Interferon (IFN)-mediated antiviral activity against the hepatitis C (HCV) through microRNAs (microRNAs).

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Abstract

**Background:** MicroRNAs are a class of small non-coding RNA molecules that function through post-transcriptional regulation of gene expression by a process termed RNA interference (RNAi), and that also have to prominence as critical regulators in a wide array of mechanisms of cell physiology.

**Aim of the work:** The study will attempt to evaluate the expression of several microRNAs in peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C (CHC) at 12 hours after the first injection of pegylated interferon in comparison with healthy controls.

**Patients and methods:** forty patients with chronic hepatitis C virus infection (CHCV), their age ranged between (20-56) years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, after 12 hours of the first interferon injection, and twenty healthy individuals were included to serve as controls. All the patients and controls were subjected to the following history, clinical examination, abdominal ultrasonography and collection of samples for routine laboratory investigations. CBCs and Taqman quantitative RT-PCR for MicroRNAs expression analysis of miR-128a, miR 196a, miR-196b, miR 296.

**Results:** Our study revealed that the microRNAs had a higher levels of expression in cases of CHCV infection.

**Conclusion:** Our study concluded that there’s a highly significant increase in expression levels of IFN-induced microRNAs were observed in patients of microRNAs-128a, 196a, 196b, 296.

**Recommendations:** The future use of miR inhibitors or mimics and / or siRNAs might be useful for the development of diagnostic and therapeutic strategies aimed at the recovering of protective innate responses in HCV infections.

**Key Words:** Interferon regulated microRNAs, (128a, 196a, 196b, 296 microRNAs modulated by HCV replicon clones.

**Introduction**

The hepatitis C (HCV) is a major public health problem and a leading cause of chronic liver disease (1). The currently recommended therapy of chronic HCV infection is the combination of a pegylated interferon and ribavirin (2). Recently an even more direct link between IFN and miRNAs has emerged (3). It has
been suggested that miRNAs may be an effector in the classical vertebrate innate immune system (4). There is increasing evidence that miRNAs may have an important function in viral replication and may be used by host cells to control viral infection (5), (6).

MicroRNAs (miRNAs) are an important class of small non-coding RNA molecules that have recently come to prominence as critical regulators in a wide array of mechanisms of cell physiology (5), (6). Due to different viral, environmental and host factors, a sustained virological response is achieved in about 50% of patients infected with HCV genotype 1 and in about 80% of patients infected with HCV genotypes 2 or 3 (7), (8). MiRNAs are a class of small, naturally occurring RNA molecules that play critical roles in modulating numerous biological pathways by regulating gene expression. The knowledge that miRNA expression is dysregulated in many pathological disease processes, including cancer (9). Recently, a new class of small (19-25 nucleotides) noncoding RNAs, microRNAs (miRs or miRNAs), has been linked to several human disease, including cancer. MicroRNAs are involved in temporal and tissue-specific eukaryotic gene regulation, either by translated inhibition or exonucleolytic mRNA decay, targeting through imperfect complementarity, the 3'– untranslated region (3' – UTR) of the mRNA. Since their ability to potentially target any human mRNA, it is likely that microRNAs are involved in almost every biological process, including cell regulation, cell growth, apoptosis, cell differentiation, and stress response (10).

MicroRNAs (miRNAs) mark a new paradigm of RNA-directed gene expression regulation in a wide spectrum of biological systems. These small non-coding RNAs can contribute to the repertoire of host-pathogen interaction during viral infection. This interplay has important consequences, both for the virus and the host. There have been reported evidences of host-cellular miRNAs modulating the expression of various viral genes, thereby playing a pivotal role in the host-pathogene interaction network (11).

