothrombin time (PT)].
2 Anti HCV antibodies (third generation) by microparticle enzyme immunoassay (MEIA) technology on Axsym was performed for all participants. Positive cases followed by RT-PCR (Reverse transcription polymerase chain reaction) for confirmation of HCV infection and estimation of total viremia [18].
3 Quantitative determination of alpha-fetoprotein (AFP) Using chemiluminescence technique on Cobas e 411

from Roche (Germany) [19].

4 Quantitative determination of serum miR-122 expression level using q RT- PCR assay.

5 Quantitative determination of human serum Bcl-w expression level using q RT- PCR assay.

Samples collection:

Approximately 7 mL of venous blood was taken from the subjects and healthy controls. Five ml of each blood sample was transferred to plain vacutainer tubes, the sample allowed to clot for 30 minutes and then centrifuged for 15 minutes at 3000 rpm. The clear supernatant serum was separated from the clot and some used for liver function tests and AFP, and some was stored in aliquots at-80°C formiR-122 and Bcl-w determination. The remaining 2mL was invacutainer trisodium citrate tube for determination of prothrombin time. All laboratory studies were performed at National Liver Institute, department of clinical biochemistry.

2.1. Quantitative Determination of Serum miR-122 Expression Level Using q RT-PCR Assay

2.1.1. Total RNA Extraction: According to the Manual by Qiagen

Frozen serum samples were thawed.Then200 µl from serum was added to 1 ml QIAzol Lysis Reagent. The mixture was mixed.200 µl chloroform was added to the tube containing the homogenate and closed securely. The tube was vortexed. The tube containing the homogenate was placed on the bench top at room temperature for 2–3 min. The homogenate was centrifuged for 15 min at 12,000 x *g* at 4°C. After centrifugation, the sample was separated into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The upper aqueous phase was transferred to a new collection tube. 1.5 volumes of 100% ethanol were added and mixed.Up to 700 µl of the sample was Pipetted, into an RNeasy Mini spin column in a 2 ml collection tube. The flow-through was discarded.700 µl Buffer RWT was added to the RNeasy Mini spin column. The lid was closed gently and centrifuged for 15 s at ≥8000 x *g* to wash the column. The flow through discarded.500 µl Buffer RPE was pipetted onto the RNeasy Mini spin column. The lid gently was closed and centrifuged for 15 s at ≥8000 x *g* to wash the column. The flow through was discarded. The RNeasy Mini spin column was placed into a new 2 ml collection tube, and the old collection tube was discarded with the flow-through. The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube. 30–50 µl RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane. The lid was closed gently and centrifuged for 1 min at ≥8000 x g to elute the total RNA. Then the total RNA was aliquoted into two aliquots; one for RT- PCR of miR-122 and the other one for RT-PCR of Bcl-w.

2.1.2. Reverse Transcription (RT) and Quantitative PCR (qPCR) [20]

1-Prepare the RT reaction master mix: The expression levels of miRNA-122 and its house keeping gene miR-16 were measured by quantitative RT-PCR. To prepare the RT master mix using the TaqMan® MicroRNA Reverse transcription Kit components:20X or 60 X RT primers were diluted to a 5X working stock solution using 0.1X TE buffer. The primer sequence for miR-122 gene was the following: Forward 5' -GACAAGCCTGGCTACTGTGTT-3'. And Reverse5'-GTGGCCCATCTTGTCCTTC-3'.miR-16was used as internal control. The primer sequence of miR-16 gene was as follow: Forward 5'-TAGCAGCACGTAAATATTGGCG-3'. And Reverse 5'-TGCGTGTCGTGGAGTC- 3'.

In a polypropylene tube, the RT master mix was prepared as follow: 0.15 µl100mM dNTPs, 1.00 µl MultiScribe™ Reverse Transcriptase, 50 U/µl, 1.50 µL10× Reverse Transcription Buffer, 0.19 µl RNase Inhibitor, 20 U/µl and 4.16 µl Nuclease-free water in a final volume of 7.00 µl. Each 15-µL RT reaction consists of 7 µl master mix, 3 µl of 5 X RT primers, and 5 µl RNA sample. Mixed gently and centrifuged to bring the solution to the bottom of the tube.

2-Perform reverse transcription (cDNA Synthesis):To program the thermal cycler: 30 min at 1°C, 30 min at 42°C, 5 min at 85°C, and then maintained at 4°C.The reaction volume was set to 15.0 µl. The reaction tube or plate was loaded into the thermal cycler. Then the RT run was started.

3-Prepare the q PCR reaction mix:A sterile 1.5-mL microcentrifuge tube was obtained for each sample. And the following components were pipetted into each tube: 1.00 µl TaqMan® Small RNA Assay (20X), 1.33 µl Product from RT reaction, 10 µl TaqMan® Universal PCR Master Mix II (2X) and 7.67 µl Nuclease-free water in a total volume20 µl. The tube was capped and inverted several times to be mixed. Then the tube was centrifuged briefly.

4-Prepare the PCR reaction plate: 20 µl of the complete q PCR reaction mix was transferred into each of three wells on a 48-, or 96-, or 384-well plate. The plate was sealed with the appropriate cover. Then the plate was centrifuged briefly, and was loaded into the instrument.

