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MicroRNA expression in chronic Hepatitis C patients treated with combined pegylated interferon alpha and ribavirin therapy

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Abstract

The role of microRNAs (miRNA) in many diseases, including chronic hepatitis, represents an interesting research field. The current work aimed to study the effect of combination therapy (pegylated interferon alpha-2b plus ribavirin) on microRNA-128 (miR-128) and microRNA-296 (miR-296-5p) expression in patients with chronic hepatitis C. Also, we aimed to investigate the potential value of both types of microRNAs in differentiation between responders and non-responders either in the pre-treatment or post-treatment groups. Sixteen patients with chronic hepatitis C were compared to 10 healthy, age and sex matched controls. Venous blood samples were withdrawn before initiation of combination therapy and 12 weeks later and qPCR was used for gene expression assays. Comparison of pre-treatment group to the control group showed significant miR-128 down-regulation, while miR-296-5p showed a non-significant downregulation. Regarding post-treatment group, there was non-significant upregulation of miR-128, while miR-296-5p was significantly downregulated compared to either pre-treatment group or the control group. Comparative studies of miR-128 and miR-296-5p in responders versus non-responders showed non-significant fold change either in the pre- or post-treatment groups. It could be concluded that chronic hepatitis C as well as the combination therapy modulate miR-128/296 expression. Until now, miRNAs studied were not useful indicators to differentiate between responders and non-responders to combination therapy either in pre- or post-treatment group.

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Introduction

Egypt has the highest prevalence of hepatitis C in the world and chronic hepatitis C viral infection (HCV) is the main cause of liver cirrhosis and cancer**.** HCV isolates, based on genetic differences, can be classified into six genotypes (1-6) where genotype 4 predominates in Egypt (1).

Early virological response (EVR) means the decrease of serum HCV RNA by two logs or more at treatment by week 12. Patients who did not reach an EVR failed to achieve sustained virological response (SVR). The absence of an EVR is the most important means of identifying non-responders (2).

Recently, it was reported that strategies for treating HCV infection have been improved by newly discovered direct antiviral agents (DAAs), which inhibit viral enzymes (e.g., protease and polymerase inhibitors). The current standard therapy for CHC is triple therapy with pegylated IFN, ribavirin, and one of the HCV protease or polymerase inhibitors. Therefore, even in the presence of DAAs, interferon (IFN) will continue as the backbone of combination therapy, since viral resistant mutants emerge rapidly if DAA is used alone. Despite extensive examination of the biological and clinical effects of IFN in patients with chronic hepatitis C, the prediction of treatment responses in individual patients remains difficult (3).

MicroRNAs (miRNAs) are important class of small non-coding RNA molecules that have recently come to prominence as regulators of cell physiology and pathology including viral infection. Previous studies reported that miRNAs and viral RNA interaction may be achieved through a wide array of mechanisms. In this regard, miRNA encoded by mammalian viruses may down regulate both viral and cellular genes. Moreover, cellular miRNA may either silence viral RNAs, and /or control the expression of viral necessary cellular proteins (4)

Subjects and methods

The current study was conducted in Department of Medical Biochemistry, in cooperation with Molecular Biology and Biotechnology Unit and Biochemical Endocrinology Unit, after approval of the study scheme by ethical committee, Faculty of Medicine, Benha University. During the period from May to October 2012, sixty three male patients were selected from Hepatic Center, Benha Fever Hospital. Additional 10 apparently healthy subjects were recruited as a control group. Their ages ranged 23-56 years (y) with mean value 35.65 y. All patients received doses of combination therapy including pegylated IFN alfa-2b (1.5µg/kg/week) plus weight-based ribavirin (10.6 mg/kg/day). Prior to initiation of treatment all patients were reactive to HCV antibodies test and showed positive HCV PCR results. The patients were followed up 12 weeks after the start of combination treatment. Based on PCR results, they were subclassified into responders (54/60, 90%) and non-responders (6/60, 10%) group. The responders group included 10 randomly selected patients whose PCR results changed to negative**,** while, the non-responders group included 6 patients whose PCR results failed to decrease by at least 2 logs (5).