**Patients and methods**

Forty patients with chronic hepatitis C virus infection (CHCV), their age between (20-56) years, selected from the National Hepatology and Tropical Medicine Research Institute were included in this study, after 12 hours of the first interferon injection, and twenty healthy individuals were included to serve as controls, with IFN alpha *in-vitro*-treatment. All patients have anti-HCV antibodies, HCV RNA in serum, evidence of chronic hepatitis on liver biopsy, elevated levels of aminotransferase above the upper limit, serum albumin, bilirubine, and
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Prothrombine time within normal limit with negative history of drug abuse, non reactive HBsAg, with exclusion of other chronic disease and pregnancy. There was no clinical signs of decompensated liver disease. All the patients and controls were subjected to the following history, clinical examination, abdominal ultrasonography and collection of samples. A 5 ml whole blood was drawn by venipuncture into edeta coated tube. Taqman quantitative RT-PCR for microRNAs were estimated for expression analysis of miR-128a, miR-196a, miR-196b, miR 296 from patients with CHC and healthy individuals were quantified by a real time 5’ exonuclease RT-PCR Taqman assay which include the following step: miRNA isolation: microRNA was isolated from collected whole peripheral blood of CHCV patients using specific mirVana™ miRNA isolation kit (Ambion ®) with catalog number (AM 1560) according to manufacturer instruction. cDNA synthesis: was synthesized by reverse transcription of extracted microRNA using the specific TaqMan MiRNA Reverse Transcription (RT) kit (Applied Biosystems, USA) briefly, each RT reaction contained 5 ng of extracted total miRNA, 3 uL of TaqMan MicroRNA assays, 1.50 uL of RT 10x buffer, 0.25 mM each of dNTPs, 3.33 U/uL Multiscribe reversetranscriptase and 0.25 U/uL RNase inhibitor. The 15 uL reactions were incubated in a Biometra T3 Thermocycler (MMedical, Italy) in a 96-well plate for 30 minutes at 16 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, and then held at 4 °C. Real time-PCR Taqman assay: Real time PCR was performed following standard protocol of the manufacturer in Step One Plus Real Time PCR system ( Applied Biosystem, USA) and threshold cycles (CT) were calculated using Sequence Detection Software (SDS v1.2, Applied Biosystem, USA). All primer and probes of each miRNA investigated were present in the TaqMan microRNA assays purchased from Applied Biosystems. The 20 uL reaction included 1.33 uL RT product, 10 uL of TaqMan Universal PCR Master and 1 uL of TaqMan MicroRNA assays. The reactions were incubated in a 96-well optical plate at 95 °C for 10 minutes, following by 40 cycles of 95°C for 15 s and 60 °C for 1 minute. The relative expression levels of each miRNA were measured using the constitutively expressed RNU6B as endogenous control the expression of each miRNA relative to RNU6B was determined using the arithmetic formula (2 –Δ Ct ) or (2 –ΔΔ Ct) according to the supplier’s guidelines (Applied Biosystems) reference (12).

Statistical analysis: Analysis of data of all patients was done by IBM comuter using SPSS (Statistical Program for social science version 12) as follows: Description of
quantitative variables as, mean, SD and range. Description of qualitative variables as number and percentage. Unpaired t-test was used to compare quantitative variables, in parametric data (SD < 50 % mean), (13).

**Results**

The study included 40 patients with CHCV infection and 20 healthy volunteers with IFN alpha *in-vitro* treatment.

The Real time-PCR expression level of microRNAs 128a against the hepatitis C virus in (PBMCs) peripheral blood mononuclear cells, 12 hours after the first interferon injection had a higher level compared to the microRNAs in (PBMCs) from healthy individuals after *in-vitro* interferon-mediated antiviral with statistically significant difference (P< 0.001) by using unpaired t-test, table (1), graph (1), fig (A).

![Fig (A) - Amplification blot curves for quantitative real time PCR of miRNA 128a (upper arrow) samples (lower arrow) control.](image)

The Real time-PCR expression level of microRNAs 269 against the hepatitis C virus, 12 hours after the first interferon injection in (PBMCs) had a higher level compared to the microRNA in (PBMCs) from healthy individuals after *in-vitro* interferon-mediated antiviral with statistically significant difference (p< 0.001) by using unpaired t-test, table (2), graph (2), fig (B).
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The Real time-PCR expression level of microRNAs 169A against the hepatitis C virus, 12 hours after the first interferon injection in (PBMCs) had a higher level compared to the microRNAs in (PBMCs) from healthy individuals after *in-vitro* interferon-mediated antiviral with statistically significant difference (P < 0.001) by using unpaired t-test, table (3), graph (3), fig (C).

Fig (B) - Amplification blot curves for quantitative real time PCR of miRNA 269 (upper arrow) samples (lower arrow) control.

Fig (C) - Amplification blot curves for quantitative real time PCR of miRNA 196a (upper arrow) samples (lower arrow) control.
Fig (D) - Amplification blot curves for quantitative real-time PCR of miRNA 196b (upper arrow) samples (lower arrow) control. The Real time-PCR expression level of microRNA 169b against the hepatitis C virus, 12 hours after the first interferon injection in (PBMCs) had a higher level compared to the microRNAs in (PBMCs) from healthy individuals after in-vitro interferon-mediated antiviral with statistically significant difference (P<0.001) by using unpaired t-test, table (4), graph (4), fig (D).

Table (1) Comparison between CHCV patients and controls as regard microRNAs 128a.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases</th>
<th>Controls</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=10</td>
<td>N=5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SD</td>
<td>1.8±0.5</td>
<td>0.81±0.01</td>
<td>4.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Range</td>
<td>0.96-2.6</td>
<td>0.79-0.83</td>
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</tr>
</tbody>
</table>

This table shows that CHCV patients had a higher expression level of microRNAs 128a as compared to controls with statistically significant difference by using unpaired t-test.

Graph (1): Quantitative expression level of microRNAs 128a in (PBMCs) from patients with CHCV in comparison to the healthy controls (code: 1.00= patients& 2.00=controls).
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Table (2) Comparison between CHCV patients and controls as regard microRNAs 296.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases N=10</th>
<th>Controls N=5</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean±SD</strong></td>
<td>312.9±46</td>
<td>131.7±1.1</td>
<td><strong>8</strong></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>217-385</td>
<td>130.7-133</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This table shows that CHCV patients had a higher expression level of microRNAs 296 as compared to controls with statistically significant difference by using unpaired t-test.