5-Set up the experiment or plate document and run the plate: Thermal Cycling Conditions: All reactions were run on the 7500 (Applied Biosystems, USA) using the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The run was started. Melting curve analysis was performed at the end of PCR cycles in order to validate the specificity of the expected PCR product. Relative quantitation (RQ) of miR-122 was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method [21].

2.2. Quantitative Determination of Human Serum Bcl-w Expression Level Using q RT- PCR Assay

2.2.1. cDNA Reaction Preparation: According to the Manual by Applied Biosystems

To prepare The 2X RT Master Mix (Per 20 µL reaction): The volume of components needed to prepare the required number of reaction was calculated by referring to the following: 2.0 µL 10X RT Buffer, 0.8 µL 25X dNTP Mix (100mM), 2.0 µL 10X RT Radom Primers, 1.0 µL MultiScribe ™Transcriptase, 1.0 µL RNase Inhibitor, 3.2µL Nuclease-free

H2O, in a total volume 10.0 µL per reaction. The PCR primers for Bcl-w gene were the following sequence: Bcl-w forward 5`CACCCAGGTCTCCGATGAAC3`. and reverse 5`TTGTTGACACTCTCAGCACAC3`. The housekeeping gene β-actin was used as endogenous control. β-actin forward and reverse primers were 5`AGCGAGCATCCCCCAAAGTT 3`. And 5`GGGCACGAAGGCTCATCATT 3`; respectively.

To prepare the cDNA Reverse Transcription reactions: 10 µL of 2XRT master mix was pipetted into each well of 96-well reaction plate.10 µL of RNA sample (previously obtained from total RNA extraction step) was pipetted into each well, pipetting was done up and down two times to mix. The plate was sealed. The plate was briefly centrifuged to spin down the contents and to eliminate any bubbles. To program the thermal cycling conditions: The thermal cycler was programmed using the conditions below: 25°C for 10 min., 37°C for 120 min., 85°C for 5 min. and then maintained at 4°C.

2.2.2. Quantitation of Bcl-w by q RT PCR: [22]

A sterile 1.5-mL microcentrifuge tube was obtained for each sample. And the following components were pipetted into each tube:12.5 µl Power SYBR® Green PCR Master Mix (2X),1 µl (12.5p mol) forward primer, 1 µl (12.5 p mol) reverse primer, 5 µl template and 5.5 µl water in a total volume 25 µl. The tube was capped and inverted several times to be mixed. The tube was centrifuged briefly. Thermal cycling conditions for quantitation: 10 min. at 95°C (DNA polymerase activation) and PCR cycling step (40 cycles) 15 sec. at 95 ºC (denature), followed by 1 min. at 60°C (anneal/extend). Relative expression (RQ) of Bcl-w was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method [21].

Statistical analysis was carried out using SPSS version 20. Values were expressed as mean±SD, Kruskal-Waills and Mann-Whitney tests were used. Spearman's correlations were done between different parameters. ROC curves analyses were done to evaluate the value of circulating miR-122 level for discriminating patients with HCC from HCV. X^2 was obtained by Chi-Square test. P value was considered significant when ≤ 0.05.

3. Results

Eighty four individuals were enrolled in the study. Sixty four patients were admitted to National liver Institute Menofiya University, Tropical medicine and Oncology departments. Twenty healthy volunteers were also included. The studied subjects were classified into: Group I: 20 patients with HCV infection. They were (60% males, 40% females) with age ranged from 31 to 71 years old. Group II: 44 patients with hepatocellular carcinoma (HCC), who were associated with HCV infection. They were (63.6 % males, 36.4 females) with age ranged from 35 to 74 years old. Group III: 20 healthy volunteers with no liver disease. They were (70 % males and 30 % females) with age ranged from 31 to 69 years old. The different stages of liver fibrosis (F0-F4) were assessed by fibroscan and were distributed among patient groups as follow: In HCV group [F0=2(10%), F1=4(20%), F2=4(20%), F3=4(20%), and F4=6(30%]. And in HCC group [F0=0 (0%), F1=0(0%), F2=8(18.2%), F3=14(31.8%) and F4=22(50%)].

Results obtained presented in tables 1 - 6 and figures 1-11. Table 1 and figure 1 show demographic data of subjects enrolled in the study. A highly significant increase in ALT, AST, ALP and total bilirubin were detected in both patient groups compared to controls. While, serum albumin, prothrombin concentration were significantly decreased. AFP serum levels were significantly increased in all patient groups as compared to control group, its levels in HCC patients was significantly higher than HCV patients without HCC.