Patients were selected according to the inclusion and exclusion criteria of National Committee for Control of Viral Hepatitis in Egypt (Egyptian National Control Strategy for Viral Hepatitis, 2008-2012).

Sampling:

Venous blood sample (8.5 ml) was withdrawn from each subject and divided into four parts, after signing a written informed consent. The first part (2.0 ml) was collected into ethylene diamine tetra-acetic acid (EDTA) vacutainer for determination of CBC. The second part (4.0 ml) was allowed to clot and centrifuged. The serum separated was used for determination of AST, ALT, albumin, creatinine, HCV antibodies and later determination of HCV (RNA) by real time PCR. The third part (2.0 ml) was withdrawn into tube with tri-sodium citrate solution (concentration 3.5 %) in a ratio of 9:1 for determination of prothrombin time (PT). The fourth part (0.5 ml) was stored in RNA protect Animal Blood tubes (Qiagen, Germany) at –80°C till further processing. Another venous blood sample (8.5ml) was taken after 12 weeks from initiation of combination therapy. The sample was divided as mentioned before.

According to regulations of National Committee for Control of Viral Hepatitis in Egypt, all subjects were subjected to full history taking, complete physical examination, abdominal ultrasonography scanning, ECG, fundus examination, routine biochemical investigations and histopathological examination of liver biopsies (Egyptian National Control Strategy for Viral Hepatitis, 2008-2012).

Biochemical investigations:

CBC was performed by automated hematology analyzer Sysmex XS-1000i (Sysmex, Japan), biochemical assay of fasting serum glucose (6)**,** serum TSH level (7)**,** serum creatinine (8)**,** serum ANA titre (9) and Bilharzial antibodies by Indirect Heme-Agglutination assay (IHA) (10)**.** Also, Liver function tests including serum alanine amino transferase (ALT)(11), asparate amino transferase (AST) (11), serum alkaline phosphatase (12)**,** serum albumin (13), serum total bilirubin (14), serum alpha fetoprotein (15) by Microtech spectrophotometer (Vital Scientific, Netherlands) and plasma prothrombin time (16) by Behring Fibrin timer II from (Behring, Germany). In addition, hepatitis markers were assayed including hepatitis B surface antigen (HBsAg) by enzyme immunoassay (17)**,** HCV antibodies by enzyme immunoassay (18)**.**

Determination of HCV-RNA viral load:

QIAamp Viral RNA mini Kits were used for viral RNA extraction by Qiacube automatic extractor (Qiagen, Germany). This was followed by quantitative RT-PCR by absolute quantitation method using artus HCV RG RT-PCR Kit (Qiagen, Germany). Four quantitation standards were used to generate standard curves on StepOne real time PCR system (Applied Biosystems, USA). Calculation of results was performed using the following equation:

Result (IU/ml) = Result (IU/µl) x Elution Volume (µl) / Sample Volume (ml)

The manufacturers' instructions were followed throughout all steps.

Molecular Analysis of Gene Expression of Circulating miR-128 and miR-296-5p:

The RNeasy® Protect Animal Blood kit (Qiagen, Germany) was used according to the manufacturer's instructions to separate total RNA including miRNA from blood samples stored in RNA protect Animal Blood Tubes. This was immediately followed by reverse transcription of miRNA template in G-storm thermal cycler (Thermo, England), using miScript II RT Kit (Qiagen, Germany) according to the manufacturer's instructions. In order to detect the gene expression levels of mir-128 and mir-296-5p, we performed qPCR by the 7900HT Fast Real-Time PCR System (Applied Biosystems, Singapore). Target-specific miScript Primer Assays (forward primers) and the miScript PCR Starter Kit (Qiagen, Germany) were used for qPCR, following the manufacturer's instructions. Data were analyzed by the RQ manager program 1.2 ABI SDS software (ABI 7900HT), the relative quantities of the target genes were normalized against standard endogenous control RNU-6B. Fold expression changes were calculated using the equation $2^{-\Delta\Delta CT}$(19)**.**

Statistical analysis:

The collected data were computed and statistically analyzed using SPSS program version 16 software for windows (SPSS Inc, Chicago, ILL Company). Independent and dependent variables were expressed as mean ± SD, median and range. Data were compared using Wilcoxon -Mann-Whitney for non-parametric results. p value <0.05 was considered significant.