Graph (2): Quantitative expression level of microRNAs 296 in (PBMCs) from patients with CHCV in comparison to the healthy controls (code: 1.00=patients & 2.00=controls).
Table (3) Comparison between CHCV patients and controls as regard microRNAs 169a.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (N=10)</th>
<th>Controls (N=5)</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>8.2±1.7</td>
<td>6.2±0.13</td>
<td>2.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Range</td>
<td>6.02-10.5</td>
<td>6-6.3</td>
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</tbody>
</table>

This table shows that CHCV patients had a higher expression level of microRNAs 169a as compared to controls with statistically significant difference by using unpaired t-test.

Graph (3): Quantitative expression level of microRNAs 169a in (PBMCs) from patients with CHCV in comparison to the healthy controls (code: 100=patients & 2.00=controls).
Table (4) Comparison between CHCV patients and controls as regard microRNAs 196b.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases N=10</th>
<th>Controls N=5</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>1.3±0.3</td>
<td>0.28±0.01</td>
<td>6.9</td>
<td>&lt;0.001</td>
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<tr>
<td>Range</td>
<td>0.84-1.90</td>
<td>0.26-0.29</td>
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</table>

This table shows that CHCV patients had a higher expression level of microRNAs 196b as compared to controls with statistically significant difference by using unpaired t-test.

Graph (4): Quantitative expression level of microRNAs 196b in (PBMCs) from patients with CHCV in comparison to the healthy controls (code: 1.00=patients& 2.00=patients).

**Discussion**

In the last years microarry technology provided a comprehensive analysis of alteration in gene expression induced by HCV and revealed important processes of virus-host interactions(14-18). Interestingly, microarry studies indicated that HCV stimulates the endogenous Type I Interferon (IFN-a/B) pathway as suggested by activation of IFN-stimulated genes (ISGs) (19-26). Recently, it has been proposed that also microRNAs (miRs), a class of small
non-coding regulatory RNAs, are involved in the antiviral pathway induced by IFN- B treatment. The synthetic introduction of five IFN-B-induced miRs into HCV replicon cells may stimulate the antiviral effect of IFN-B blocking HCV replication and infection. These five miRs (miR-196, miR-296, miR-351, miR-431 and miR-448) likely induced an antiviral state either through alteration of gene expression and / or directly targeting HCV RNA, as was demonstrated for two of them (miR-196 and miR-448) (3).

In the present study, we found that CHCV patients had a higher expression levels of microRNAs (miR-128a, miR-196a, miR-196-b, Mir-296) than the controls.

Burni, et al. (27) postulated that the expression profile revealed that 16 out of 24 miRs were modulated in HCV replicon clone 21-5. Analysis in HCV replicon clones 22-6 and 21-7 indicated that 3 out of 16 miRs, (miR-128a, miR-196a, and miR-142-3P) were modulated in concerted fashion in all three HCV clones. Microarray analysis revealed that 37 out of 1981 genes, predicted targets of the 3 miRs, showed an inverse expression relationship with the corresponding miR in HCV clones, as expressed for true targets. Classification of the 37 genes by Panther System indicated that the dataset contains genes involved in biological processes that sustain HCV replication and / or in pathways potentially implicated in the control of antiviral response by HCV infection. These results are in agreement with our results of (miR-128a, miR-196a).

PBMCs from patients with CHCV had a greater expressions of {miR-1, miR-30, miR-128, miR-196, miR-296} compared with healthy controls, with the exception of miR-196 (28-31), these findings are in agreement with our study results for (miR-128a, and miR-296) but against (miR-196a, and miR-196b) that the present study had a higher expression levels of miR-196a, miR-196b than the healthy controls.

Scagnolari et al. (12) observed that when levels of the miRNAs were measured 12 hours after the first IFN injection, increases in expression levels of IFN-induced miRNAs were observed in 25-50% of patients depending on the type of miRNA examined. Those results are in correlation with our results.

In the present work the twenty healthy controls blood samples were exposed to IFN alpha in-vitro treatment that leads to a transcriptional induction of the four
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miRNAs (miR-128a, miR-196a, Mir-196b, miR-296).

Hubbell et al. (32), Roos et al. (33) postulated that miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296) involved in IFN-mediated antiviral activity against HCV, are expressed in PBMCs collected from healthy individuals after in-vitro IFN alpha treatment, and that their expression in such cells may be induced by IFN-alpha to varying degrees specifically, greater increase in miR-1 and miR-128, these results in agreement with our results.

Scagnolari et al. (12) discovered that the IFN alpha in-vitro treatment of PBMCs leads to a transcriptional induction of the expression of several miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296), these results are in correlation with our results.

REFERENCES