Table 1. *Demographic characteristics of subjects enrolled in the study.*

Parameter	GI HCV N=20	GII HCC N=44	GIII Control N=20
T. blirubin (mg/dl)			
Mean	1.37	2.99	0.68
SD	0.63	2.26	0.1
F	51		
P	<0.001*		
Sig. with	2,3	1,3	1,2
ALT (U/L)			
Mean	85.1	81.41	25.1
SD	38.73	46.14	5.04
F	45.47		
P	<0.001*		
Sig. with	3	3	1,2
AST (U/L)			
Mean	81.2	107.5	28.3
SD	37.4	48.8	5.41
F	26.9		
P	<0.001*		
Sig. with	2,3	1,3	1,2
ALP (U/L)			
Mean	258.3	462	145.1
SD	76.4	236.6	27.6
F	57.3		
P	<0.001*		
Sig. with	2,3	1,3	1,2
Albumin (g/dl)			
Mean	3.68	3.16	4.55
SD	0.58	0.43	0.32
F	66.3		
P	<0.001*		
Sig. with	2,3	1,3	1,2
PT (Sec.)			
Mean	12.88	15.8	11.27
SD	1.5	2.9	0.3
F	31.57		
P	<0.001*		
Sig. with	2,3	1,3	1,2
AFP (ng/ml)			
Mean	8.18	1964.3	2.67
SD	5.25	1955.5	1.2
F	65.3		
P	<0.001*		
Sig. with	2,3	1,3	1,2

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, PT: Prothrombin time, AFP, Alpha-fetoprotein, SD: Standard Deviation, F: Test Value, P: Over all P value, P is considered significant when P ≤ 0.05.

Serum miR-122 expression level (RQ) in HCV group (GI) was significantly elevated than HCC (GII) (P=0.025). While, serum Bcl-w expression level (RQ) in HCC group (GII) was highly significantly elevated than HCV (GI) (P<0.001).As represented in figures 3, 4 and showed in table 2.

Mean expression level of miR-122 (RQ) was not significantly elevated in significant fibrotic group compared to early fibrotic group of patients (p=0.152). As represented in figure 2, and showed in table 3. Serum miR-122 level was increased with increasing of fibrosis stage until F3 and then slight drop in its level occur in F4. (Data not shown)

The data from patient groups (HCV and HCC) showed that miR-122 expression levels were positively correlated with ALT, AST, and ALP, total viremia and fibrosis stage. And it was in a negative significant correlation with Prothrombin concentration and Bcl-w. As represented in figures 5, 6, 7, 8, 9 and 10 and showed in tables 4, 5.

Table 2. *miR-122, Bcl-w and total viremia among patient groups.*

Parameter	GI HCV N=20	GII HCC N=44
miR-122 (RQ) Copies/ml		
Mean	9.12	5.68
SD	7.83	4.16
F	-------	
P	0.025*	
Sig. with	2	1
Bcl-w (RQ) Copies/ml		
Mean	0.26	0.55
SD	0.21	0.28
F	-------	
P	<0.001*	
Sig. with	2	1
Total viremia (Copies/ml x103)		
Mean	996.21	1490.43
SD	743.51	838.3
F	-------	
P	*0.017	
Sig. with	2	1

SD: Standard Deviation, F: Test Value, miR-122: micro RNA-122, P is considered significant when P ≤0.05

Table 3. *miR-122 (RQ) level among early fibrosis group and significant fibrosis group.*

Parameter	Early Fibrosis (F0-F1) (n = 6)	Significant Fibrosis (F2-F4) (n=58)
miR-122 (RQ) (Copies/ml)		
Mean	2.8	7.16
SD	1.32	5.86
F	--------	
P	0.152	

SD: Standard Deviation, F: Test Value, P: Over all P value, P is considered significant when P ≤ 0.05

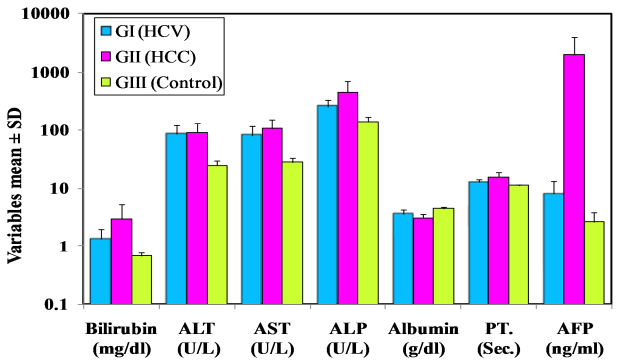

Figure 1. *Comparison of mean values of liver function tests and AFP among different studied groups.*

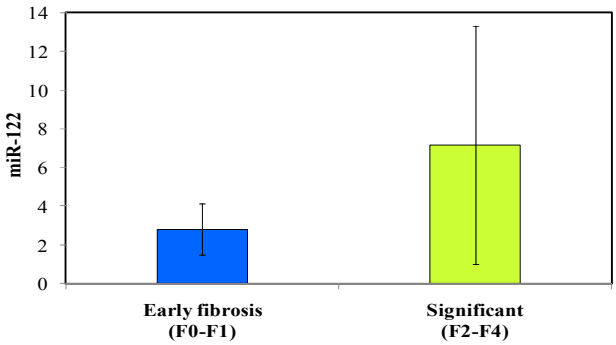

Figure 2. *Comparison of mean values of miR-122 among early fibrosis group and significant fibrosis group.*