Results

The demographic data, clinical, biochemical and pathological laboratory findings of the studied groups are summarized in Tables (1 and 2). Regarding miR-128, our results showed non-significant upregulation in the post-treatment patients as compared to either controls or pre-treatment patients (p>0.05 each), Table (3). However, comparison of pre-treatment group with the control group revealed that it was significantly downregulated (p<0.05), Table (3).

Furthermore, the expression of miR-296-5p in the post-treatment group was decreased compared to that of the control group and the pre-treatment with significant statistical difference (p<0.05 each). On the other hand, miR-296-5p in the pre-treatment group showed non-significant downregulation as compared to the control group (p>0.05), Table (3).

Finally, comparison of miR128/296 between responders and non- responders, revealed non-significant differences in pre- and post-treatment groups, figure (1).

Table (1): Age, weight, BMI, habits, bilharziasis, ultrasonography and biopsy findings of the studied groups

Studied parameters	Controls (n.=10)	Patients (n.=16)	
		Pre-treatment	Post-treatment
Age (year) mean±SD	38.50±10.32	33.88±9.13	33.88±9.13
Weight (kg)	81.90±8.53	81.31±10.16	79.44±10.03
BMI (kg/m^2)	26.17±2.72	26.91±2.47	26.32±2.70
Habit (Smoker/Non-smoker)	3/7	1/15	1/15
U/S (CLD/BIL/SPL/CIR)	-	4/3/2/1	4/3/2/1
Biopsy (F1A1/F1A2/F2A1/F2A2/F3A2)	-	5/1/4 /1/5	5/1/4 /1/5

BMI: body mass index, U/S: ultrasonography, CLD: chronic liver disease, Bil: Bilharziasis, SPL: splenomegaly, CIR: cihrrosis

Table (2): Biochemical laboratory findings of the studied groups

Studied parameters	Controls (n.=10)	Patients (n.=16)	
		Pre-treatment	Post-treatment
Hemoglobin (g/dl)	14.85±0.71	14.71±1.28	11.68±1.34
Leucocytes (X10^3/mm^3)	5.67±1.61	6.21±1.69	4.03±1.23
Platelets (X10^3/mm^3)	190.90±30.25	210.12±39.38	177.40±43.66
ANC (X103/mm^3)	4.21±1.62	2.45±0. 84	1.69±0.76
Bilharziasis (-ve/+ve)	10/0	11/5	11/5
ALT (U/l)	23.90±9.94	54.69±19.10	31.88±9.29
AST(U/l)	26.10±10.98	44.88±18.60	33.56±6.41
ALP (U/l)	92.74±51.32	138.81±50.57	132±46.54
Albumin (g/dl)	4.45±0.43	4.24±0.44	4.09±0.46
Total bilirubin (g/dl)	0.77±0.13	0.68±0.21	0.84±0.28
PT (sec)	12.28±0.84	13.01±0.93	13.37±0.54
AFP (ng/ml)	3.20±1.23	2.40±1.22	2.12±0.91
FSG (mg/dl)	90.80±12.99	87.81±18.60	87.88±17.45
TSH (µIU/ml)	1.81±0.57	1.80±0.78	2.40±0.67
Creatinine (mg/dl)	0.81±0.19	0.91±0.15	0.87±0.16
HCV Viral load (IU/ml)	-	372980±470241	69337±131036

ANC: Absolute neutrophil count, ALT: Alanine aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline phosphatase, PT: Prothrombin time, AFP: Alpha fetoprotein, FSG: Fasting serum glucose, TSH: Thyroid stimulating hormone

Table (3): Fold change of miR-128 and miR-296-5p expression in the studied groups

	Controls (n.=10)	Pre-treatment (n.=16)	Post-treatment (n.=16)
miR-128 median (range)	18.30 (12.78 - 24.75)	14.40 (10.06 - 76.78)†	16.39(6.24- 66.71)
miR-296-5p median (range)	188.60 (90.90 - 384.17)	142.61 (6.24 - 481.10)	37.22 (8.44- 454.79)†, ‡

†: significant as compared to control group, ‡: significant as compared to pre-treatment group