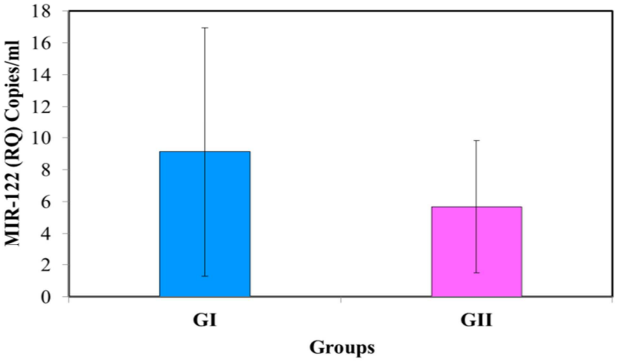

Figure 3. *Comparison of mean values of miR-122 among HCV group and HCC group.*

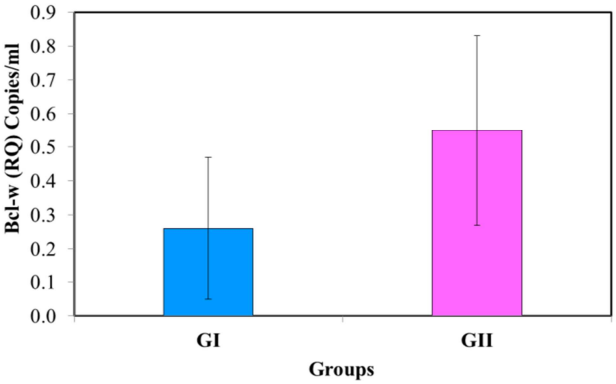

Figure 4. *Comparison of mean values of Bcl-w among HCV group and HCC group.*

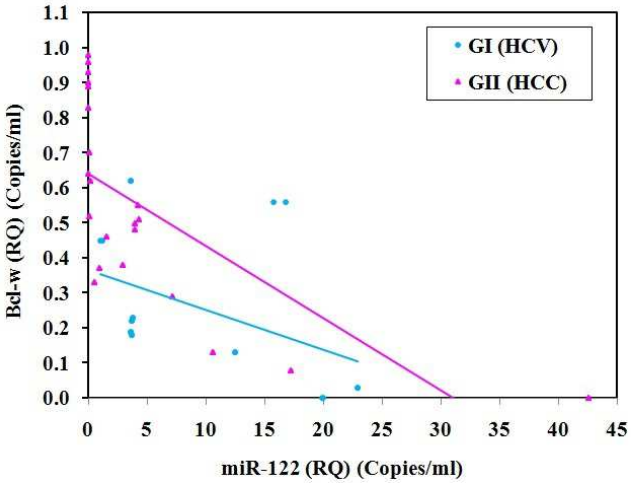

Figure 5. *Correlation betweenmiR-122 and Bcl-w in HCVandHCC groups (r= -.585, p=0.007) and(r = -0.542, p=<0.001) respectively.*

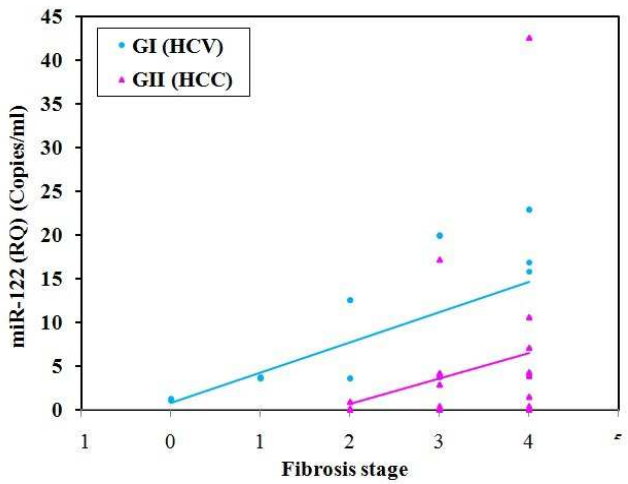

Figure 7. *Correlation between miR-122 and Fibrosis stage in HCV and HCC groups(r=0.778, p=, 0.001) and (r= 0.356, p=0.018) respectively.*

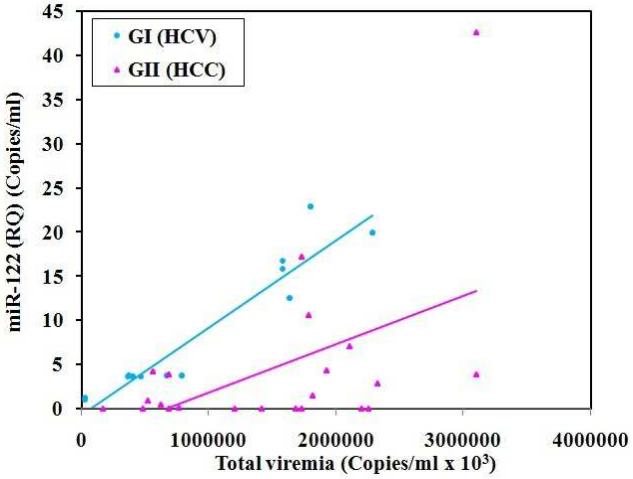

Figure 6. *Correlation between miR-122 and total viremia in HCV and HCC groups (r=0.913, p= <0.001) and (r=0.465, p= 0.001) respectively.*