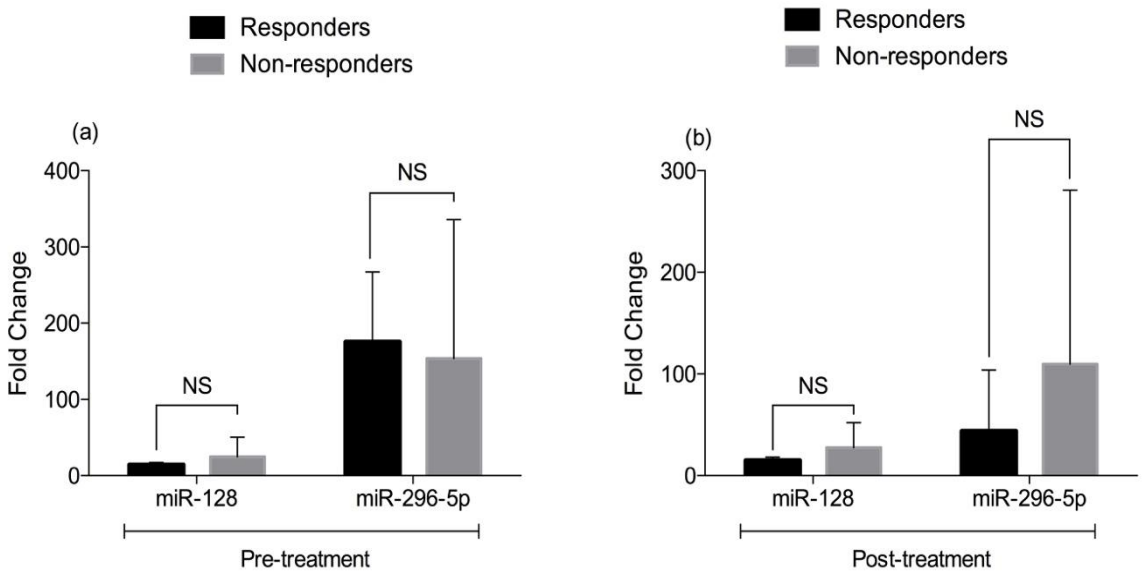

Figure (1): Fold change of miR-128 and miR-296-5p in responders versus non-responders in studied groups. NS: non-significant

DISCUSSION

Chronic hepatitis C represents a growing health problem worldwide that has attracted more attention recently. About 3% of the world's population (130–170 million people) have chronic HCV infection (20). In Egypt, there were 9 million people infected with HCV in 2010 (21). Globally, about 3–4 million people are infected per year, and more than 350,000 people die every year from hepatitis C-related diseases (22,23). In the last decade, HCV-related HCC showed about 3-fold increase. Next decade, HCV morbidity and mortality are likely to reach their peak (24,25).

Antiviral therapy is used to obtain a sustained virological response (SVR). In the last decade, treatment with pegylated interferon - α 2a or 2b (pegIFN) combined with weight-based ribavirin (RBV) for 48 weeks (in genotype 4) has been considered the standard of care (SOC) for HCV treatment. With pegIFN/RBV treatment, SVR rates were 40–50% (26); however, it is costly and has side effects. The factors that determine the likelihood of achieving SVR are called predictors of response. Depending on the time of evaluation, they can be classified as viral- or host-related, or as pre- or on-therapy (27). MicroRNAs have been shown to function as regulators of gene expression

through RNA interference (RNAi) which has a vital role in antiviral immune response in mammalian viruses (28).

The current work aimed to study the effect of combination (pegIFN/RBV) therapy on microRNA-128 and microRNA-296 in patients with chronic hepatitis C. Also, to verify that; these 2 types of microRNAs might be useful to differentiate between responders and non-responders either in the pre- treatment or post- treatment group.

Our results showed that miR-128 and miR-296-5p were expressed in whole blood collected from healthy individuals (Table: 3). A previous study reported that miRNAs were expressed in peripheral blood mononuclear cells (PBMNCs) from healthy volunteers and HCV patients (4). In addition, it was found that miRNAs were expressed in PBMNCs from healthy subjects (29).