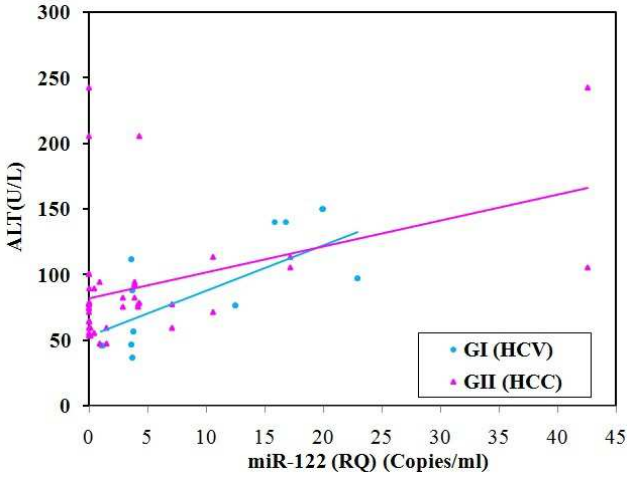

Figure 8. *Correlation between miR-122 and ALTin HCV and HCC groups (r=0.603, p=, 0.005) and (r=0.424, p=0.004) respectively.*

Table 4. *Correlation of miR-122, AFP, total viremia, fibronectin and fibrosis stage, in HCV infected patients.*

Parameters	miR-122 (RQ)		AFP		Total viremia		Fibronectin		Fibrosis stage	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
T. Bilirubin	-0.116	0.627	0.467*	0.038	-0.055	0.819	0.079	0.741	0.111	0.640
Albumin	0.094	0.692	-0.436	0.054	0.033	0.889	-0.497*	0.026	-0.512*	0.017
ALT	0.603*	0.005	0.103	0.666	0.739*	<0.001	0.612*	0.004	0.644*	0.002
AST	0.548*	0.012	0.079	0.741	0.697*	0.001	0.733*	<0.001	0.694*	0.001
ALP	0.633*	0.003	0.224	0.342	0.745*	<0.001	0.660*	0.002	0.675*	0.001
PT	-0.499*	0.025	-0.236	0.316	-0.626*	0.003	-0.513*	0.021	-0.610*	0.004
Bcl-w (RQ)	-0.585*	0.007	-0.055	0.819	-0.703*	0.001	-0.357	0.122	-0.297	0.203
miR-122 (RQ)	1.000	-	-0.359	0.120	0.913*	<0.001	0.669*	0.001	0.778*	<0.001
AFP	-0.359	0.120	1.000	-	-0.170	0.475	-0.297	0.204	-0.124	0.603
Total viremia	0.913*	<0.001	-0.170	0.475	1.000	-	0.678*	0.001	0.706*	0.001
Fibronectin	0.669*	0.001	-0.297	0.204	0.678*	0.001	1.000	-	0.885*	<0.001

AFP: Alpha-fetoprotein, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase. PT: Prothrombin time, ALP: Alkaline phosphatase, P is considered significant when P ≤ 0.05.

Table 5. *Correlation of miR-122, AFP, total viremia, fibronectin and fibrosis stage, in HCC patients.*

Parameters	miR-122 (RQ)		AFP		Total viremia		Fibronectin		Fibrosis stage	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
T.Bilirubin	-0.127	0.410	0.371*	0.013	0.021	0.890	-0.117	0.451	0.156	0.313
Albumin	-0.299*	0.049	-0.031	0.842	-0.425*	0.004	-0.323*	0.033	-0.375*	0.044
ALT	0.424*	0.004	0.271	0.075	0.298*	0.050	0.492*	0.001	0.308*	0.042
AST	0.441*	0.003	0.082	0.598	0.470*	0.001	0.559*	<0.001	0.337*	0.025
ALP	0.706*	<0.001	-0.067	0.667	0.405*	0.006	0.409*	0.006	0.416*	0.015
PT	-0.357*	0.017	0.003	0.983	-0.376*	0.012	-0.336*	0.026	-0.315*	0.048
Bcl-w (RQ)	-0.542*	<0.001	0.083	0.592	-0.309*	0.041	-0.286	0.060	-0.123	0.425
miR-122 (RQ)	1.000	-	-0.241	0.115	0.465*	0.001	0.579*	<0.001	0.356*	0.018
AFP	-0.241	0.115	1.000	-	-0.271	0.075	0.041	0.790	0.030	0.847
Total viremia	0.465*	0.001	-0.271	0.075	1.000	-	0.566*	<0.001	0.163	0.291
Fibronectin	0.579*	<0.001	0.041	0.790	0.566*	<0.001	1.000	-	0.299*	0.049

AFP: Alpha-fetoprotein, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase. PT: Prothrombin time, ALP: Alkaline phosphatase, P is considered significant when $P \leq 0.05$.

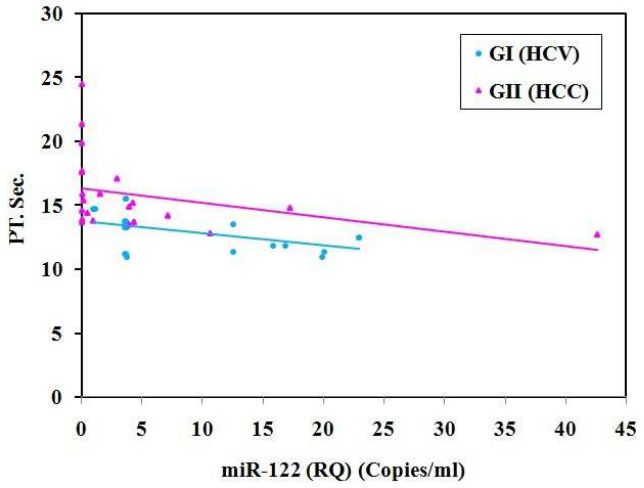

Figure 9. *Correlation between miR-122 and PT in HCV and HCC groups (r= -0.499, p=, 0.025) and (r=-0.357, p=0.017) respectively.*

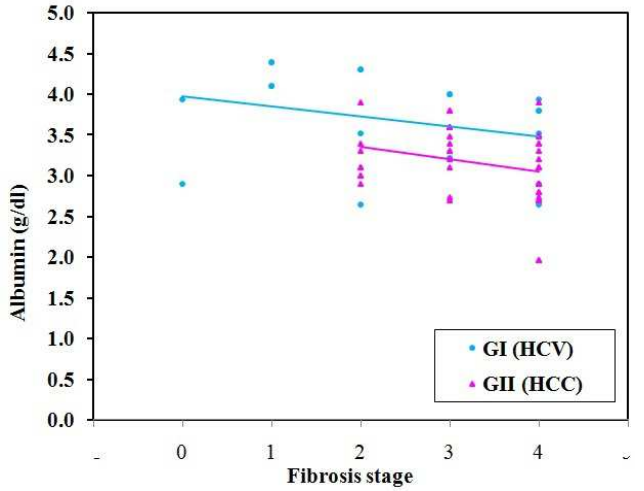

Figure 10. *Correlation between fibrosis stage and albumin in HCV and HCC groups (r= -0.512, p=, 0.017) and (r=-0.357, p=0.044) respectively.*

Receiver Operating Characteristic (ROC) Curve analysis

ROC curve analysis was designed for miR-122 in HCV infected patients against HCC group of patients. ROC curve was performed as cut off for disease progression. The area under the curve and the best cut off values (c.o.v.) and youden index of miR-122 level was obtained from the sensitivity and specificity % in progression of malignancy [23].

The ROC curve analysis showed that miR-122 presented a significant area under the curve of 0.705 and achieved sensitivity (63.64%) and specificity (75%) as represented in figure 11 and showed in table 6. Table 7 represented the risk estimate at miR-122 cut off value (3.85), after calculating the positive and negative predictive values. A significantly elevated relative risk by 5.25 folds more for developing malignancy after HCV infection.

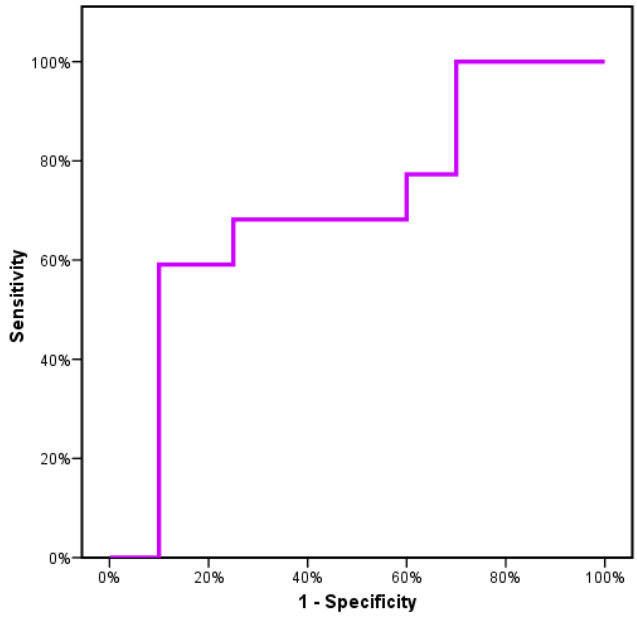

Figure 11. *ROC curve for miR-122 to diagnose HCC patients.*

Table 6. *Different diagnostic values of miR-122 obtained from Roc curve.*

Biochemical parameter	ROC Area under the curve	Sig	Youden's index	Sensitivity	Specificity	Cut off value
miR-122	0.705*	0.009	0.491	63.64	75.0	≤3.85

Youden index = (Specificity + Sensitivity) - 1

Table 7. *Frequency distribution and risk estimate at miR-122 cut off value in HCV infected patients and HCC patients.*

Parameter	Group	Cut off value	PPV	NPV	X^2	OR	95% CI	P
miR-122	HCV HCC	≤3.85	84.85	48.39	8.218*	5.250	1.607 – 17.153	0.007*

X^2: obtained by chi-square test. PPV: positive predictive value, NPV: negative predictive value, P: is considered significant ≤ 0.05, OR: Odds Ratio, 95%CI: 95%confidence interval

4. Discussion

Chronic HCV infection causes normally quiescent hepatocytes to divide repeatedly, leading to fibrosis, cirrhosis and occasionally progression to hepatocellular carcinoma (HCC) [24]. Liver cirrhosis is the most common complication of chronic HCV infection. Once cirrhosis is established, the risk of developing HCC is increased [25].