MiR-128 showed significant downregulation while miR-296-5p showed non-significant decrease of expression in the pre- treatment group compared to the control group (Table: 3).

This could be explained by the fact that hepatitis C virus is a poor inducer of interferon (30). Type I IFN system is the most potent arm of innate antiviral immunity. Interferon alpha has potent antiviral and antiproliferative properties, but does not act directly against HCV. Instead, interferon alpha creates a non-specific antiviral environment within the hepatocyte by inducing interferon-stimulated genes. On exposure to HCV, the infected cell release interferon which binds to the neighboring cell membrane receptors. Interferon signals mainly through the Janus kinase - signal transducer and activator of transcription (Jak-STAT) pathway. Stat1, Stat2, interferon regulatory factor-3 (IRF-3), IRF-9 and protein kinase RNA-activated (PKR) are important effectors in this pathway. Finally, interferon induces genes encoding for proteins which promote infected cell apoptosis, inhibit viral replication and viral and cellular proteins synthesis (31).

On the other hand, HCV like other viruses seems to have evolved several mechanisms to avoid the host innate immune response (32). It was found that HCV proteins specifically stimulates the IFN induction pathway during the first 12 hours of infection, but inhibits it thereafter (30) through:

Firstly, HCV uses its proteins to degrade Stat1 leading to disruption of Jak-Stat pathway (33)**.** ***Secondly,*** HCV inhibits IRF-3 a protein that induces IFN gene transcription (34).

Finally, HCV inhibits protein kinase R (PKR) due to sequence similarity between HCV envelope (E2) protein and phosphorylation sites of PKR and its target, the translation initiation factor (eIF2α). Thus, E2 blocks PKR inhibitory effect on protein synthesis and cell growth (35).

Type I IFNs regulate the host miRNAs which inhibits HCV replication and infectivity**.** Clearly, endogenous INF-α when decreases, leads to subsequent inhibition of interferon stimulated genes transcription including the genes coding for miRNAs (36).

The current data are in accordance with a previous report that showed modulation of 3 miRNAs including miR-128a which showed downregulation in 3 HCV replicon clones (37). Moreover, they predicted the gene targets of the 3 miRNAs. Interestingly, those targets may be involved in

pathways to sustain HCV replication and / or to control the antiviral response by the virus itself.

However, contradictory to our results, Scagnolari and colleagues reported that expression of miR-128 and miR-296-5p showed non-significant increase in the pre-treatment compared to the control group (4). A More recent study, revealed a significant increase in miR-128 and miR-296-5p in the pre-treatment compared to the control group (29). Such controversies may be attributed to different sample types and the small sample size in our study.

In addition, our results showed non-significant upregulation of miR-128 while significant dowregulation of miR-296-5p in the post treatment compared either with the pre-treatment group or with the control group (Table: 3).

The results of miR-128 were in agreement with two former studies, while those of miR-296-5p were contradictory to both of them (4,29).

Scagnolri and his group (4), separated PBMNCs from CHC patients before and 12 hours after initiating pegylated interferon alpha therapy. They noticed significant increase in miR-128 and miR-296-5p in the post treatment compared with pre-treatment group. They attributed such upregulation to induction by interferon alpha (4,29). To our mind, blood samples were taken 12 weeks after initiation of combination therapy in this work which may explain the opposing results of miR-296.

Furthermore, such contradiction might be attributed to the viral ability to exert inhibitory effect against interferon induction and the function of its effector molecules (38), and the ability of HCV to produce infinite genetic variants adds to the resistance mechanisms against interferon treatment (39).

Comparative studies of miR-128 and miR-296-5p in responders versus non-responders showed non-significant differences either in the pre-treatment or in the post-treatment groups (Figure: 1). These results support previous findings which reported that HCV infection and combination therapy might have a more profound effect on liver tissue expression of microRNAs rather than on PBMNCs (4).

Conclusion

miR-128 and miR-296-p expression may be altered by chronic hepatitis C as well as the combination therapy. Moreover, both studied miRNAs were not useful indicators to differentiate between responders and non-responders to pegIFN/RBV therapy pre- and post-treatment groups. However, our study was limited by the small sample size and further studies on larger scale are recommended.

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