AFP is a serum glycoprotein that was first recognized as a marker for HCC more than 40 years ago and has been described to detect preclinical HCC. The fetal yolk sac and fetal liver generate high levels of AFP, which decline to<10 ng/ml within 300 days of birth [26]. Our results showed that AFP serum levels were significantly increased in all patients groups as compared to control group, its levels in HCC patients was significantly higher than in HCV patients without HCC. This finding is in agreement with yang *et al.,* 2005 [27] and Scholz *et al.*, [28]. A study from Thailand found that HCC patients with AFP >400 ng/ml tend to have greater size, bilobar involvement, portal vein thrombosis and decreased survival [29]. Liver diseases other than HCC are accompanied by high levels of AFP. 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 marker [30].

Nowadays, the search for noninvasive biomarkers for the diagnosis of diseases has become a rapidly growing area of clinical research. Micro RNA expression has been studied at cellular level and in tissues in patients with HCC. MicroRNAs (miRNAs) are small non-coding RNAs that inhibit messenger RNAs (mRNAs) by binding to their 3′ untranslated regions (UTRs). miRNA are predicted to repress up to 1/2 of all human genes post-transcriptionally through translational arrest and/or mRNA degradation [31].

MiRNAs regulate diverse functions, including cell proliferation and apoptosis, and they are dysregulated in cancers, including HCC [32]. Micro RNAs are important mediators of HBV and HCV infection as well as liver disease progression, and therefore could be potential therapeutic target molecules [33].

Various microRNAs are now being investigated in hepatitis C virus infection with the most popular one being micro RNA-122, micro RNA-122 is the most abundant micro RNA in the liver [13] where it has many important biological roles, such as in fatty acid metabolism, and circadian rhythms under normal conditions [34]. Micro RNA-122 was found to interact with HCV RNA enhancing its replication [35]. It was suggested that miR-122 triggers HCV replication by post-transcriptional repression of heme oxygenase enzyme [36]. Blockage of miRNA -122 has been considered as a therapeutic approach against chronic hepatitis C [37].

Since progression of fibrosis varies in patients, distinguishing between rapid from slow fibrosis would allow a better tailoring of the treatment [38]. To date, this is best achieved by follow-up liver biopsies or repeat measurement of liver stiffness. Both methods have their limitations. Biopsy due to its invasiveness may lead to relevant complications [39], while liver stiffness measurement has the drawback of questionable or lacking results in patients with ascites, obesity, cholestasis, or hepatic inflammation and a considerable overlap in patients with low or moderate fibrosis [40]Serological biomarkers could offer a noninvasive alternative for assessment of fibrosis in chronic liver disease. Yet, their diagnostic accuracy has still limitations.

The present work aimed to assess whether serum levels of miR-122 can be used not only as a novel non invasive biomarker for liver injury and liver function capacity but also can be used to discriminate patients with HCC from those with HCV. The present work also was carried out to examine whether circulating levels of miR-122 correlate with liver disease severity in HCV patients with and without HCC, and can potentially serve as circulating biomarker for liver disease stage assessment.

The current study revealed a significant positive correlation between miR-122 expression levels and fibrosis stage in HCV and HCC groups (p= < 0.001, 0.018 respectively). This finding was in agreement with Cermelli *et al.*, 2011 [41] who reported that miRNA-122 and miRNA-34a levels positively correlated with disease severity and stated that serum levels of miRNA-34a and miRNA-122 may represent novel noninvasive biomarkers for diagnosis of histological disease severity in patients with CHC or NAFLD. While, another study by Trebicka *et al.*, 2013 [42] concluded that circulating miR-122 levels correlated negatively with increasing stages of fibrosis.

Bihrer *et al.*, 2011 [43] reported that sera from patients with

CHC contained higher levels of miRNA -122 than sera from healthy controls. They add that the serum level of miRNA -122 strongly correlates with serum ALT levels and with necroinflammatory activity in patients with CHC, but not with fibrosis stage and functional capacity of the liver. The conflict here is that in the present study, serum miR-122 levels correlates with fibrosis stage and liver function capacity.

Moreover, in our study Micro RNA-122 expression level were further elevated in patients with HCC. This finding was in agreement of Varnholt *et al.*, 2008 [44] who examined the microRNA expression profiles in a large set of 52 human primary liver tumors consisting of premalignant dysplastic liver nodules and hepatocellular carcinomas by quantitative real-time polymerase chain reaction. All patients were infected with hepatitis C, and most had liver cirrhosis. They stated that miRNA-122, miRNA100, and miRNA -10 were over expressed whereas miR-198 and miR-145 were up to 5-fold down-regulated in hepatic tumors compared to normal liver parenchyma. They concluded that miR-122 was strongly up-regulated in dysplastic and malignant liver nodules in the large sample set, and they suggested that miRNA-122 might down regulate target mRNA of undetermined tumor suppressor genes and thus lead to increased tumor growth.

A study by Zhang Y *et al.*, [45] reported that circulating miR-122 levels were elevated in patients with chronic hepatitis B viral (HBV) infection, and correlate with liver histologic stage, inflammation grades and ALT activity. Another study by Xu J *et al.*, [46] reported that serum miR-122 levels were also higher in patients with Chronic HBV infection than in patients with HCC. We are reporting here similar results for miR-122 in patients with HCV infection with and without HCC suggesting that the increase in circulating levels of miR-122 is common to chronic liver disease of all etiologies.

A Study by Köberle *et al*., 2013 [47]; In Germany reported that serum miR-122 and miR-1 levels did not significantly differ between patients with and without HCC. The conflict here is that in our study serum miR-122 expression levels differ significantly between patients with HCV and patients with HCC associated with HCV (P=0.025).

Our data from patient groups (HCV and HCC) showed that miR-122 expression levels were positively correlated with ALT, AST, and ALP; i.e. parameters of liver cell damage; and negatively correlated with prothrombin time and albumin, indicators of liver functions. These results were in agreement with Ezzat *et al.*, 2014 [48] and Köberle *et al*., 2013 who reported that; serum miRNA -122 correlated with clinical chemistry parameters of hepatic necroinflammation, liver function and synthetic capacity [47].

The study depicted the correlation of serum miR-122 level with HCV viral load, ALT, and AST activity in patient's sera. Besides, viral RNA also showed a positive correlation with ALT and AST activity; While HCV viral RNA is significantly associated with miR- 122 and likewise miR-122 showed a positive correlation with ALT and AST activity. These observations further enhance the diagnostic importance ofmiR-122. Apparently, miR-122 is quite stable and more sensitive in the serum whereas ALT and AST enzymes are comparatively less stable [49]. Moreover, quantification of viral RNA is quite expensive and time consuming procedure. Hence, these findings authenticate miR-122 as a promising biomolecule to investigate HCV infection and monitor liver dysfunctions.

Contrary to our findings of miR-122 positively correlate with total viremia, in HCV and HCC groups (P= <0.001,0.001 respectively), other authors have reported that in CHC patients, miR-122 levels correlated with fibrosis stage and inflammation activity but didn't correlate with HCV viral load [41].

The only validated target gene of miR-122, a liver-specific miRNA that is down-regulated in HCC, is CCNG1. Here, we report the modulating effects of miR-122 on Bcl-w expression. The results of Bcl-w targeting by miR-122 may have implications in the pathogenesis of HCC.

Our data reported that up-regulation of miR-122 expression in HCV patients with and without HCC was accompanied by down-regulation of Bcl-w expression level in both groups but the down regulation in HCV group without HCC was significantly higher than HCC group (P=<0.001). Our results were in agreement with Lin *et al*. [50]; In Taiwan who suggested that decreased miR-122 expression helps cells evade cell death, a cardinal feature of cancer cells. Therefore, the reduced levels of miR-122 in HCC group enhance anti-apoptotic activity through an increase in the Bcl-w/Bax ratio. Also our results were in agreement with Kumar *et al*., [51] who reported that, miR-122 was frequently down-regulated in HCC; in contrast, its target genes cyclin G1 and Bcl-w were up-regulated.

In the present study, we observed lower levels of miR-122 in patients with more advanced fibrosis (F4), indicating that reduced serum miR-122 is most likely the result of reduced release from heptocytes. This indicates that in patients with severe liver fibrosis, the miR-122 serum levels might be a marker for hepatic functional capacity, whereas at earlier stages of liver disease, the serum miR-122 levels is mainly an indicator of necroinflammatory activity and cell death in the liver. As release from damaged hepatocytes might be the major source of hepatocyte–derived miRs [52]. It is conceivable that in patients with severe fibrosis who lost a big proportion of hepatocytes and thus have less functional hepatic capacity, the release of miRs upon damage might be lower than in patients with higher amounts of healthy liver tissue.

Our results indicated that there was not a significant difference in miR-122 levels between patients with early fibrosis and patients with significant fibrosis (p=0.152). Contrary to our results, Cermelli *et al.* [41] reported that serum miR-122 levels were significantly increased in the CHC-advanced group compared to the CHC-early group.

The current study reported that although miR-122 expression levels in HCV group were significantly elevated than HCC group (p=0.025), its levels were higher in patients with significant fibrosis when compared to those with early fibrosis, this may be due to that 70% of HCV patients were

included in a group of patients with significant fibrosis.

The diagnostic performance of the Roc curve, in the present study, by examining the different studied parameters; revealed that miR-122 was found to be a significant prognostic marker for the progression of HCC associated with HCV infection (sensitivity 63.64% and specificity 75%). This finding makes miR-122 beside AFP; are good markers for detecting the progression of hepatocellular carcinoma after HCV infection.

5. Conclusion

MiR-122 can be used as novel biomarker for liver injury and liver function capacity and may be used to discriminate patients with HCC from HCV. Also, miR-122 may represent novel non invasive biomarker for assessment of liver disease severity in patients with chronic hepatitis C virus.

Overall, the prospect of using miR-122 as prognostic marker is of interest. Larger patient cohorts with differential fibrosis states will have to be analyzed to further test the utility of circulating miR-122 as biomarker for detection or monitoring of hepatocellular carcinoma and liver fibrosis.

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